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INHIBITION OF PROLIFERATION OF HUMAN LEUKAEMIA 60 CELLS BY DIETHYL ESTERS OF GLYOXALASE INHIBITORS *IN VITRO*

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Abstract—Diethyl esters of the glutathione *S*-conjugate *S*-*p*-bromobenzylglutathione, an inhibitor of glyoxalase I, and *S*-*p*-nitrobenzoxycarbonylglutathione, an inhibitor of glyoxalase II, induced growth arrest and toxicity in human leukaemia 60 cells in culture. The median growth inhibitory concentration IC_{50} values were 8.3 μM (95% C.I. 7.0–9.9 μM) for *S*-*p*-bromobenzylglutathione diethyl ester and 56 μM (95% C.I. 36–86 μM) for *p*-nitrobenzoxycarbonylglutathione. Monoethyl ester and unesterified derivatives were inactive. The diethyl ester derivatives were also toxic to mature human neutrophils under the same culture conditions where the respective median toxic concentration IC_{50} values were 39.7 (95% C.I. 35.4–44.5 μM) and 127 (95% C.I. 123–132 μM) μM. Diester derivatives may be of future interest in studying the cytotoxicity of glutathione *S*-conjugates and for the development of cytotoxic anti-tumour agents.

S-Conjugates of glutathione are formed non-enzymatically and enzymatically in biological systems during the metabolism of drugs and environmental chemicals [1]. Enzymatic formation involves the glutathione transferase (EC 2.1.5.18) series of enzymes which catalyse the conjugation of a wide range of aromatic halides with reduced glutathione: for example, *S*-2,4-dinitrophenylglutathione is formed from 1-chloro-2,4-dinitrobenzene [2–4]. The formation of *S*-conjugates of glutathione is the initial step in the mercapturic acid pathway, leading to the ultimate excretion of mercapturic acid derivatives of glutathione, $CH_3CO\text{-}NHCH(CO_2H)CH_2\text{-}SR$ [5]. *S*-Conjugates of glutathione are formed intracellularly and are expelled from cells by carrier-facilitated, ATP-dependent or -independent processes [6].

S-Conjugates of glutathione may themselves be toxic although, to date, this has been difficult to prove because a readily available and generally applicable procedure for delivering the conjugate into cells was unknown [7, 8]. Rather, the precursor substrate of glutathione transferase was added to cell systems and the mediation of toxicity by the corresponding glutathione conjugate was implied. *S*-Conjugates of glutathione have been shown to be competitive inhibitors of glutathione-dependent enzymes: glutathione transferase itself [9], glutathione reductase [10], glyoxalase I [11, 12] and glyoxalase II [13–15]. This was demonstrated from kinetic studies of the effect of *S*-conjugates of glutathione on the purified enzymes. It is not clear whether inhibition of glutathione-dependent enzymes is a mediating factor in the putative cytotoxicity of *S*-conjugates of glutathione.

Inhibitors of glyoxalase I have long been considered as prospective anti-proliferative, anti-tumour agents [16, 17]. Glyoxalase I is a component of the glyoxalase system which catalyses the formation of methylglyoxal to D-lactate via the intermediate, *S*-D-lactoylglutathione (Fig. 1). The glyoxalase system is present in the cytosol of all cells [18]. The glyoxalase pathway is a major metabolic

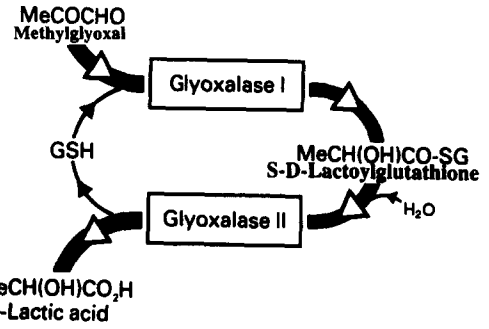

Fig. 1. The glyoxalase system.

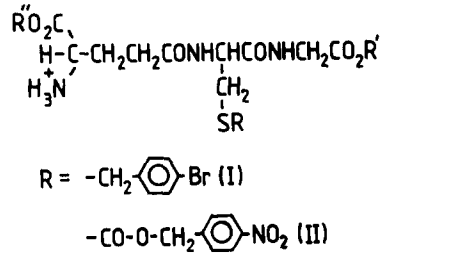

Fig. 2. Structures of compounds investigated. *S*-*p*-Bromobenzylglutathione, R = Br—⟨⟩—CH_2—; *S*-*p*-nitrobenzoxycarbonylglutathione, R = NO_2—⟨⟩—CH_2-O-CO—; monoethyl esters R′ = Et—, R″ = H; diethyl esters R′ and R″ = Et.

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route for the clearance of methylglyoxal and glyoxalase I is a highly efficient enzyme [18]. Methylglyoxal has anti-proliferative activity at high concentrations [19–21]. Inhibition of glyoxalase I would be expected to lead to the accumulation of methylglyoxal in cells and cytotoxicity. *S*-Conjugates of glutathione are competitive inhibitors of glyoxalase I [11, 12]. The most effective inhibitor of glyoxalase I studied to date is *S*-*p*-bromobenzylglutathione which has a K_i value of 0.27 μM for the human red blood cell enzyme [12].

It has been suggested recently that inhibitors of glyoxalase II may also be cytotoxic, anti-proliferative agents [22]. Glyoxalase II catalyses the hydrolysis of *S*-D-lactoylglutathione to D-lactate and reduced glutathione [18] (Fig. 1). It has been proposed that the intracellular accumulation of *S*-D-lactoylglutathione may be toxic [22]. The most effective inhibitor of glyoxalase II studied to date is *S*-*p*-nitrobenzoxycarbonylglutathione which has a K_i value of 6.5 μM with the bovine liver enzyme but may also inhibit glyoxalase I [13].

Despite the availability of potent inhibitors of glyoxalase enzymes, poor cytotoxic activity has been observed which was probably due to their inability to cross the plasma membrane of cells and reach the cytosolic glyoxalase I target [16]. Reduced glutathione itself also does not readily cross biological membranes [23]. Anderson *et al.* [24] reported that the monoethyl ester derivative of reduced glutathione, γ-L-glutamyl-L-cysteinylethylglycinate, entered into cells and was hydrolysed by a cytosolic esterase to reduced glutathione. Therefore, esterification of *S*-*p*-bromobenzylglutathione and *S*-*p*-nitrobenzoxycarbonylglutathione may give ester derivatives which can deliver the corresponding *S*-

Table 1. ^{1}H NMR spectroscopy of *S*-conjugates of glutathione and their ethyl esters

Compound	*S*-*p*-Bromobenzylglutathione			*S*-*p*-Nitrobenzoxycarbonylglutathione		
	Unesterified	Monoethyl	Diethyl	Unesterified	Monoethyl	Diethyl
Assignment						
Glycyl						
2-H (2H)	3.70	3.72	3.86	3.68	3.60	3.62
2-NH	8.62	8.80	—	8.64	9.11	8.48
($J_{2,N}$	5.56	5.56	—	5.56	5.16	5.95)
Cysteinyl						
2-H	4.48	4.50	4.47	4.50	4.49	4.55
2-NH	8.38	8.40	—	8.52	8.54	8.32
3A-H	2.76	2.74	2.82	3.37	3.33	3.32
3B-H	2.54	2.50	2.65	3.02	3.01	3.01
($J_{2,N}$	8.33	8.34	—	8.73	8.34	8.33)
($J_{2,3A}$	4.77	4.77	5.16	4.76	7.54	4.46)
($J_{2,3B}$	9.52	9.53	8.34	9.32	9.69	8.73)
($J_{3A,3B}$	−13.90	−13.50	−13.89	−13.89	−13.29	−13.89)
γ-Glutamyl						
2-H	3.33	3.55	3.95	3.31	3.75	3.79
3A-H	1.90	1.93	2.10	1.89	1.90	1.85
3B-H	1.90	1.93	2.10	1.89	1.77	1.65
4-H (2H)	2.31	2.33	2.46	2.30	2.30	1.83
($J_{2,3}$	6.39	6.35	6.90	6.74	4.37	4.96)
($J_{3A,3B}$	—	—	—	—	−12.80	−14.09)
($J_{3A,4}$	6.75	6.95	6.56	7.34	7.44	7.34)
($J_{3B,4}$	6.75	6.95	6.56	7.34	7.44	8.92)
Benzyl						
1-H (2-H)	3.70	3.79	3.70	5.39	5.40	5.39
o-H (2-H)	7.27	7.27	7.25	7.63	7.65	7.63
m-H (2-H)	7.48	7.49	7.46	8.23	8.24	8.23
($J_{o,m}$	8.34	8.34	8.33	8.73	8.73	8.74)
Other						
Ethyl (glycyl)						
1-H (2H)	—	4.05	4.11	—	4.06	4.06
2-H (3H)	—	1.15	1.18	—	1.17	1.16
($J_{1,2}$	—	7.15	7.14	—	7.15	7.14)
Ethyl (glutamyl)						
1-H (2H)	—	—	4.22	—	—	4.07
2-H (3H)	—	—	1.24	—	—	1.17
($J_{1,2}$	—	—	7.14	—	—	5.15)

NMR spectra were recorded at 270 MHz on a Joel EX 270 NMR spectrometer.

Spectra were recorded in d_6-dimethyl sulphoxide except the diethyl ester of *S*-*p*-bromobenzylglutathione which was recorded in 90% (v/v) CD_3CN in D_2O.

conjugate of glutathione into the cytosol of cells and to their putative enzyme targets.

The effects of *S-p*-bromobenzylglutathione, *S-p*-nitrobenzoxycarbonylglutathione, and monoethyl and diethyl ester derivatives (Fig. 2) on the growth and viability of human leukaemia 60 (HL60) cells in culture, and on the viability of mature human neutrophils in culture were investigated. The diethyl ester derivatives of *S-p*-bromobenzylglutathione and *S-p*-nitrobenzoxycarbonylglutathione were found to induce growth arrest and toxicity in HL60 cells, and toxicity in mature human neutrophils, although less effectively in the latter. The monoethyl ester and unesterified derivatives were inactive.

MATERIALS AND METHODS

Reduced glutathione, Dowex 1, Trypan blue, the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate and dimethyl sulphoxide were purchased from the Sigma Chemical Co. (Poole, U.K.). *p*-Bromobenzylbromide, *p*-nitrobenzylchloroformate, hydrogen chloride gas and d_6-dimethyl sulphoxide were purchased from the Aldrich Chemical Co. (Poole, U.K.). Tissue culture medium RPMI 1640 and foetal calf serum were purchased from Gibco Europe (Paisley, U.K.). Methanol (HPLC grade) was purchased from Rathburn Chemicals (Walkerburn, U.K.). Silica gel 60 F_{234} TLC plates were purchased from BDH Chemicals (Poole, U.K.).

S-p-Bromobenzylglutathione and S-p-nitrobenzoxycarbonylglutathione esters. *S-p*-Bromobenzylglutathione was synthesized by the method of Vince *et al.* [11] and *S-p*-nitrobenzoxycarbonylglutathione was synthesized by the method of Bush and Norton [13]. Mono- and diethyl esters (Fig. 2) of the *S*-conjugates of glutathione were synthesized by acid-catalysed esterification in ethanol [25] and purified by chromatography on Dowex 1, formate form with methanol as eluent [26]. They were characterized by ^1H and ^{13}C NMR, fast atom bombardment (FAB) mass spectrometry, melting point and TLC analysis, summarized in Tables 1–3 (cf. NMR data for reduced glutathione esters [25, 27]).

Cell culture conditions. Human promyelocytic leukaemia HL60 cells were incubated at 37° in RPMI 1640 media containing 10% foetal calf serum under an atmosphere of 5% CO_2 in air, 100% humidity [28]. Cells were seeded at an initial density of 5×10^4/mL and incubated with 1–500 μM glutathione derivative. Stock solutions of the compounds were

Table 2. ^{13}C NMR spectroscopy of *S*-conjugates of glutathione and their ethyl esters

Compound	*S-p*-Bromobenzylglutathione			*S-p*-Nitrobenzoxycarbonylglutathione		
	Unesterified	Monoethyl	Diethyl	Unesterified	Monoethyl	Diethyl
Assignment						
Glycyl						
C-1	171.9	169.5	170.3	171.0	169.4	170.2
C-2	41.1	40.8	41.9	41.3	40.9	40.9
Cysteinyl						
C-1	170.2	170.9	171.0	169.9	169.7	169.8
C-2	53.1	52.3	53.5	53.2	53.3	53.4
C-3	34.3	34.3	33.8	26.8	31.6	29.6
γ-Glutamyl						
C-1	170.6	171.4	172.1	170.5	170.4	172.1
C-2	52.0	51.9	53.4	52.0	51.5	51.5
C-3	26.8	26.5	26.6	31.5	31.6	31.6
C-4	32.9	33.0	32.1	32.8	32.8	32.8
C-5	170.9	171.5	174.0	172.1	172.0	174.7
Benzyl						
C-1	52.0	52.3	53.4	67.5	67.4	67.4
C-1 (Ph)	119.8	119.9	121.3	143.0	143.0	143.0
C-2,6 (Ph)	131.1	131.2	132.0	123.8	123.7	123.7
C-3,5 (Ph)	131.2	131.3	132.5	129.0	128.9	128.8
C-6	138.0	138.0	138.8	147.4	147.3	147.3
Other						
S-Carbonyl	—	—	—	169.9	169.7	169.5
Ethyl (Glycyl)						
C-1	—	60.5	62.3	—	60.4	60.3
C-2	—	14.1	14.3	—	14.1	14.1
Ethyl (Glutamyl)						
C-1	—	—	63.6	—	—	60.5
C-2	—	—	14.4	—	—	14.1

Spectra were recorded in d_6-dimethyl sulphoxide except the diethyl ester of *S-p*-bromobenzylglutathione which was recorded in 90% (v/v) CD_3CN in D_2O.

Table 3. Analytical and yield data for *S-p*-bromobenzylglutathione and *S-p*-nitrobenzoxycarbonylglutathione ethyl esters

Compound	FAB mass spectrometry M + 1 (*m/z*)*	TLC R_f value	Melting point§ (°C)	Yield‖ (%)
S-p-Bromobenzylglutathione				
Monoethyl ester	504 & 506	0.69†	176–178	45
Diethyl ester	532 & 534	0.83†	107–109	35
S-p-Nitrobenzoxycarbonylglutathione				
Monoethyl ester	515	0.71‡	134–138	34
Diethyl ester	543	0.81‡	108–111	20

* FAB mass spectra were recorded on a Kratos MS50 FAB mass spectrometer with samples dissolved in a glycerol matrix.
TLC on silica gel with mobile phase 1-propanol:acetic acid:water, †10:5:1, ‡10:5:5.
§ Melting points at decomposition.
‖ Monoethyl and diethyl esters were isolated from the same preparation.

prepared in dimethyl sulphoxide and diluted into the growth medium such that the final concentration of dimethyl sulphoxide did not exceed 5 mM, a concentration which is reported [29] to not induce differentiation or toxicity in HL60 cells and did not do so in control incubations studied here. Cell viability was judged by the ability of cells to exclude Trypan blue [30]. The differentiation of HL60 cells to neutrophil-like cells was estimated from the development of maturation-dependent activity of the superoxide-forming enzyme NADPH oxidase, by staining for the superoxide-mediated reduction of nitroblue tetrazolium to formazan, in the presence of phorbol ester [31].

Isolation and culture of human neutrophils. Mature human neutrophils were isolated from venous blood and separated from other leukocytes on a discontinuous Percoll density gradient [32]. The purity of the preparation was >95%, the predominant contaminating leukocytes being eosinophils, and viability was >99%. The isolated neutrophils were washed twice in phosphate-buffered saline and incubated for 20 min at a cell density of 2×10^6 cells/mL in cell culture medium (RPMI 1640 containing 10% foetal calf serum) prior to the addition of an equal volume of glutathione *S*-conjugate derivative dissolved in culture medium (the compound was initially prepared as a stock solution in dimethyl sulphoxide such that the final concentration of dimethyl sulphoxide in the culture was <3.5 mM). Neutrophils (1×10^6/mL) were incubated with and without the glutathione-*S*-conjugate for 24 hr. Cell viability was assessed by Trypan blue exclusion.

Data analysis. Median growth inhibitory concentration IC_{50} values were calculated by linear regression of response–log ([glutathione S-conjugate]) data pairs in the 50% growth inhibition region and interpolation using the regression equation.

RESULTS

Growth inhibition and toxicity of diethyl esters of S-p-*bromobenzylglutathione and* S-p-*nitrobenzoxycarbonylglutathione to HL-60 cells in culture*

HL60 cells were incubated with *S-p*-bromobenzylglutathione, *S-p*-nitrobenzoxycarbonylglutathione and the monoethyl and diethyl ester derivatives (1–500 μM) for 4 days. No significant inhibition of growth or toxicity was found on exposure to *S-p*-bromobenzylglutathione, *S-p*-nitrobenzoxycarbonylglutathione and their monoethyl ester derivatives at concentrations ≤ 500 μM. However, the diethyl ester derivatives of *S-p*-bromobenzylglutathione and *S-p*-nitrobenzoxycarbonylglutathione produced a decrease in viable cell number with median effective concentrations IC_{50} values of 8.3 (95% C.I. 7.0–9.9; N = 12) and 56 (95% C.I. 36–86; N = 9) μM, respectively (Fig. 3a and b).

S-p-Bromobenzylglutathione diethyl ester and *S-p*-nitrobenzoxycarbonylglutathione diethyl ester were toxic to HL60 cells, as judged by the Trypan blue exclusion test (Fig. 4a and b). The growth curves of HL60 cells treated with the diesters show an inhibition of proliferation in the first day of culture (Fig. 5a and b). Thereafter, the surviving residual cells proliferate at a rate similar to control, untreated cells. This suggests that *S-p*-bromobenzylglutathione diethyl ester and *S-p*-nitrobenzoxycarbonylglutathione diethyl ester induce growth arrest and are toxic to HL60 cells, with the major response occurring within the initial 24 hr of culture.

Toxicity of S-p-*bromobenzylglutathione diethyl ester and* S-p-*nitrobenzoxycarbonylglutathione diethyl ester to human neutrophils in culture*

Human neutrophils were incubated with *S-p*-bromobenzylglutathione diethyl ester and *S-p*-nitrobenzoxycarbonylglutathione diethyl ester in RPMI with 10% foetal calf serum for 24 hr. These compounds were toxic, as judged by the Trypan blue exclusion tests, with median toxic concentration IC_{50} values of 39.7 (95% C.I. 35.4–44.5 μM; N = 16) and 127 (95% C.I. 123–132 μM; N = 8) μM, respectively.

DISCUSSION

Glutathione *S*-conjugates have been studied for

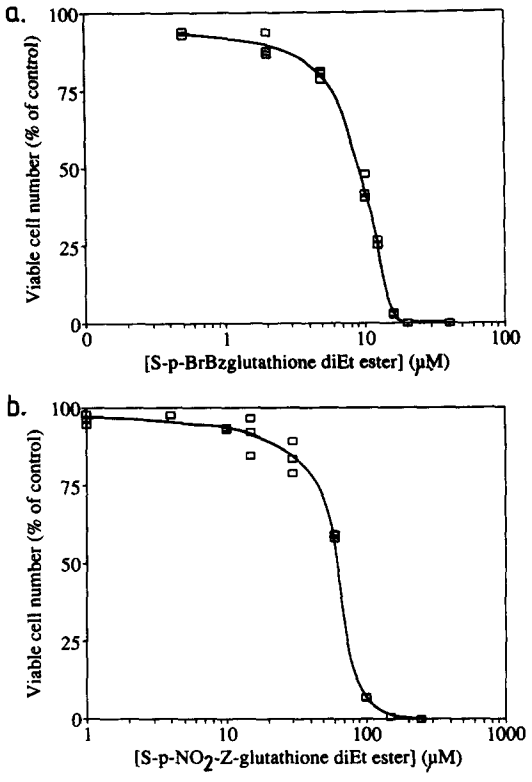

Fig. 3. Effect of *S-p*-bromobenzylglutathione diethyl ester and *S-p*-nitrobenzoxycarbonylglutathione diethyl ester on HL60 viable cell number. HL60 cells were cultured in the absence and presence of the title compounds at the concentration indicated under the conditions described in Materials and Methods, and the effect on HL60 cell growth evaluated as the viable cell number: the percentage of viable cells in control incubations after 3 days of culture. (a) *S-p*-Bromobenzylglutathione diethyl ester, (b) *S-p*-nitrobenzoxycarbonylglutathione diethyl ester.

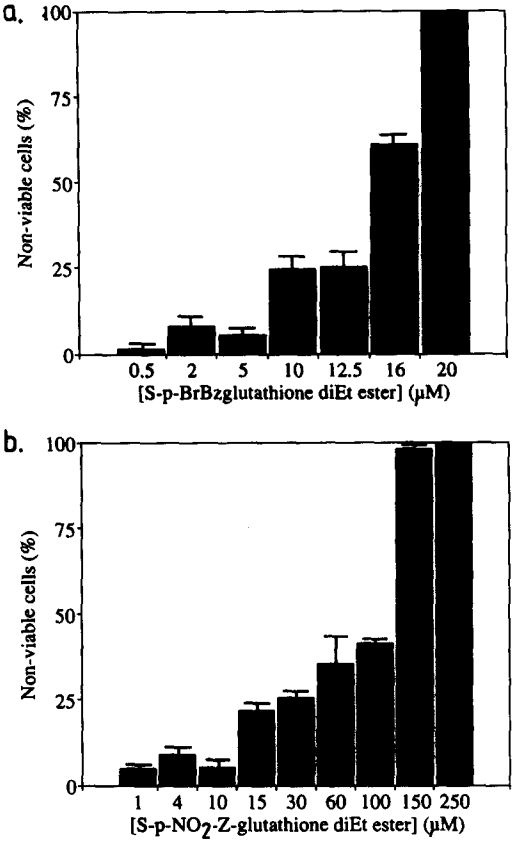

Fig. 4. Effect of *S-p*-bromobenzylglutathione diethyl ester and *S-p*-nitrobenzoxycarbonylglutathione diethyl ester on HL60 cell viability. HL60 cells were cultured in the absence and presence of the title compounds at the concentration indicated under the conditions described in Materials and Methods, and HL60 cell viability was evaluated as the percentage of cells *not excluding* Trypan blue after 3 days of culture. (a) *S-p*-Bromobenzylglutathione diethyl ester, (b) *S-p*-nitrobenzoxycarbonylglutathione diethyl ester. Data are means ± SD of six determinations.

many years as intermediates in the metabolism of aromatic compounds and substrate analogue, competitive inhibitors of glutathione-dependent enzymes [1–15]. The inhibition of enzymes thought to be involved in the control of cell proliferation (glyoxalase I and glyoxalase II) has suggested that glutathione *S*-conjugates may have anti-proliferative activity [13, 16, 17]. The inhibition of enzymes involved in the detoxification of xenobiotic compounds (glutathione transferase) and cellular resistance to oxidative damage (glutathione reductase, glutathione peroxidase), has suggested that glutathione *S*-conjugates may be key intermediates in the mediation of toxicity of xenobiotic agents where persistence and oxidative stress are sequelae of the toxicity [1–10]. It was expected, therefore, that further investigation of the cellular effects of glutathione *S*-conjugates would lead to the development of novel anti-proliferative anti-tumour agents and further understanding of the development of cytotoxicity induced by the metabolism of xenobiotic compounds. However, this expectation has generally not been realised in practise, which is probably due to the failure to deliver glutathione *S*-conjugates into cells where their expected pharmacological effects and receptors are located.

The toxicity of *S-p*-bromobenzylglutathione to murine lymphocytic leukaemia L1210 cells *in vitro* has been studied but no detectable toxicity was found [16]. We have found similar low toxicity of *S-p*-bromobenzylglutathione and *S-p*-nitrobenzoxycarbonylglutathione to HL60 cells in culture. Reduced glutathione and *S*-conjugates of glutathione do not readily cross the plasma membrane from the extracellular medium and enter the cell cytosol [23], and indeed, may be metabolized by γ-glutamyltransferase to *S*-conjugates of cysteinylglycine [33], *en route* to secretion as mercapturic acid derivatives [5]; this may account for the lack of toxicity of the unesterified compounds studied herein. Monoethyl ester and diethyl ester derivatives of glutathione were found to be highly resistant to cleavage by γ-glutamyltransferase [24].

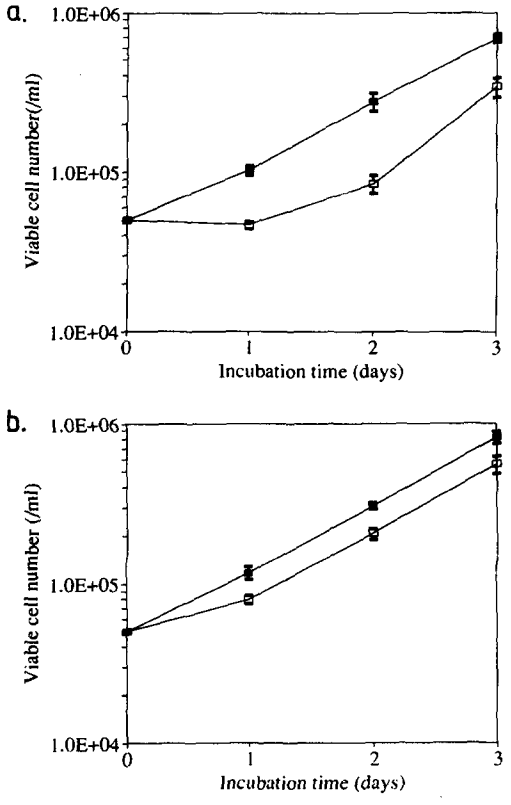

Fig. 5. Effect of *S-p*-bromobenzylglutathione diethyl ester and *S-p*-nitrobenzoxycarbonylglutathione diethyl ester on HL60 cell growth. HL60 cells were cultured in the absence and presence of the title compounds at the concentration indicated under the conditions described in Materials and Methods, and HL60 cell viability was evaluated as the cell concentration *not excluding* Trypan blue after the periods of culture indicated. (a) *S-p*-Bromobenzylglutathione diethyl ester, (b) *S-p*-nitrobenzoxycarbonylglutathione diethyl ester. Data are means ± SD of six determinations.

The monoethyl ester derivative of reduced glutathione may be used to supplement cellular levels of reduced glutathione, apparently by entry of the monoester into the cell cytosol and de-esterification by a non-specific esterase [24]. However, all components in solution of monoester derivatives of reduced glutathione and glutathione-*S*-conjugates are charged at physiological pH and, therefore, whilst monoesterification may deliver reduced glutathione into cells (possibly by an amino acid uptake channel or similar), it may not be generally applicable for the delivery of *S*-conjugates of glutathione into cells.

The diethyl ester derivative of reduced glutathione is expected to possess only one charged group, the γ-L-glutamyl ammonium group, with a pK_a of *ca.* 9.2. Therefore, a component of the diester derivative will be uncharged and may partition into the plasma membrane and out into the cytosol. Diethyl ester derivatives of *S-p*-bromobenzylglutathione and *S-p*-nitrobenzoxycarbonylglutathione were found to be toxic to HL60 cells in culture. The diethyl ester derivatives were also toxic to human neutrophils in culture. The ratio IC_{50} (neutrophils)/IC_{50} (HL60 cells) was *ca.* 4.8 for *S-p*-bromobenzylglutathione diethyl ester and *ca.* 2.3 for *p*-nitrobenzoxycarbonylglutathione diethyl ester. For these compounds, there appears to be limited selectivity of toxicity to proliferating tumour cells relative to corresponding mature, differentiated cells of similar lineage. The mechanisms of the growth inhibition and cytotoxicity of *S-p*-bromobenzylglutathione diethyl ester and *S-p*-nitrobenzoxycarbonylglutathione diethyl ester are not known. A probable mechanism of action is penetration of the diethyl ester into the cell cytosol, de-esterification by non-specific esterase, and inhibition of glutathione-dependent enzymes by the unesterified analogues. Particularly important enzymes in this respect are glyoxalase I and glyoxalase II.

The inhibition of human red blood cell glyoxalase I and glyoxalase II, purified to homogeneity, by *S-p*-bromobenzylglutathione, *S-p*-nitrobenzoxycarbonylglutathione and monoethyl and diethyl ester derivatives has been studied. *S-p*-Bromobenzylglutathione was found to be a competitive inhibitor of glyoxalase I with a K_i value (mean ± SD) of 0.16 ± 0.04 μM but was a very poor inhibitor of glyoxalase II (500 μM compound gave only very poor inhibition). *S-p*-Nitrobenzoxycarbonylglutathione was a competitive inhibitor of both glyoxalase I, with a K_i value of 3.12 ± 0.88 μM, and a competitive inhibitor of glyoxalase II with a K_i value of 1.10 ± 0.07 μM. Generally, the monoethyl and diethyl esters are poor and ineffective inhibitors of glyoxalases. Further details of the inhibition of human glyoxalases by these compounds and the purification of human glyoxalases to homogeneity are to be published (Allen RE, Lo TWC and Thornalley PJ, submitted for publication). Preliminary evidence from HPLC analysis of HL60 cell extracts suggests that the diethyl ester derivatives enter cells and are therein de-esterified to the corresponding monoester and unesterified analogues. The relative potency of *S-p*-bromobenzylglutathione diethyl ester and *S-p*-nitrobenzoxycarbonylglutathione diethyl ester suggests that a prospective mechanism of action may be inhibition of glyoxalase I, rather than inhibition of glyoxalase II, by the unesterified analogue formed intracellularly. Further investigation of the mechanism of action of these compounds is in progress.

S-p-Bromobenzylglutathione diethyl ester and *S-p*-nitrobenzoxycarbonylglutathione diethyl ester represent novel cytotoxic agents which may have importance in anti-tumour drug development and evaluation of the pharmacological and toxicological effects of glutathione *S*-conjugates. Meister and co-workers [23, 24] suggested and experimentally demonstrated that monoethyl esterification of reduced glutathione potentiated its pharmacological effects expressed intracellularly by delivery of reduced glutathione into cells. The diethyl ester derivative of reduced glutathione has also been prepared and was found to be more effective than the monoester in increasing the cytosolic concentration of reduced glutathione [34]. Our investigation suggests that diethyl esterification

(and *not* monoethyl esterification) potentiates the pharmacological activity of *S*-*p*-bromobenzylglutathione and *S*-*p*-nitrobenzoxycarbonylglutathione. Diethyl esterification may be a generally applicable modification to potentiate the pharmacological effects of glutathione *S*-conjugates.

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