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INHIBITION OF GROWTH OF HUMAN LEUKAEMIA 60 CELLS BY *S*-2-HYDROXYACYLGLUTATHIONES AND MONOETHYL ESTER DERIVATIVES

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Abstract—*S*-2-Hydroxyacylglutathione derivatives were found to induce growth arrest and toxicity in human leukaemia 60 cells in culture. *S*-D-Lactoylglutathione was the most effective with a median inhibitory concentration IC_{50} of 82 μM (95% C.I. 65–105 μM). No similar toxicity was induced by reduced glutathione and/or the corresponding aldonic acid (500 μM) in human leukaemia 60 cells, nor by *S*-D-lactoylglutathione (500 μM) in mature human neutrophils under the same culture conditions. Monoethyl ester derivatives of the *S*-2-hydroxyacylglutathiones were prepared and also induced growth arrest and toxicity but were less effective than the corresponding unesterified compounds. *S*-2-Hydroxyacylglutathione derivatives also inhibited the incorporation of [^{3}H]thymidine into DNA early in the development of toxicity: for *S*-D-lactoylglutathione, the median inhibitory concentration was 74 μM (95% C.I. 47–116 μM). The mechanism of the inhibition of human leukaemia cell growth by *S*-D-lactoylglutathione and other *S*-2-hydroxyacylglutathione derivatives is unknown but appears to be mediated by the inhibition of DNA synthesis.

S-D-Lactoylglutathione is the physiological intermediate of the glyoxalase system which catalyses the conversion of methylglyoxal to D-lactate [1]. The glyoxalase system comprises two enzymes, glyoxalase I and glyoxalase II, and a catalytic amount of reduced glutathione. Glyoxalase I (EC 4.4.1.5) catalyses the formation of *S*-D-lactoylglutathione from the hemithioacetal formed non-enzymatically from methylglyoxal and reduced glutathione:

$$MeCOCHO + GSH \rightleftharpoons MeCOCH(OH) - SG \xrightarrow{\text{GLYOXALASE I}} MeCH(OH)CO - SG.$$

Glyoxalase II (EC 3.1.2.6) catalyses the hydrolysis of *S*-D-lactoylglutathione to D-lactate, reforming the reduced glutathione consumed in the glyoxalase I-catalysed reaction step:

$$MeCH(OH)CO - SG + H_2O \xrightarrow{\text{GLYOXALASE II}} MeCH(OH)CO_2H + GSH.$$

The glyoxalase system is present in the cytosol of all cells and is not normally found in extracellular fluids. *S*-D-Lactoylglutathione does not readily cross biological membranes and hence its locus of biological activity is normally restricted to the cytosol and cytoplasmic face of cell membranes [1].

Exogenous *S*-D-lactoylglutathione added to human leukaemia 60 (HL60) cells in culture induced growth arrest and toxicity [2]. The toxicity was characterized by an increase in the percentage of cells in the G_0-G_1 phase of the cell cycle and a decrease in the percentage of cells in the S and G_2-M phases. The mechanism of the inhibition of HL60 cell proliferation by *S*-D-lactoylglutathione is unknown.

S-D-Lactoylglutathione (and other *S*-2-hydroxyacylglutathione derivatives) may be cleaved by γ-glutamyltransferase to *S*-D-lactoylcysteinylglycine which rearranges to *N*-lactoylcysteinylglycine (or generally *N*-2-hydroxyacylcysteinylglycine) [3]. γ-Glutamyltransferase occurs bound onto the external surface of cell plasma membranes and free in plasma [4]. Since γ-glutamyltransferase activity was found on HL60 cells and in foetal calf serum [2], we suggested that *S*-D-lactoylglutathione may be metabolized in the culture medium by this route [2]. We therefore sought a vehicle for delivery of *S*-D-lactoylglutathione into cells and a modification to confer resistance to cleavage by γ-glutamyltransferase to stabilize *S*-D-lactoylglutathione in the extracellular medium.

Anderson *et al.* [5, 6] have demonstrated that the monoethyl ester derivative of reduced glutathione, γ-glutamylcysteinylethylglycinate, crosses the plasma membrane, entering the cytosol. It is therein de-esterified, effectively delivering reduced glutathione into the cell cytosol. The monoester derivative was also resistant to cleavage by γ-glutamyltransferase.

In this report, we describe further studies on the anti-leukaemic activity of *S*-D-lactoylglutathione. *S*-2-Hydroxyacylglutathione analogues of *S*-D-lactoylglutathione (Fig. 1) were found to have similar anti-leukaemic activity but were less effective than *S*-D-lactoylglutathione. These compounds also inhibited DNA synthesis in HL60 cells. Monoethyl ester derivatives (ethyl glycinate esters) were prepared and they were also toxic but with IC_{50} values significantly higher than the corresponding unesterified analogue. No similar toxicity was induced in corresponding differentiated cells, mature human neutrophils, under similar culture conditions.

MATERIALS AND METHODS

Materials. *S*-Glycolylglutathione, *S*-D-lactoyl-

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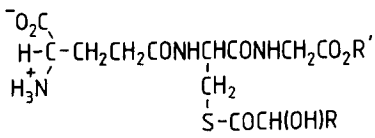

Fig. 1. Structure of *S*-2-hydroxyacylglutathiones and their monoester derivatives. *S*-Glycolylglutathione, R = H; *S*-D-lactoylglutathione, R = —CH_3; *S*-L-glyceroylglutathione, R = —CH_2OH; *S*-D-mandelylglutathione, R = —Ph. Monoethyl ester derivatives, R′ = Et.

glutathione, *S*-L-glyceroylglutathione and *S*-D-mandelylglutathione were prepared and purified as described [7]. Trypan blue, 12-*O*-tetradecanoylphorbol-13-acetate, nitroblue tetrazolium, reduced glutathione and D-lactic acid were purchased from the Sigma Chemical Co. (Poole, U.K.). Tissue culture medium RPMI 1640 and foetal calf serum were purchased from Gibco Europe (Paisley, U.K.). HPLC cartridges (Nova-Pak octadecylsilica ODS, 4 μm, 0.8 × 10 cm) and pre-columns of the same material (0.8 × 1.0 cm) were purchased from Waters-Millipore U.K. (Watford, U.K.). γ-Glutamyltransferase (bovine kidney, 8.2 U/mg, where 1 U catalyses the formation of 1 μmol of *p*-nitroaniline from L-γ-glutamyl-*p*-nitroanilide per min at pH 8.5 and 25°) was purchased from Sigma.

Monoethyl esters of *S*-2-hydroxyacylglutathiones were prepared and purified by methods similar to those recently described for reduced glutathione monoethyl ester [8]. The monoethyl esters were synthesized by acid-catalysed esterification in aqueous ethanol (5 mL of 80% ethanol containing 3 M HCl and 100 mg of *S*-2-hydroxyacylglutathione, stirred for 4 hr at 25°). The product was purified by preparative reverse phase HPLC [8] using an ODS (6 nm pore size; 6 μm particle size) cartridge (25 mm × 10 cm) with a guard cartridge (25 mm × 1 cm) in a 25 × 10 radial compression module (Waters-Millipore). The mobile phase was 13.8 mM formic acid, pH 2.83, with isocratic elution at a flow rate of 10 mL/min (*S*-D-lactoylglutathione, *S*-glycolylglutathione and *S*-L-glyceroylglutathione) or a linear gradient of 0–80% methanol over 20 min (*S*-D-mandelylglutathione). The sample loading was 25 mg and the eluate was monitored at 233 nm. Fractions containing the monoethyl ester products were eluted in the retention time intervals 13–26 min (*S*-D-lactoylglutathione), 6–12 min (*S*-glycolylglutathione), 9–15 min (*S*-L-glyceroylglutathione) and 13–15 min (*S*-D-mandelylglutathione).

S-2-Hydroxyacylglutathione derivatives were characterized by ^1H and ^{13}C NMR spectroscopy, and purity analysed by TLC and analytical reverse phase HPLC, summarized in Tables 1–3.

Reaction of S-2-*hydroxyacylglutathione derivatives with γ-glutamyltransferase.* The rate of the γ-glutamyltransferase-catalysed conversion of *S*-2-hydroxyacylglutathione to the corresponding *N*-2-hydroxyacylcysteinylglycine was determined by measuring the rate of formation of thiol groups, detected by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [3]. *S*-2-Hydroxyacylglutathione derivative (10–500 μM) was incubated in 100 mM Tris–phosphate buffer, pH 7.4 and 37°, containing 1 mM DTNB and 20 mM glycylglycine. γ-Glutamyltransferase (0.5 U/mL) was added and the absorbance monitored at 412 nm. The initial rate of formation of *N*-acylcysteinylglycine was deduced, assuming a 1 : 1 stoichiometric reaction with DTNB, forming 2-nitro-5-mercaptobenzoic acid with an extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ [9].

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 [2]. Cells were seeded at an initial density of 4.5×10^4/mL and incubated with 1–500 μM *S*-2-hydroxyacylglutathione derivative for up to 4 days.

For DNA synthesis measurements, HL60 cells (5.0×10^4/mL) were incubated with the concentration of *S*-2-hydroxyacylglutathione in RPMI 1640 with 10% fetal calf serum for 2 hr. [^3H]-Thymidine (Amersham International, Amersham, U.K.; 2.5 μCi) was added and the incubation continued for 1 hr. The DNA was then extracted [10] and counted.

Isolation and culture of human neutrophils. Neutrophils were isolated from venous blood from healthy human donors and separated from other contaminating leukocytes on a discontinuous Percoll density gradient as described [11]. The purity of the preparation was >95%, the predominant contaminating leukocytes being eosinophils, and viability was >99%. Cells were washed twice in phosphate-buffered saline and incubated for 20 min at a cell density of 2×10^6/mL in cell culture medium (RPMI 1640 containing 10% foetal calf serum) prior to the addition of an equal volume of freshly prepared *S*-D-lactoylglutathione solution (1 mM) in culture medium. Cell viability was assessed by Trypan blue exclusion. Experiments were performed in quadruplicate (4 hr incubations) or quintuplicate (24 hr incubations).

Data analysis. Kinetic data from studies of the reaction of *S*-2-hydroxyacylglutathione derivatives with γ-glutamyltransferase were analysed by non-linear regression to the Michaelis–Menten equation using the ENZFITTER programme (Biosoft, Cambridge, U.K.). Median growth inhibitory concentration IC_{50} values were calculated by linear regression of data around the 50% biological effect region of the log[*S*-2-hydroxyacylglutathione]–biological response curve and substitution of 50% response in the regression equation. The IC_{50} values and 95% confidence intervals are given. The significance of difference between the IC_{50} values for *S*-D-lactoylglutathione and all other *S*-2-hydroxyacylglutathiones studied, and between *S*-2-hydroxyacylglutathiones and the corresponding *S*-2-hydroxyacylglutathione monoethyl ester, are given. The slopes of the logit [$=\ln(I/(100 - I))$, where I = percentage decrease in viable cell number] versus ln([*S*-2-hydroxyacyl-glutathione]/μM) plot are also reported.

RESULTS

Growth inhibition of human leukaemia 60 *cells by* S-2-*hydroxyacylglutathione derivatives*

It was previously reported that *S*-D-lactoyl-

Table 1. ^{1}H NMR spectroscopy of monoethyl esters of *S*-2-hydroxyacylglutathiones

Assignment	*S*-Glycolylglutathione	*S*-D-Lactoylglutathione	*S*-L-Glyceroylglutathione	*S*-D-Mandelylglutathione
Glycyl				
2A-H	3.83	3.73	3.86	3.67
2B-H	3.83	3.73	3.86	3.57
($J_{2A.2B}$	—	—	—	−17.47)
Cysteinyl				
2-H	4.38	4.36	4.49	4.32
3A-H	3.31	3.17	3.31	3.23
3B-H	3.07	2.92	3.10	2.97
($J_{2.3A}$	5.16	5.16	4.76	5.56)
($J_{2.3B}$	7.94	8.14	7.74	7.95)
($J_{3A.3B}$	−14.29	−14.29	−13.89	−14.09)
γ-Glutamyl				
2-H	3.61	3.40	3.62	3.50
3-H (2H)	1.97	1.85	1.99	1.85
4-H (2H)	2.34	2.22	2.34	2.16
($J_{2,3}$	6.35	6.35	5.96	6.35)
($J_{3,4}$	10.32	7.74	7.54	7.74)
Ethyl				
1-H (2H)	4.04	3.94	4.07	3.97
2-H (3H)	1.09	0.99	1.12	1.03
($J_{1,2}$	7.15	7.14	7.14	7.14)
Other				
2-H (2H)	4.15	2-H 4.14 3-H (3H) 1.10 $J_{2,3}$	2-H 4.27 3-H (2H) 3.69 $J_{2,3}$	2-H 5.16 Ph 7.24

NMR spectra of *S*-2-hydroxyacylglutathione monoethyl esters (10 mg/mL) were recorded in 100 mM NaD_2PO_4/Na_2DPO_4, pD 7.0, in D_2O.

Table 2. ^{13}C NMR spectroscopy of monoethyl esters of *S*-2-hydroxyacylglutathiones

Assignment	*S*-Glycolylglutathione	*S*-D-Lactoylglutathione	*S*-L-Glyceroylglutathione	*S*-D-Mandelylglutathione
Glycyl				
C-1	173.6	173.9	173.5	173.9
C-2	40.9	40.6	40.9	40.7
Cysteinyl				
C-1	170.7	170.5	170.8	170.6
C-2	53.5	53.2	53.5	53.4
C-3	25.6	25.2	25.5	25.5
γ-Glutamyl				
C-1	171.8	171.4	171.8	171.5
C-2	52.3	51.8	52.2	52.0
C-3	28.2	28.2	28.6	28.5
C-4	30.6	30.4	30.6	30.6
C-5	173.5	173.1	174.2	173.4
Ethyl				
C-1	62.0	61.2	62.0	61.9
C-2	12.7	12.4	12.7	12.7
Other	C-1 204.0 C-2 66.7	C-1 206.7 C-2 72.7 C-3 18.9	C-1 204.4 C-2 77.3 C-3 62.7	C-1 204.2 C-2 78.6 C-1(Ph) 137.0 C-2,6(Ph) 128.4 C-3,5(Ph) 126.4 C-4(Ph) 128.5

NMR spectra of *S*-2-hydroxyacylglutathiones (10 mg/mL) were recorded in 100 mM NaD_2PO_4/Na_2DPO_4, pD 7.0, in D_2O.

Table 3. Chromatographic analysis of *S*-2-hydroxyacylglutathione derivatives and their monoethyl esters

	TLC R_f value		HPLC retention time (min)	
	Unesterified	Monoethyl ester	Unesterified	Monoethyl ester
S-D-Lactoylglutathione	0.39	0.56	7.0	12.6
S-Glycolylglutathione	0.36	0.53	4.9	10.2
S-L-Glyceroylglutathione	0.33	0.48	4.3	9.5
S-D-Mandelylglutathione	0.54	0.65	13.8	19.4

TLC analysis of *S*-2-hydroxyacylglutathione derivatives and their monoester products was performed on silica gel plates with a mobile phase of *n*-butanol/acetic acid/water, 10:5:5, with detection by ninhydrin in ethanol (0.2%). Data shown are the means of four determinations with a standard deviation of <0.01.

S-2-Hydroxyacylglutathione derivatives and their monoesters were analysed by analytical reverse phase HPLC on an ODS (6 nm pore size; 4 μm particle size) cartridge (8 mm × 10 cm) in an 8 × 10 radial compression module (Waters-Millipore). The mobile phase was 10 mM H_3PO_4/NaH_2PO_4, pH 2.83, with a linear gradient of 0–50% methanol over 20 min at a flow rate of 2 mL/min. The eluate was monitored at 233 nm. The sample was *S*-2-hydroxyacylglutathione derivative (1 mg/mL, 10 μL injection).

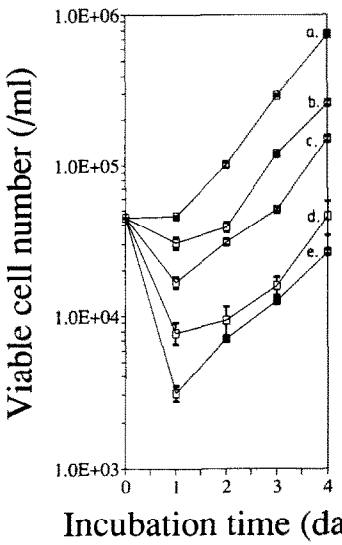

Fig. 2. Effect of *S*-2-hydroxyacylglutathione derivatives on human leukaemia 60 cell growth. Cells were seeded at an initial density of 4.5×10^4/mL and incubated with *S*-acylglutathione derivative (500 μM). Key: (a) control, (b) + *S*-D-mandelylglutathione, (c) + *S*-glycolylglutathione, (d) + *S*-L-glyceroylglutathione and (e) + *S*-D-lactoylglutathione. Data are the means ± SEM of a minimum of eight experiments.

glutathione induced growth arrest and toxicity to human leukaemia cells in culture [2]. The growth curves of HL60 cells in the absence and presence of *S*-D-lactoylglutathione and other *S*-2-hydroxyacylglutathiones, *S*-L-glyceroylglutathione, *S*-D-mandelylglutathione and *S*-glycolylglutathione, are presented in Fig. 2. *S*-2-Hydroxyacylglutathiones produced a decrease in viable cell number, relative to the control, throughout the incubation. The viable cell number after 24 hr was lower than the initial seeding density (*S*-D-lactoylglutathione −93%, *S*-L-glyceroylglutathione −83%, *S*-glycolylglutathione −63% and *S*-D-mandelylglutathione −32%), confirming that the *S*-2-hydroxyacylglutathiones were toxic to HL60 cells, although only 2.5% of cells stained positive with Trypan blue at this time (data not shown). However, cell debris was visible, as reported earlier for *S*-D-lactoylglutathione-treated HL60 cells [2]. After 24 hr, the residual surviving cells attained rapid growth kinetics similar to control, untreated cells, between days 2–4 of culture. This suggests, therefore, that the surviving cells had retained their clonogenicity.

All the *S*-2-hydroxyacylglutathiones studied decreased the viable cell number when added to cultures of HL60 cells. *S*-D-Lactoylglutathione was the most potent: the median effective dose for decrease in viable cell number for 4 day cultures was 82 μM (Fig. 3). No differentiation of HL60 cells was induced by *S*-2-hydroxyacylglutathiones at the concentrations studied (≤500 μM). The median inhibitory concentration IC_{50} values for *S*-2-hydroxyacylglutathiones are presented in Table 4. *S*-D-Lactoylglutathione had the lowest IC_{50} value of the *S*-2-hydroxyacylglutathione derivatives studied. There was no significant growth arrest and/or toxicity induced in HL60 cells by reduced glutathione (≤500 μM); corresponding aldonic acids, D-lactate, L-glycerate, D-mandelate and glycolate (≤500 μM); or reduced glutathione and aldonic acid (≤500 μM each) (data not shown).

Logit plots for the dose–response relationship for *S*-2-hydroxyacylglutathione derivatives and their monoethyl esters produced a linear transformation of the concentration–response data where the slopes were in the range 2.33–3.68 (Table 4).

Effect of S-D-*lactoylglutathione on the viability of human neutrophils in culture*

Mature human neutrophils incubated in culture medium with and without 500 μM *S*-D-lactoylglutathione for 4 hr gave cell viability values of >99%, and incubated for 24 hr gave cell viability

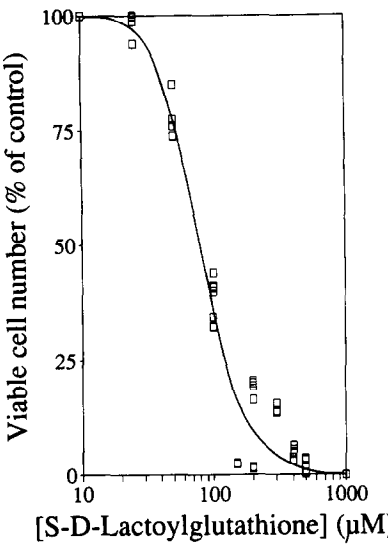

Fig. 3. Effect of S-D-lactoylglutathione on human leukaemia 60 cell growth. Cells were seeded at an initial density of 5×10^4/mL and incubated for 4 days with the concentration of S-D-lactoylglutathione indicated. The IC_{50} value was 82 (95% C.I. 65–104) μM (N = 13).

values of 92 ± 1% and 91 ± 1%, respectively. In corresponding cultures of HL60 cells, there was a >95% decrease in viable cell number.

Effect of monoesterification of S-2-*hydroxyacylglutathiones*

S-2-Hydroxyacylglutathione derivatives were confirmed to be substrates of γ-glutamyltransferase. Apparent K_M values (μM) and relative V_{max} values (%) determined from N observations were: S-D-lactoylglutathione 532 ± 62, 100 ± 7 (N = 24), S-glycolylglutathione 256 ± 55, 65 ± 15 (N = 17), S-L-glyceroylglutathione 256 ± 56, 91 ± 11 (N = 15) and S-D-mandelylglutathione 271 ± 23, 87 ± 7 (N = 15). Ethyl esterification on the glycyl residue of these compounds conferred resistance to cleavage: there was no detectable activity of S-2-hydroxyacylglutathione monoethyl esters with γ-glutamyltransferase (data not shown).

S-2-Hydroxyacylglutathione monoesters inhibited growth of HL60 cells in culture but were less effective than the corresponding unesterified S-2-hydroxyacylglutathione, giving higher IC_{50} values (Table 4). There was no significant growth arrest and/or toxicity induced in HL60 cells by reduced glutathione monoethyl ester (<500 μM), or glutathione monoethyl ester with corresponding aldonic acid together (500 μM each) (data not shown).

Inhibition of DNA synthesis by S-2-*hydroxyacylglutathione derivatives*

The rate of DNA synthesis in HL60 cells in culture, as judged by [^{3}H]thymidine incorporation, was inhibited by S-D-lactoylglutathione. The median inhibitory concentration IC_{50} value was 74 μM (95% C.I. 47–116; N = 10) (Fig. 4), which was not significantly different from the IC_{50} value of S-D-lactoylglutathione determined by viable cell number ($P > 0.05$). This was measured between the second and third hour of culture when there was no significant decrease in cell viability. Other S-2-hydroxyacylglutathione derivatives and their monoethyl esters also inhibited DNA synthesis but less effectively than S-D-lactoylglutathione (data not shown).

DISCUSSION

Methylglyoxal, S-D-*lactoylglutathione, glyoxalases and cell growth*

The glyoxalase system was implicated in the control of cell growth by Egyud and Szent-Gyorgyi [12] where methylglyoxal was thought to be a growth-inhibiting substance and glyoxalase I, which catalyses the metabolism of methylglyoxal, a growth-promoting factor. However, an effect of the glyoxalase intermediate, S-D-lactoylglutathione, on cell growth was not considered, until recently [2]. S-D-Lactoylglutathione is formed intracellularly and is not normally found in extracellular fluids (below the limit of detection of current assays: <0.1 μM).

Table 4. Median toxic concentrations of S-2-hydroxyacylglutathione derivatives and their monoesters to HL60 cells

	IC_{50} (μM) Mean, 95% C.I. (N)	Slope of logit plot Mean ± SD (N)
S-D-Lactoylglutathione	82, 65–104 (13)	2.53 ± 0.22 (48)
S-D-Lactoylglutathione monoethyl ester	208, 141–307 (26)*†	2.56 ± 0.26 (26)
S-Glycolylglutathione	154, 143–165 (12)*	3.66 ± 0.15 (29)
S-Glycolylglutathione monoethyl ester	201, 162–249 (21)*†	3.34 ± 0.22 (33)
S-L-Glyceroylglutathione	124, 82–187 (24)*	3.03 ± 0.14 (39)
S-L-Glyceroylglutathione monoethyl ester	138, 75–252 (24)*‡	3.68 ± 0.18 (74)
S-D-Mandelylglutathione	238, 179–316 (25)*	2.39 ± 0.09 (40)
S-D-Mandelylglutathione monoethyl ester	268, 233–308 (25)*†	2.33 ± 0.15 (29)

* $P < 0.01$ relative to the value for S-D-lactoylglutathione.
† $P < 0.01$ relative to the value for the corresponding unesterified derivative.
‡ $P > 0.05$ relative to the value for the corresponding unesterified derivative.

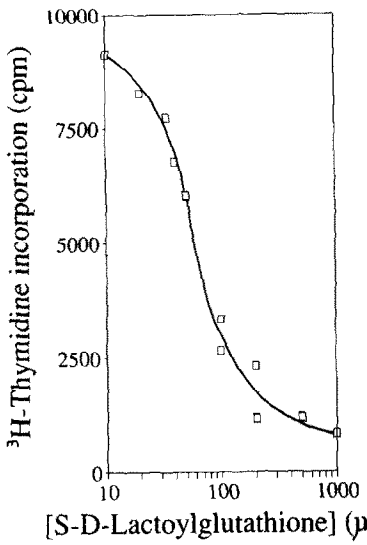

Fig. 4. Effect of *S*-D-lactoylglutathione on [^{3}H]thymidine incorporation into DNA in HL60 cells. HL60 cells (5.0×10^4/mL) were incubated with the concentration of *S*-2-hydroxyacylglutathione incubated in RPMI 1640 with 10% foetal calf serum for 2 hr. [^{3}H]Thymidine (2.5 μCi) was added and the incubation continued for 1 hr. The DNA was then extracted [10] and counted. The IC_{50} value was 74 μM (95% C.I. 47–116 μM) (N = 10).

Addition of exogenous *S*-D-lactoylglutathione (10–500 μM) to the extracellular medium of cell systems therefore poses an abnormal metabolic challenge to the cells. From the investigation described, this challenge was countered effectively without effect on cell viability by mature human neutrophils, but not by HL60 cells.

Growth inhibition and toxicity of S-2-*hydroxyacylglutathione derivatives to human leukaemia* 60 *cells*

All of the *S*-2-hydroxyacylglutathione derivatives studied inhibited the growth of HL60 cells. Corresponding aldonic acid, reduced glutathione, and reduced glutathione and aldonic acid together were without similar effect, although higher concentrations of reduced glutathione and L-lactate are reported to inhibit tumour cell growth *in vitro* [13–15]. *S*-2-Hydroxyacylglutathione derivatives also induced similar growth inhibition and toxicity in erythroleukaemia K562 cells (T. W. C. Lo and P. J. Thornalley, unpublished observation). The inhibition of HL60 cell growth was characterized by a decrease in viable cell number below the initial value after 24 hr and thereafter a return of residual cells to exponential growth. This suggests that *S*-2-hydroxyacylglutathiones induced toxicity in HL60 cells within the initial 24 hr of culture. Although there was evident cytotoxicity (the viable cell number was less than the cell number at seeding), little increase in the percentage of non-viable cells was found, as judged by Trypan blue exclusion. This is similar to our previous finding with *S*-D-lactoylglutathione-treated HL60 cells where it was reported that cell debris could be observed in cell cultures by microscopic examination and by flow cytommetric analysis [2].

The IC_{50} values for the *S*-2-hydroxyacylglutathione derivatives were in the order *S*-D-lactoylglutathione < *S*-L-glyceroylglutathione < *S*-D-mandelylglutathione < *S*-glycolylglutathione, which is the inverse order of the relative V_{max} values for the reaction of these compounds with γ-glutamyltransferase but does not correlate with the relative V_{max}/K_M values. The logit transformation of dose–response curves gave linear correlated data with slopes in the range 2.33–2.68. The significance of this is not clear but may reflect some amplification of the inhibition of cell growth following drug–receptor binding.

Monoethyl ester derivatives of *S*-2-hydroxyacylglutathiones had significantly higher IC_{50} values than the corresponding unesterified derivatives. Mono-esterification, therefore, partially prevented the inhibition of proliferation of HL60 cells induced by *S*-2-hydroxyacylglutathione derivatives. Since the monoethyl ester derivatives were not substrates of γ-glutamyltransferase, this may suggest that the anti-proliferative effect of *S*-2-hydroxyacylglutathione derivatives was mediated, in part, by the γ-glutamyltransferase product, *N*-2-hydroxyacylcysteinylglycine. This remains to be evaluated.

Monoethyl esterification of *S*-2-hydroxyacylglutathione derivatives may facilitate cellular penetration, with de-esterification to the corresponding *S*-2-hydroxyacylglutathione and hydrolysis by glyoxalase II, and thereby promote the degradation of *S*-2-hydroxyacylglutathione derivatives to innocuous products. Preliminary measurements of the concentration of *S*-D-lactoylglutathione in HL60 cells incubated with *S*-D-lactoylglutathione monoester suggested that it did not give rise to a significant increase in the cellular concentration of *S*-D-lactoylglutathione (data not shown).

S-D-Lactoylglutathione (and other *S*-2-hydroxyacylglutathione derivatives) also inhibited the incorporation of [^{3}H]thymidine into DNA early in the development of toxicity. At this time, there was no detectable decrease in cell viability and no change in the cellular pool of thymidine (data not shown). The IC_{50} values for *S*-D-lactoylglutathione determined by viable cell number and inhibition of thymidine uptake were not significantly different (83 and 74 μM, respectively; $P > 0.05$). This suggests that the synthesis of DNA was inhibited by *S*-2-hydroxyacylglutathione treatment and that inhibition of DNA synthesis may be a key feature of the development of cytotoxicity.

Mature, differentiated cells, neutrophils, were resistant to toxicity induced by *S*-D-lactoylglutathione. However, *S*-D-lactoylglutathione was not without effect on neutrophil function. We have previously reported that high concentrations (1–5 mM) of *S*-D-lactoylglutathione inhibited chemotaxis in response to the chemotactic peptide *N*-formylmethionylleucylphenylalanine [16] and influenced the kinetics of phorbol ester-activated secretion of specific granules [17], consistent with an effect on the functional response of neutrophils but without significant toxicity. Lower concentrations of *S*-D-lactoylglutathione (10 μM) potentiated phorbol

ester-activated secretion [17] and our recent research suggests a similar concentration (20 μM) potentiates chemotaxis to *N*-formylmethionylleucylphenylalanine (R. E. Allen and P. J. Thornalley, unpublished observation).

The anti-proliferative activity of *S*-D-lactoylglutathione was unexpected. The activity of glyoxalase II in human tumour cells in culture was found to be low but increased during differentiation [18] (as found in other proliferating cell types undergoing maturation: regenerating liver and developing embryo [19, 20]). Glyoxalase II activity also increased in human tumour cells in culture during serum deprivation-induced growth arrest [21]. The capacity of cells to metabolize *S*-D-lactoylglutathione characteristically increased during differentiation and growth arrest, and the concomitant cellular concentration of *S*-D-lactoylglutathione decreased [22]. HL60 cells have a relatively high cytosolic concentration of *S*-D-lactoylglutathione [22].

The anti-proliferative effect of *S*-D-lactoylglutathione and other *S*-2-hydroxyacylglutathione derivatives on proliferating cells remains an intriguing development which may lead to the discovery of novel therapeutic anti-proliferative agents.

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