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Antimalarial activity *in vitro* of the glyoxalase I inhibitor diester, *S-p*-bromobenzylglutathione diethyl ester

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Abstract—*S-p*-Bromobenzylglutathione diethyl ester induced toxicity in the malarial parasite *Plasmodium falciparum* in infected human red blood cells in culture. The median inhibitory concentration, IC_{50}, was 4.77 ± 0.12 μM (N = 10) for incorporation of [^{3}H]hypoxanthine in nucleotide synthesis and 5.20 ± 0.1 μM (N = 10) for incorporation of [^{14}C]isoleucine into protein. The prospective mechanism of action is inhibition of glyoxalase I by the de-esterified metabolite, *S-p*-bromobenzylglutathione, and accumulation of the cytotoxic substrate methylglyoxal.

The glyoxalase system catalyses the conversion of methylglyoxal to D-lactate, via the intermediate *S*-D-lactoylglutathione. It comprises two enzymes, glyoxalase I and glyoxalase II, and a catalytic amount of reduced glutathione [1]. 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:

$$MeCOCHO + GSH \leftrightharpoons MeCOCH(OH)\text{-}SG$$

$$\xrightarrow{\text{Glyoxalase I}} MeCH(OH)CO\text{-}SG.$$

Glyoxalase II (EC 3.1.2.6) catalyses the hydrolysis of *S*-D-lactoylglutathione to D-lactate and reforms the reduced glutathione consumed in the glyoxalase I catalysed reaction.

$$MeCH(OH)CO\text{-}SG + H_2O \xrightarrow{\text{Glyoxalase II}} MeCH(OH)CO_2^- + H^+ + GSH.$$

Methylglyoxal is formed by the non-enzymatic elimination of phosphate from dihydroxyacetonephosphate and glyceraldehyde-3-phosphate in human red blood cells [2]. The glyoxalase system is found in the cytosol of all cells, including red blood cells and malarial parasites, and is the major pathway for the metabolism of methylglyoxal and D-lactate formation therein (D-lactate is not further metabolized in red blood cells and malarial parasites) [3, 4].

The glyoxalase pathway can be viewed as essentially a metabolic pathway for the detoxification of methylglyoxal, a potentially noxious α-oxoaldehyde unavoidably formed from triosephosphates in the Embden-Meyerhof pathway [2]. Indeed, this is implicit in its metabolic activity. This efficient methylglyoxal metabolizing system [5] normally maintains only a low steady-state concentration of methylglyoxal (50–100 nM) in red blood cells [6], avoiding the toxicity associated with high concentrations of methylglyoxal, which is particularly pronounced in proliferating cells [7]. The dependence of the malarial parasite on anaerobic glycolysis and the high glycolytic rate of the parasite suggest that it may be particularly sensitive to pharmacological inhibition of glyoxalase I and consequent methylglyoxal-induced toxicity.

The rate of D-lactate formation in human red blood cells increases by *ca.* 10–20-fold upon infection with *Plasmodium falciparum*, the increasing D-lactate formation originating from methylglyoxal formed in the parasite; no parasite D-lactic dehydrogenase has been detected [4]. This indicates that concomitant with parasitic infection, there is a marked increase in flux through the glyoxalase pathway. Human red blood cell glyoxalase I has been purified and characterized [8]. Glyoxalase I from red blood cells had a K_M of 204 μM for methylglyoxal-reduced glutathione hemithioacetal whereas the *P. falciparum* enzyme K_M is *ca.* 38 μM. There was *ca.* two times as much glyoxalase I in a red blood cell as in the parasite [4]. The high rate of methylglyoxal formation by parasites suggests that inhibitors of glyoxalase I delivered into infected red blood cells may induce selective toxicity to the parasite.

Substrate analogue inhibitors of glyoxalase I have been developed [9]. *S-p*-Bromobenzylglutathione is a potent, competitive inhibitor of human red blood cell glyoxalase I: its inhibition constant, K_i, is 0.16 μM [10]. However, until recently no associated potent anti-malarial activity was detected. *S-p*-Bromobenzylglutathione, like other glutathione-*S*-conjugates, does not readily cross cell plasma membranes [11] and is cleaved in the extracellular medium to inactive products by γ-glutamyl transferase [12]. Recently, we prepared the diethyl ester derivative of *S-p*-bromobenzylglutathione, α-ethyl-γ-L-glutamyl-*S-p*-bromobenzyl-L-cysteinyl-ethylglycinate. *S-p*-Bromobenzylglutathione diethyl ester is resistant to cleavage by γ-glutamyl transferase, crosses cell plasma membranes and is de-esterified in the cytosol by non-specific esterase [13]. It had potent anti-leukaemia activity: the median effective dose, IC_{50}, for human leukaemia 60 cells was 8 μM [11].

In this study, we describe the toxicity of *S-p*-bromobenzylglutathione diethyl ester to *P. falciparum in vitro*.

Materials and Methods

Materials. *S-p*-Bromobenzylglutathione diethyl ester was prepared and characterized as described [13]. A stock solution in dimethylsulphoxide (500 mM) was prepared. The effect of *S-p*-bromobenzylglutathione diethyl ester (1 μM–1 mM) on red blood cell viability was studied by measuring leakage of haemoglobin, lipid peroxidation and reduced glutathione concentration, as described previously [14, 15], in red blood cell suspensions (25%, v/v) in Krebs–Ringer phosphate buffer, pH 7.4 at 37° for 2 hr.

Malaria cultures. *S-p*-Bromobenzylglutathione diethyl ester was tested *in vitro* against synchronized *P. falciparum* (strain C10) using a [^{3}H]hypoxanthine and [^{14}C]isoleucine uptake assay [16]. A stock solution of *S-p*-bromobenzylglutathione diethyl ester (50 mM) was prepared in dimethylsulphoxide. Drug sensitivity was determined using drug dilutions prepared in microtitre plates to which isotope and infected erythrocytes were added. The total culture volume was 200 μL/well, with 2% haemocrit and 12% ring stage parasites. After incubation for 28 hr, the plates were harvested onto glass fibre paper, washed with water and processed for scintillation counting.

Time course studies were undertaken using 8 mL cultures (2% haematocrit, 5% rings) containing *S-p*-bromobenzylglutathione diethyl ester and isotopes. At

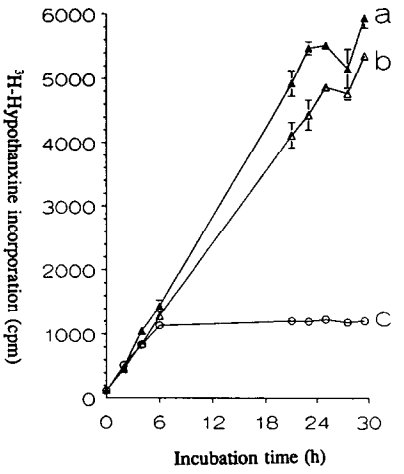

Fig. 1. Effect of *S-p*-bromobenzylglutathione diethyl ester on [^{3}H]hypoxanthine incorporation into nucleotides in RNA and DNA in *P. falciparum*-infected human red blood cells *in vitro*; 12% ring stage parasites at time zero. (a) Control, (b) + dimethylsulphoxide, (c) + *S-p*-bromobenzylglutathione diethyl ester (6 μM) with dimethylsulphoxide. Data are means ± SD of three experiments.

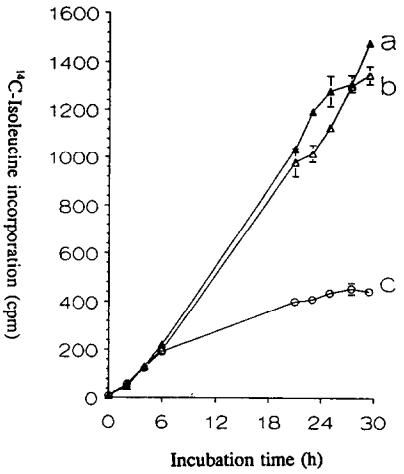

Fig. 2. Effect of *S-p*-bromobenzylglutathione diethyl ester on [^{14}C]isoleucine incorporation into protein in *P. falciparum* infected human red blood cells *in vitro*; 12% ring stage parasites at time zero. (a) Control, (b) + dimethylsulphoxide, (c) + *S-p*-bromobenzylglutathione diethyl ester (6 μM) with dimethylsulphoxide. Data are means ± SD of three experiments.

various times, duplicate 200 μL samples were removed and frozen in a microtitre plate until harvested.

The effect of *S-p*-bromobenzylglutathione diethyl ester on red blood cells under the culture conditions was investigated by incubation of uninfected red blood cells (2% haemocrit) in culture medium with *S-p*-bromobenzylglutathione (20 μM) for 24 hr and estimation of haemolysis by determination of extracellular haemoglobin [14].

Results and Discussion

S-p-Bromobenzylglutathione diethyl ester had no demonstrable toxicity to red blood cells (20%, v/v) in short-term (2 hr) culture at concentrations ≤1 mM: there was no detectable leakage of haemoglobin, decrease in reduced glutathione or increase in lipid peroxidation in *S-p*-bromobenzylglutathione-treated cells, relative to controls. It was also not toxic to red blood cells (2% haematocrit) over 24 hr in culture at 0.5–20 μM (data not shown). However, when *S-p*-bromobenzylglutathione diethyl ester was added to human red blood cells infected with the malarial parasite *P. falciparum in vitro*, incorporation of tritiated nucleotides into DNA and RNA from [^{3}H] hypoxanthine and incorporation of [^{14}C]isoleucine into protein were inhibited (Figs 1 and 2). With 6 μM *S-p*-bromobenzylglutathione diethyl ester, both effects developed after 6 hr. This lag period is apparently required for *S-p*-bromobenzylglutathione diethyl ester to deliver cytotoxic metabolites into infected cells: red blood cell and parasite penetration by the diethyl ester and de-esterification to form the potent glyoxalase I inhibitor *S-p*-bromobenzylglutathione may occur during this period. The dose–response data suggest that the lag phase decreases at higher concentrations of *S-p*-bromobenzylglutathione diethyl ester (see below). Thereafter, the incorporation of tritiated nucleotides into DNA and RNA from [^{3}H]-hypoxanthine ceased and the incorporation of isoleucine into protein continued at a much diminished rate. Reverse-phase HPLC analysis of red blood cells incubated with *S-p*-bromobenzylglutathione diethyl ester gave chromatographic peaks of the diethyl ester, monoethyl ester and unesterified analogues, indicating that *S-p*-bromobenzylglutathione enters red blood cells and is therein de-esterified (cf. reduced glutathione monoethyl ester) [17].

The dependence of protein synthesis and nucleotide synthesis in the infected cells on the concentration of *S-p*-bromobenzylglutathione diethyl ester, as judged by the incorporation of [^{14}C]isoleucine into protein and incorporation of [^{3}H]hypoxanthine into DNA and RNA in the initial 24 hr of culture, was investigated. Count data were fitted by non-linear regression to the logistical equation, $C = C_{max} \times IC_{50}{}^n/(IC_{50}{}^n \pm [BBDE]^n)$ where C and C_{max} are the counts (cpm) of nucleic acid or protein precipitates in the presence and absence of *S-p*-bromobenzylglutathione diethyl ester at the concentration [BBDE], IC_{50} is the median inhibitory value and n is the logistical regression coefficient for N data points (Fig. 3).

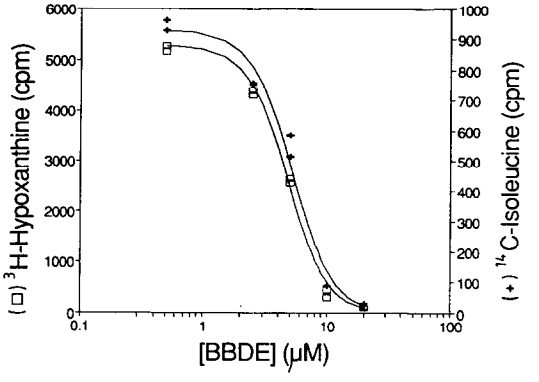

Fig. 3. Concentration–response curves for the effect of *S-p*-bromobenzylglutathione diethyl ester on [^{14}C]isoleucine incorporation into protein and [^{3}H]hypoxanthine incorporation into nucleotides in RNA and DNA in *P. falciparum*-infected human red blood cells *in vitro*. For nucleotide synthesis, $IC_{50} = 4.77 \pm 0.12$ μM and $n = 2.75 \pm 0.19$ (N = 10); for protein synthesis, $IC_{50} = 5.20 \pm 0.01$ and $n = 2.66 \pm 0.01$ (N = 10). The mean control counts were for [^{3}H]hypoxanthine incorporation 5270 cpm and for [^{14}C]isoleucine incorporation 928 cpm. Cultures were initiated as 5% ring stage in 2% haematocrit.

For nucleotide synthesis, the IC_{50} value was 4.77 ± 0.12 μM and $n = 2.75 \pm 0.19$ (N = 10); and for protein synthesis, the IC_{50} value was 5.20 ± 0.01 μM and $n = 2.6 \pm 0.01$ (N = 10). The finding that the logistical regression coefficient was >1 suggested that there is a cooperative or amplified toxic response involved in the mechanism of parasite killing.

The mechanism of action of *S-p*-bromobenzylglutathione diethyl ester is not known. *S-p*-Bromobenzylglutathione formed by de-esterification in the red blood cell, and possibly in the parasite too, inhibits glyoxalase I leading to the accumulation of methylglyoxal to toxic levels. Methylglyoxal binds and modifies guanine, induces single-strand breaks, and crosslinks DNA and protein and A-T base pair-rich regions of double-stranded DNA [18–21]. Methylglyoxal-mediated effects on parasite DNA, RNA and protein synthesis may mediate the anti-malarial activity of *S-p*-bromobenzylglutathione diethyl ester which is commensurate with the selectivity of toxicity to infected red blood cells.

Agents such as *S-p*-bromobenzylglutathione diethyl ester with both anti-proliferative and anti-tumour activity (with putative mechanisms of action related to inhibition of DNA, RNA and protein synthesis) are not generally considered to be suitable candidates for chemotherapy of malaria because of the expected toxicity to myeloid tissue and other host proliferating cells [22], although studies of the toxicity to the human T4 lymphocyte cell line CEM-SS showed decreased toxicity with a median growth inhibitory concentration of *ca.* 34 μM (P. J. Thornalley, unpublished observations). The selectivity of this type of compound may be improved by exploiting structural differences of the host and parasite glyoxalase I enzymes and optimizing delivery of the diester derivative into the parasite.

Glyoxalase I inhibitor diesters are a novel type of cytotoxic agent which are mechanistically well suited to inducing biocidal activity in organisms dependent on Embden-Meyerhof glycolysis.

Department of Chemistry and Biological Chemistry
University of Essex
Wivenhoe Park
Colchester CO4 3SQ, and
†National Institute for Medical Research
Mill Hill
London, U.K.

PAUL J. THORNALLEY*
MALCOLM STRATH†
R. J. M. WILSON†

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* Corresponding author.