Glutathione S–Transferase Activity of Human Erythrocytes Incubated in Aqueous Solutions of Five Antimalarial Drugs

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ABSTRACT

Levels of human erythrocyte glutathione S-transferase (GST) activity were ascertained in the presence of separate increasing concentrations (0.2, 0.4, 0.6, and 0.8 mg% w/v) of five antimalarial drugs {Pyrimethamine/Sulphadoxine (PS), Halofantrine-HCl (H-HCl), Quinine, Artemether/umefantrine (AL) and Chloroquine Phosphate (CP)} *in vitro.* Determination of erythrocyte GST activity was carried out by spectrotrophotometric method with the standard substrate (1-chloro-2, 4-dinitrobenzene) and co-substrate (reduced gluthathione) at maximum absorbance (λ_{max}) = 340 nm. Human erythrocyte GST activity ranged between 3.27 ± 0.13 and 3.40 ± 0.05 iu/gHb. The addition of increasing concentrations of four antimalarial drugs to assay mixture engendered decreased levels of GST activity in a concentration dependent manner, which was in the order: CP > AL > Quinine > PS. Specifically, 0.8 mg% CP exhibited the highest capacity to cause decreased GST activity from values of 3.41 ± 0.06 to 2.18 ± 0.09 iu/gHb, representing 33.7% relative inhibition of GST activity. In contrast, concentrations of H-HCl between 0.2 and 0.6 mg% caused elevation of GST activity above the values of the control samples. However, the increased levels of GST activity was not significantly different ($p > 0.05$) compared with the control samples. The five antimalarial drugs exhibited variable capacities to alter human erythrocyte GST activity, which suggest the capability of these drugs to perturb the redox status of human erythrocytes.

Keywords: Antimalarials, 1-chloro-2, 4-dinitrobenzene, erythrocytes, glutathione-S transferase.

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INTRODUCTION

Purification and characterization of glutathione S-transferase (GST; EC. 2.5.1.18) from human erythrocyte reported the molecular weight to be 47,500 Dalton, composed of two subunits of the same apparent molecular weight.[1;2] The enzyme is active with a variety of compounds bearing an electrophilic centre. The erythrocyte GST has low isoelectric unit (PI) of 4.50[1;3] as compared with alkaline PI values for human liver enzymes of 7.80, 8.25, 8.55, 8.75 and 8.80 for alpha (α), beta (β), gamma (γ), delta (δ) and epsilon (ε) respectively.[3;4]

The ubiquitous enzyme, GST, is primarily involved in the neutralization of harmful exogenous or endogenous compounds by enzymatic conjugation with the scavenger peptide- gluthathione and/or by direct binding to non-substrate ligands.[5;6] Other functions of GST include protection against oxidative damage to lipids and nucleic acids, as well as metabolism of some steriods and leucotrienes.[6] The enzyme is represented by a family of cytosolic proteins bearing dimeric structures, the polymorphic expression of which has been widely studied in human tissues and wide series of cells.[6-8] The soluble GSTs are divided into distinct classes based on similarities in their primary structures and substrate specificities. In human erythrocytes, GST is present in large amount in two forms; a highly cationic enzyme designated with the Greek letter rho (ϱ), which accounts for < 5% of the total enzyme correponding to the P form.[1;9;10] Because of the absorbance and overlapping properties of erythrocyte GST isoforms with the ϱ form, the GST P1-1 dimer is often considered the sole GST enzyme in the erythrocyte.[6;10]

Although Ahmad and Srivastava[11] reported the selective inhibition of malarial parasites GST activity by various classes of inhibitors that showed potential therapeutic benefits, chemotherapeutic agents interact with structural and functional constituents of human

erythrocytes, specifically, haemoglobin, diverse enzymes and membrane components. Therefore, the present *in vitro* study ascertained the capcity of five antimalarial drugs {Pyrimethamine/Sulphadoxine (PS), Halofantrine-HCl (H-HCl), Quinine, Artemether /Lumefantrine (AL) and Chloroquine Phosphate (CP)} to interfere with human erythrocyte GST activity.

MATERIALS AND METHODS

Antimalarial Drugs: Five (5) antimalarial drugs were used in this study: Pyrimethamine/Sulphadoxine (mixture ratio; 5:1 w/w) (SWISS SWIPHA) Pharmaceutical Nigeria Ltd), Artemether/Lumefantrine (mixture ratio; 1:6 w/w) (Beijing NORVATIS Pharmaceutical Company, Beijing, China), Chloroquine Phosphate (MAY and BAKER, Pharmaceutical Company Nigeria, Plc), Halofantrine-HCl (SMITHKLINE BEECHAM Laboratories Pharmaceutical Company, France) and Quinine (BDH, UK). Five percent (5.0% w/v) stock solution of the five antimalarial drugs was prepared by dissolving 2.5 g of each drug in 50 ml of distilled water. Serial dilutions were made to obtain corresponding concentrations of 0.8, 0.6, 0.4 and 0.2 mg% (w/v).

Collection and Preparation of Blood Samples: A total of twenty-five (25) blood samples of human erythrocyte HbAA genotype were collected via venepuncture from clinical confirmed healthy and non-malarious male volunteers between the ages of 18 and 35 yr, in accordance with the ethical principles that have their origins in the Declaration of Helsinki.

Blood volume of 5 ml was collected from each donor who had been screened for glucose-6-phosphate dehydrogenase deficiency and stored in EDTA tubes. The erythrocytes were washed by centrifugation method as described by Tsakiris *et al.,*.[12] Within 2 hr of collection of blood samples, portions of 1.0 ml of the samples were introduced into centrifuge test tubes containing 3.0 ml of buffer solution pH = 7.4:250 mM tris (hydroxyl methyl) amino ethane–HCl (Tris-HCl)/140 mM NaCl/1.0 mM $MgCl_2$/10 mM glucose). The erythrocytes were separated from plasma by centrifugation at 1200 x *g* for 10 min and washed three times by the same centrifugation method with the buffer solution. To remove platelets and leucocytes, the sediment was re-suspended in 3 ml of phosphate-buffered saline (PBS) solution, pH 7.4, and passed through a column (3.5 cm in a 30 ml syringe) of cellulose-microcrystalline cellulose (ratio w/w 1:1) as described by Kalra *et al.,*.[13] The eluted fraction was passed twice through a new column of cellulose-microcrystalline cellulose (ratio 1:1 w/w) to obtain erythrocyte suspension sufficiently devoid of leucocytes and platelets. The erythrocytes were finally re-suspended in 1.0 ml of this buffer and stored at 4 °C. The washed erythrocytes were lysed by freezing/thawing as described by Galbraith and Watts,[14] and Kamber *et al.,*.[15] The erythrocyte haemolysate was used for dtermination of GST activity.

Determination of Haemoglobin Concentration: A modified method of Baure,[16] as described by Chikezie,[17] based on cyanomethaemoglobin reaction was used for the determination of haemoglobin concentration (g/dl). A 0.05 ml portion of erythrocyte haemolysate was added to 4.95 ml of Drabkin reagent (100 mg NaCN and 300 mg $K_4Fe(CN)_6$ per liter). The mixture was left to stand for 10 min at room temperature and absorbance read at λ_{max} = 540 nm (UV-Visable Spectrophotometer, Jenway 6405) against a blank. The absorbance was used to evaluate haemoglobin concentration by comparing the values with the standards.[17]

Determination of Erythrocyte Haemolysate Glutathione S-transferase Activity: GST activity was assayed by monitoring the conjugation of 1-chloro-2,4dinitro benzene (CDNB) with glutathione (GSH) at λ_{max} = 340 nm at 37 °C.[5]

$$CDNB + GSH \rightarrow CDNB - S - glutathione$$

The enzyme assay was according to methods of Habig *et al.,*[5] with minor modifications.[18] The 1.0 ml in 2% ethanol enzyme assay mixture contained 0.5 mM CDNB (0.02 ml), 1.0 mM GSH (0.05 ml), 0.68 ml of distilled water and 100 mM phosphate buffer (K_2HPO_4/KH_2PO_4; pH = 6.5) (0.2 ml).

The CDNB was pre mixed with the phosphate buffer before use. The phosphate buffer-CDNB mixture was pre-incubated for 10 min at 37 °C and the reaction started by adding GSH, followed immediately by aliquot (0.05 ml) of the haemolysate. The rate of increase in absorbance at λ_{max} = 340 nm was measured for 10 min at 37 °C against a blank solution containing the reaction mixture, in which the haemolysate was substituted with distilled water. For the test analyses, the enzyme assay was carried out in the presence of 0.68 ml of the five (5) separate antimalarial drugs at different concentrations (0.2, 0.4, 0.6 and 0.8 mg%).

Statistical Analyses: The experiments were designed in a completely randomized method and data collected were analyzed by the analysis of variance procedure while treatment means were separated by the Least Significance

Difference (LSD) incorporated in the Statistical Analysis System (SAS) package of 9.1 version (2006).

Calculation of Enzyme Activity: According to Anosike *et al*.,[18] the expression below was used to evaluate erythrocyte GST activity in international unit per gram haemoglobin (iu/gHb).

$$E_A = \frac{100 \text{ O.D/min VC}}{[Hb]\sum VH}$$

Where: E_A = Enzyme activity in iu/gHb; [Hb] = Haemolysate haemoglobin concentration (g/dl); O. D/min = Change in absorbance per min at 340 nm; $\sum$ = Millimolar extinction coefficient = 9.6 mM^{-1} cm^{-1}, in reaction in which 1 mole of GSH is oxidized; VC = Cuvette volume (total assay volume) = 1.0 ml; VH = Volume of haemolysate in the reaction system (0.05 ml).

RESULTS AND DISCUSSION

The mean (±S.D) of erythrocyte GST activity and corresponding relative activity in the presence of increasing experimental concentrations of five antimalarial drugs is presented in Table 1 below.

The control/reference values of GST activity ranged between 3.27 ± 0.13 and 3.40 ± 0.05 iu/gHb. The addition of increasing concentrations of PS, Quinine, AL and CP engendered decreased levels of GST activity in a concentration dependent manner, which was in the order: CP > AL > Quinine > PS. Specifically, 0.8 mg% CP exhibited the highest capacity to cause decreased GST activity from values of 3.41 ± 0.06 to 2.18 ± 0.09 iu/gHb, representing 33.7% relative inhibition of GST activity. In contrast, concentrations of H-HCl between 0.2 and 0.6 mg% caused elavation of GST activity above the values of the control samples. However, these increased values of GST activity was not significantly different ($p > 0.05$) compared with the control samples.

In concord with the present *in vitro* study, Ayalogu *et al*.,[19] reported that rat erythrocyte GST was inhibited *in vitro* by antimalarials, alkaloid drugs, Chloroquine and Fansidar™ (sulphdoxine + pyrimethamine). They averred that because the enzyme is an integral component of cellular redox system, agents that compromise GST activity may elevate erythrocyte oxidative stress. Therefore, decreased level of human erythrocyte GST activity is associated with perturbation of erythrocytes redox status as posited by previous researchers.[19-25] In a different study, incubation of rat liver GST with variety of chlorophenoxyalkyl acid herbicides (CPAs) resulted in a dose dependent inhibition of GST activity.[26] Furthermore, incubation of GST obtained from three aquatic species with CPAs, quinones and o-chloranil also caused inhbition of the enzyme activity *in vitro* and *in vivo* after 18 hr.[27] In another report, Davies,[28] stated that chlorothalonil binds to GST at low GSH concentration. He further asserted that the binding was irreversible and probably covalent; a classical case of irreversible non-competitive inhibition kinetics. It is worthwhile to mention that GSTs have high affinity for endogenous compounds such as bilirubin, bile acids, haemin, fatty acids and steriods.[29-33] Under this circumstance, GSTs do not form glutathione conjugates with their substrates.[30;34] Therefore, GSTs have been termed "ligandins" and the non-enzymatic substrates referred to as "non-substrate ligands".[29;35;36] This interaction most often compromised GST activity.[29;33]

In vivo studies by Ahmad and Srivastava,[11] reported that selective inhibition of malarial parasites GST by various classes of inhibitors could be viewed as a potential chemotherapeutic strategy to combat malaria. These authors also showed that purified GST from

Table 1. Human Erythrocyte Glutathione S-transferase Activity in the Presence of Five Antimalarial Drugs

	GST Activity iu/gHb/ (Relative Activity %)				
[Drug] (mg/100 ml)	0.0 (control)	0.2	0.4	0.6	0.8
PS	3.41 ± 0.06^{a} (100.0)	3.39 ± 0.13^{a} (99.4)	3.35 ± 0.06^{a,b} (98.2)	3.22 ± 0.05^{b,c} (94.4)	3.09 ± 0.16^{c} (90.6)
H-HCl	3.37 ± 0.08^{a} (100.0)	3.3 ± 0.10^{a} (100.6)	3.41 ± 0.10^{a} (101.2)	3.40 ± 0.10^{a} (100.9)	3.34 ± 0.15^{a} (99.1)
Quinine	3.27 ± 0.27^{a} (100.0)	3.04 ± 0.07^{b} (93.0)	2.43 ± 0.07^{c} (74.3)	2.41 ± 0.09^{c} (73.7)	2.39 ± 0.07^{c} (73.1)
AL	3.37 ± 0.07^{a} (100.0)	2.46 ± 0.08^{b} (73.0)	2.47 ± 0.12^{b} (73.3)	2.39 ± 0.07^{b,c} (70.9)	2.30 ± 0.08^{c} (68.2)
CP	3.29 ± 0.09^{a} (100.0)	2.44 ± 0.06^{b} (74.2)	2.29 ± 0.09^{c} (69.6)	2.19 ± 0.04^{c,d} (66.6)	2.18 ± 0.05^{d} (66.3)

The results are mean (X) ± S.D of five ($n = 5$) determinations; Means in the row with the same letter are not significantly different at $p < 0.05$ according to LSD.

Plasmodium yoelii was inhibited by compounds like protoporphyrin IX, cibacron blue, as well as by the GSH depleting agent menadione. Inhibition of GST activity potentiated the accumulation of antimalarial metabolites, which was responsible for creating a hazardous milieu in parasite, leading to death. Therefore, the selective inhibition of parasite GST in relation to the host cell by these agents showed potential therapeutic benefits in malaria chemotherapy.[37] These findings are been exploited to achieve therapeutic benefits in malaria disease.

The present *in vitro* study showed that aqueous solution of the five antimalarial drugs altered human erythrocyte GST activity, which suggests the capability of these drugs to perturb the redox status of human erythrocytes.

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