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Full Length Research Paper

Polymerization of human sickle cell haemoglobin (HbS) in the presence of three antimalarial drugs

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To study the capacity of antimalarial drugs to interfere with polymerization of sickle cell haemoglobin (HbS), *in vitro* polymerization was induced by incubating erythrocyte haemolysate of HbS with 2 g% sodium metabisulphite ($Na_2S_2O_5$) in the presence of 2 mg% concentration of three (3) separate antimalarial drugs (Coartem^{TM}, Quinine, and Chloroquine phosphate). Spectrophotometric method was used to ascertain the level of polymerization of HbS at a regular interval of 30 seconds for a period of 180 seconds at extinction coefficient (λmax) of 700nm. The level of polymerization was expressed in percentage of the absorbance of control sample at the 180th second. The three antimalarial drugs caused significant (p<0.05) reduction in HbS polymerization in the following ranges: Coartem^{TM} (17.05-31.07%), Quinine (13.95-28.85%) and Chloroquine phosphate (10.85-33.01%). These results indicated the feasibility of the three drugs to retard HbS polymerization and as apparent potential candidates for therapy and management of sickle cell disease.

Key words: Anti-malarial drugs, polymerization, sickle cell, haemoglobin, sodium metabisulphite.

INTRODUCTION

The sickling disorder was the first description of a molecular disease and accounts for the vast majority of clinical important structural disorder to be studied. The sickle cell gene (β^S) occurs widely throughout Africa, part of Asia, the Arabian Peninsula and part of Southern Europe. In Africa, there are two areas with very high frequencies of β^S. One includes Nigeria, Ghana, and the other, Gabon and Zaire (Wainscoat, 1987; Uzoegwu and Onwurah, 2003). The carrier rate in this area is 10-30% of the population and the disease accounts for approximately 80,000 infant deaths per year (Wainscoat 1987). Sickle cell disease occurs with a much lower incidence in part of Italy, Greece, the Middle East and India. Genetic studies suggests that β^S mutation may have arisen independently in Africa and Asia and that subsequent selection pressure by malaria has resulted in the observed high frequencies because heterogeneous carriers are more resistant to *P. falciparum* malaria during childhood (Weatheral et al., 1983).

Sickle cell anemia is caused by a single base mutation of adenine to thymine which results in a substitution of valine for glutamic acid at the sixth codon of the β-globin chain (Koch et al., 2002). This substitution has a profound structural consequence on haemoglobin and its biologic function. The reason for this phenomenon is the consequence of the substitution in HbS of polar glutamic acid residue with a non-polar valine molecule and thereby generates a sticky patch on the beta chain (Martin, 1983). Importantly, only this one β6 Val---Glu residue of each tetramer is in an intermolecular contact region. Specifically, the hydrophobic valine side chain appears to fit into a hydrophobic pocket formed by a β88 leucine residue and a β85 phenylalanine residue on an adjacent molecule. The normal glutamic acid would not easily fit into this pocket explaining at least part of why deoxyHbA does not polymerize. In conditions of reduced oxygen tension, HbS molecules form relatively insoluble polymer. The model described in sequential steps includes –

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Abbreviations: 5HMF, 5-Hydroxylmethyl-2-furfural; **FMC,** Federal Medical Center; **IMSUTH,** Imo State University Teaching Hospital; **PBS,** phosphate buffered saline; **LSD,** least Significant difference; **SAS,** statistical analysis system; **EDTA,** ethylene diamine tetraacetic acid; **HbS,** sickle cell haemoglobin.

nucleation, growth and subsequent alignment of the molecule into microfibrils parallel to each other with the resultant membrane deformity and damage (Bindon, 2003).

In spite of the full understanding of the pathology, physiology, and the molecular nature of the disease, a cure for sickle cell anemia still is unavailable. Strategies to treat sickle cell anemia since the early times of the disease state discovery in 1910, has focused mainly on prophylactic measures to alleviate the painful crises. While many researchers are still searching for clues to a cure, individuals can only alleviate the symptoms and prevent complications. Some common ways that sickle cell anemia can be managed are: blood transfusions, administration of painkilling drugs, intravenous fluids, oral antibiotics such as penicillin and by using the anticancer drug hydroxyurea (Bownas, 2002). Several attempts are in progress to finding new types of anti-sickling agents that specifically binds to intracellular sickle cell haemoglobin (HbS). Some anti-sickling agents that fulfill this criterion are 5-hydroxylmethyl-2-furfural (5HMF) (Abdulmalik et al., 2005), certain amino acids such as phenylalanine, lysine, and arginine (Anosike et al., 1991) and 2-imidazolines (Chang et al., 1983). Interestingly, in clinical practice, hydroxyurea is commonly used as anti-sickling agent to manage the disease, and it has been recently approved by the United States Food and Drug Administration as a drug for that purpose (Mehanna, 2001).

There are reports on the capability of some anti-malarial drugs to distort/alter certain red blood cell elements/physicochemical properties. Notable are red blood cell glutathione-S transferase activity, osmotic fragility index and level of methaemoglobin (Chikezie et al., 2009a,b; Chikezie, 2009; Chikezie et al., 2008). Therefore, the present *in vitro* study intends to ascertain the capacity of three anti-malarial drugs to interfere with HbS polymerization. The findings would provide preliminary insights into the role of these anti malarials in the sickling process, for more informed prescription and therapeutic strategies for individuals who express this genotype.

MATERIALS AND METHODS

Collection of blood samples/preparation of erythrocyte haemolysate

Five milliliters (5.0 ml) of venous blood obtained from the volunteers by venipuncture was stored in EDTA anticoagulant tubes. Blood samples were from patients attending clinics at the Federal Medical Center (FMC), Imo State University Teaching Hospital (IMSUTH), Orlu, St. John Clinic / Medical Diagnostic Laboratories, Avigram Medical Diagnostic Laboratories, and Qualitech Medical Diagnostic Laboratories. These centers are located in Owerri, Imo State, Nigeria.

The erythrocytes were washed by methods as described by Tsakiris et al. (2005). Within 2 h 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/I.0 mM $MgCl_2$/10 mM glucose). The erythrocytes were separated from plasma by centrifugation at 1200xg for 10 min, washed three times by three similar centrifugations with the buffer solution. The erythrocytes re-suspended in 1.0 ml of this buffer were stored at 4°C. The washed erythrocytes were lysed by freezing/thawing as described by Galbraith and Watts (1980) and Kamber et al. (1984). The HbS erythrocyte haemolysate was used for polymerization studies.

Anti-malarial drugs

Three (3) anti malarial drugs were used in this study: CoartemTM (Beijing NORVATIS Pharmaceutical Company, Beijing, China), Chloroquine phosphate (MAY and BAKER, Pharmaceutical Company Nigeria, Plc), and Quinine (BDH, UK). Two percent (2 mg %) (w/v) solution of the three anti malarial drugs was prepared by dissolving 2 mg of each drug in 100 ml of distilled water.

Polymerization studies of erythrocyte haemolysate

HbS polymerization was assessed by the turbidity of the solution (polymerizing mixture) by the method described by Iwu et al. (1988). The level of polymerization was ascertained by increasing absorbance of the assay mixture. A portion of 0.1 ml of the erythrocyte haemolysate containing HbS was introduced into a test tube followed by addition of 0.5 ml of Phosphate buffered saline (PBS) solution, pH = 7.4 {NaCl (9.0 g)/$Na_2HPO_4.2H_2O$ (1.71 g)/ $NaH_2PO_4.2H_2PO_4.2H_2O$ (2.43 g) per 1litre of distilled water} and 1.0 ml of distilled water. The sample was transferred into a cuvette and HbS polymerization was induced by adding 3.4 ml of 2% sodium metabisulphite solution. The change in absorbance of the assay mixture was recorded at a regular interval of 30 s for 180 s at λmax = 700 nm.

To determine the anti-sickling property of the three antimalarial drugs, the procedure above was repeated by substituting 1.0 ml of distilled water with separate 2 mg% of the three antimalarial drugs.

Calculation

Percentage polymerization = [At x 100] / [$Ac_{180}{}^{th}{}_{second}$]

Where, At = Absorbance of test/control sample at time = t second; $Ac_{180}{}^{th}{}_{second}$ = Absorbance of control sample at 180^{th} s.

Statistical/data 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 versions (2006).

RESULTS AND DISCUSSION

The change in absorbance of the control and test the samples and the corresponding percentage polymerize-tion are presented in presented in Table 1 and Figure 1, respectively. The results presented in Table 1 showed increasing absorbance of the assay mixture in the control

Table 1. Changes in absorbance of the control and test samples with time.

Time (s)	Absorbance (λ=700 nm)			
	Control	CoartemTM	Quinine	Chloroquine phosphate
0	0.00	0.00	0.00	0.00
30	0.052 ± 0.02a	0.043 ± 0.004a	0.037 ± 0.04a	0.042 ± 0.02a
60	0.103 ± 0.01b	0.071 ± 0.005a	0.074 ± 0.04a	0.069 ± 0.02a
90	0.113 ±0.02b	0.087 ± 0.008a	0.091 ± 0.04a	0.086 ± 0.02a
120	0.122 ± 0.01b	0.097 ± 0.012a	0.100 ± 0.05a	0.098 ± 0.02a
150	0.126 ± 0.02b	0.104 ± 0.015a	0.106 ± 0.05a	0.107 ± 0.02a
180	0.129 ± 0.01b	0.107 ± 0.015a	0.111 ± 0.05a	0.115 ± 0.02a

Means in the row with the same letter are not significantly different at $p< 0.05$ according to LSD.

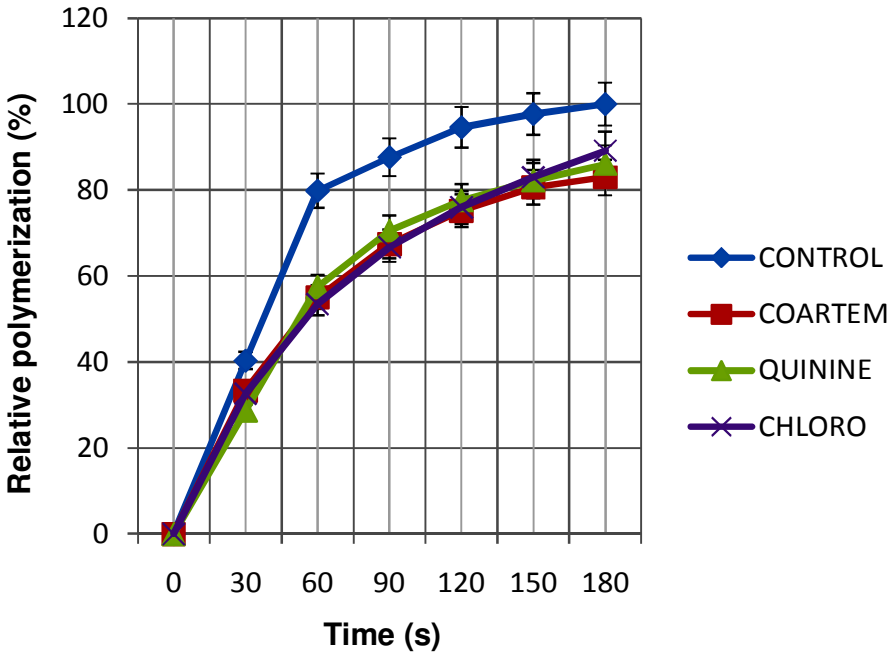

Figure 1. Percentage polymerization of HbS in the absence (Control Sample) and presence of three anti malarial drugs.

and test samples as the experimental time progressed. However, the absorbance of the polymerization mixture in the presence of the three anti malarial drugs was not significantly different ($p<0.05$) from the control sample at the 30th second. These values were indications that polymerization of HbS molecules occurred in the control sample and in the presence of the three anti malarial drugs (Figure 1). For instance, within the experimental time of 30-180 s, the relative polymerizations range between 33.33-82.95, 28.68-86.05% and 32.56-89.15% upon the introduction of Coartem, Quinine and Chloroquine phosphate, respectively.

The results presented in Table 2 showed the three anti malarial drugs caused significant ($p<0.05$) reduction in HbS polymerization in the following range: Coartem™ (17.05 - 31.07%), Quinine (13.95 - 28.85%) and Chloroquine phosphate (10.85-33.01%).

Furthermore, whereas Chloroquine phosphate caused maximum inhibition of HbS polymerization at the 60th s (percentage inhibition= 33.01 ± 0.06%), the capacity diminished at the 180th s (percentage inhibition= 10.85 ± 0.06%). Generally, there was a dwindling capacity of the three anti malarials to inhibit HbS polymerization as the experimental time approached the 180th s.

The present study showed that the level of polymerization of HbS molecules was attenuated upon the introduction of the three anti malarial drugs in the polymerizing mixture. The pattern by which these drugs effected this inhibitory action was similar to phenylalanine (Ekeke and Shode, 1990; Anosike et al., 1991), methanol and water soluble extracts of dried fish (tilapia) and dried prawn (*Astacus red*) (Nwaoguikpe and Uwakwe, 2005),

Table 2. Percentage inhibition of HbS polymerization in the presence of three anti malarial drugs with time.

Drugs/ time (s)	Percentage inhibition of polymerization					
	30	60	90	120	150	180
CoartemTM	17.31 ± 0.09	31.07 ± 0.08	23.01 ± 0.08	20.49 ± 0.06	17.46 ± 0.08	17.05 ± 0.08
Quinine	28.85 ± 0.07	28.54 ± 0.10	19.47 ± 0.07	18.03 ± 0.08	15.87 ± 0.06	13.95 ± 0.08
Chloroquine P	19.23 ± 0.08	33.01 ± 0.06	23.89 ± 0.05	19.67 ± 0.10	15.08 ± 0.11	10.85 ± 0.06

methanol and water soluble extracts of *Cyperus esculentus* (tiger nut sedge) (Monago and Uwakwe, 2009).

These observations were obvious reflection of the capability of these anti-sickling agents to bind and shield the contact points of HbS monomers required for polymerization. Although these three anti malarials have been implicated in alteration of certain red blood cell elements in such a level that compromised physiochemical integrity and functionality of these cells (Chikezie et al., 2009a,b; Chikezie, 2009; Chikezie et al., 2008), our present findings showed they exhibited anti-sickling properties.

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