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Synthesis, Characteristics and Antiplasmodial studies of the deoxy and disulphide Derivatives of Dihydroartemisinin

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Abstract Malaria parasite has developed resistance to the available and affordable drugs. The world health organization has recommended the use of artemisinin and artemisinin based combination therapy (ACT) for the treatment of chloroquine resistant uncomplicated malaria. The use of the artemisinins is challenged by short half life of the drugs due to the presence of endogenous superoxide dismutase which attacks the peroxide bond and a high rate of recrudescence. These research was aimed at isosteric structural modification of dihydroartemisinin by replacing the peroxide bond with disulphide, assessing the characteristics of the products formed and the *in vivo* antiplasmodial efficacy using mice. Pure dihydroartemisinin solution was chemically reduced using hydrogen generated *in situ* from zine pellet and dilute hydrochloric acid. Chloroform was used to extract the organic phase. The reduced product was dissolved in dimethylsulphoxide and oxidized using hydrogen sulphide gas. The melting point, boiling point as well as GC-MS properties of the original starting material dihydroartemisinin, the deoxy and disulphide derivatives were analyzed. They were also tested for their antiplasmodial efficacy using *Plasmodium berghei berghei*. The stability of the products was accessed by incubating them respectively in susperoxide dismutase dissolved in phosphate buffer. Thin layer chromatographic analysis on the three compound gave R_f values of 0.71 for pure dihydroartemisinin (DHA), 0.59 for deoxydihyroartemisinin (RDHA) and 0.61 for the disulphide-dihydroartemisinin (DDHA) respectively indicating they were different compound. The GC-MS molecular ion fragment was 284, 254 and 316 for pure DHA, RDHA and DDHA respectively. Stability tests showed that RDHA was most stable followed by DDHA while pure DHA was least stable. Acute toxicity study LD_{50} was 547.70mg/kg, 273.86mg/kg and 346.41mg/kg for DHA, RDHA and DDHA respectively. The sulphide derivative had antimalaria activity close to the reference DHA, but is more stable since it is not susceptible to the endogenous enzyme superoxide dismutase.

Keywords structural modification, dihydroartemisinin, characteristics, stability, antiplasmodial

Introduction

Malaria is a mosquito-borne infectious disease of human and other animals caused by a parasitic protozoan of the genus plasmodium. Symptoms of malaria include fever, fatigue, vomiting and headache. Symptoms usually appears ten to fifteen days after the bite of an infectious mosquito and if not properly treated uncomplicated malaria many develop to severe malaria and manifest as jaundice, seizures and death [1,2].

Malaria parasite has developed resistance to most of the available and affordable drugs. Chloroquine resistance was first reported in the Cambodia region in 1975 [3]. The world health organization has recommended the use of artermisinin derivatives and its combination therapy in the treatment of uncomplicated malaria. Artemisinin, a

sesquiterpene lactone with endoperoxide bond, derived from the Chinese plant *Artemesia annua* has the fastest rate to clear malaria parasite from the blood [4]. However the main challenge of the artemisinin and its derivatives is their short systemic haft life, which is partly attributed to the endogenous enzyme superoxide dismutase which attacks the peroxide bond [5].

This research was aimed at isosteric structural modification of the artemisinin nucleus by introducing sulphur, a hetero atom to oxygen [6], and accessing some physicochemical properties, stability and antiplasmodial potency of the deoxy and sulphide derivatives of the dihydroartemisinin formed *in vivo* using mice.

Materials and Methods

Chemicals: all chemicals used in the study were of analytical grade. Pure dihydroartemisinin was a gift from May and Baker Plc. Lagos, Nigeria. All the reagents were purchased from Sigma Aldrich-Germany or BDH Chemicals - Pool England through their Nigeria representatives. A Cecil spectrophotometer CE 7200 and GC-MS-QP2012 plus - Shemadzu Japan were used for analysis.

Animals: A total of sixty healthy Swiss albino mice of both sexes weighing between 21 – 28g were used in the study. They were maintained under standard environmental condition and had free access to food and water at the animal house, Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo.

The animals were handed as approved by the Animal Ethics Committee, University of Uyo.

Chemical Reduction of Dihydroartemisinin: Dihydroartemisinin powder 500mg was dissolved in 200ml of chloroform in a flat-bottomed flask. 10.0g of zinc dust was placed in a 500ml beaker and 100ml of dilute hydrochloric acid was added to produce hydrogen gas. The DHA solution was poured into the Zn/HCl mixture for the hydrogen gas produced to reduce the peroxide bond *in situ* [7]. The Zn-HCl-DHA mixture was stirred continuously under subdued light for two hours. To the mixture, 10ml of dilute sodium hydroxide was added and stirring continued for another 10 minutes. The mixture was transferred into a separating funnel. The chloroform phase containing the deoxydihydroartemisinin (RDHA) was collected in a beaker and kept in a dark cupboard for the chloroform to evaporate, leaving yellowish powder RDHA.

Synthesis of Sesquiterpene Lactol Endodisulphide: Deoxydihydroartemisinin 10mg was dissolved in 10ml of dimethyl-sulphoxide in a large tube. Hydrogen sulphide gas from Kipps apparatus was bubbled into the solution for two hours in a fume cupboard. Precipitate formed was allowed to settle and decanted. The residue was washed with water and allowed to dry in a dessicator.

Test for the Presence of Sulphur in the Synthesized Endodisulphide (Sodium fussion test): Small quantity of the synthesized disulphide was placed in a combustion tube and small quantity of sodium metal was added. The tube was heated to red hot for the component to fuss. The resulting solid was dissolved in water and filtered. To a potion of the filtrate few drops of sodiumnitroprusside was added and to another portion lead acetate solution was added [7].

Solubility and Melting Point Determination: About 1.0mg each of pure DHA, RDHA and DDHA were placed in a small test tube, and 1.0ml of different solvents was added and stirred to observe their solubility. Some quantity of each of DHA, RDHA, DDHA were filled into a capillary tube and inserted to a melting point apparatus to observe their melting point.

Thin Layer Chromatographic Analysis: Using different solvent combination acetone/methanol/dichloromethane in ratio 2:2:1 and chloroform/methanol/ dichloromethane in a ratio of 2:2:1, the chromatographic characteristic of the pure duhydrotemisinin, deoxydihydroartemisinin and disulphidedihydrotemisinin were determined and their R_f values noted.

GC- MS Analysis: Hyphenated technique of gas chromatography coupled with mass spectrophotometer (GC-MS), Shimadzu, Japan was employed to obtain the chromatogram. The analysis was performed at a column oven temperature of 250^{o}C and a pressure of 100.2 kpa with a total flow rate of 6.2 ml/minute. The infrared spectra were recorded on Fourier Transform infrared spectrophotometer, model 8400s shimodz, Japan.

Stability in Superoxide Dismutase: Solutions of DHA, RDHA and DDHA were prepared and their wave length of maximum absorbance was determined. They were incubated in a phosphate buffer with 0.1M superoxide dismutase at 37^{o}C, and at 30 minutes, one hour and two hours, their absorbance were determined at their respective λmax [8].

Antiplasmodial Studies

Microorganism and Innoculation: Chloroquine sensitive strain of *Plasmodium berghei berghei* (ANKA) was obtained from the National Institute of Medical Research (NIMER), Lagos in January 2017 and was maintained by sub-passage in mice.

Each mouse was inoculated with 0.3ml of infected blood containing about 1.0 x10^{7} *Plasmodium berghei* parasitized erythrocytes. The inoculums contained 5.0x10^{7} plasmodium parasitized erythrocytes per milliliter. This was prepared by determining both the percentage parasitaemia and the erythrocytes count of the donor mouse and diluting the blood with isotonic saline in proportions indicated by both determinations, red blood counts and percentage parasitaemia [9].

Determinations of Median lethal Dose (LD_{50}): The UP-AND-DOWN procedure (UDP) was used in which one animal is dosed at a time to see if the dose needs to be put up or down to achieve an estimate of the LD_{50} thereby giving the minimum number of animal a lethal dose of the test substance. Doses of the drug between 200-800mg/kg body weight was administered intraperitoneally (ip) to the mice and observed for 24 to 48 hours for signs of physical toxicity. If the mice survived the dose for the next mice was increase, if it dies, the dose is decreased. LD_{50} of 547.70mg/kg was obtained for pure DHA, 273.86 mg/kg for RDHA and 346.41 mg/kg for DDHA respectively [10].

Evaluation of Prophylactic Activity: Prophylactic activity of the synthesized products were assessed as described by Peters (1965).Twenty five mice were randomly divided into five groups of five each. They were treated orally for three consecutive days between 8-9am using 10ml/kg distilled water (negative control group A), 2.2mg/kg of DHA group B, 2.4 mg/kg of RDHA group C, 3.1mg/kg of DDHA group D and 5.0mg/kg of chloroquine group E (positive control). On the fourth day (D_3) the mice were inoculated with *Plasmodium berghei*. The parasitaemia levels were assessed by blood smear seventy two hours later.

Suppressive Activity: The Knight and Peter's four day test was adopted against chloroquine sensitive *P. berghei* in mice [11]. Twenty five mice were inoculated on the first day D_o intraperitoneally with 0.2mL of infected blood containing 1.0x10^{7} *P. berghi*- parasitized erythrocytes. They were divided into five groups of five each. They were treated orally for four consecutive days using 2.2mg/kg of DHA group B, 2.4 mg/kg of RDHA group C and 3.1 mg/kg of DDHA group D respectively. There were two control groups; the positive control group E received 5.0mg/kg body weight of chloroquine, while the negative control group A were given equal volume of sterile water.

On the fifth day, thick blood film was made from the tail blood. The film was then stained with leishman's stain to reveal parasitized erythrocytes out of 500 in a random field of the microscope The average percentage suppressive effect of parasitaemia was calculated in comparison with the controls as follows:

$$\frac{\text{Average\% parasitaemia in negative control} - \text{Average \% parasitaemia test group}}{\text{average \% parasitaemia in negative control.}}$$

Evaluation of Curative Activity: Rane's test was used to evaluate the schizonticidal activity of DHA, the synthesized RDHA and DDHA and chloroquine. This was done as described by Ryley and Peter (1970) [12]. *P. berghei* was injected intraperitoneally into 25 mice on the first day (D_o). Seventy-two hours later, (D_3) the mice were randomly divided into five groups of five mice per group and the various drugs were administered orally as follows, groups B,C and D received 2.2 mg/kg DHA, 2.8mg/kg RDHA, 4.1mg/kg DDHA respectively, while group four (E) received 5.0mg/kg chloroquine and group A were given equal volume of distilled water.

On the fifth day, thick blood film was made from tail blood. The film was stained with laishman's stain to reveal parasitized erythrocytes out of 500 in a random field of microscope. The average percentage suppression of parasitaemia was calculated in comparison with the control as follows:

$$\frac{\text{Average \% parasitaemia in negative control} - \text{Average \% parasitaemia in test group}}{\text{Average \% parasitaemia in negative control}}$$

The mean survival time (MST) in each treatment group was determined over a period of 29 days ($D_o - D_{28}$)

$$\frac{\text{No. of days survived}}{\text{Total No. of day (29)}} \times \frac{100}{1} = \text{MST}$$

Statistical Analysis: The results were expressed as mean ± standard deviation (SD). Parameters in the group were compared by one way (ANOVA) using the computer software statistical package for social sciences (SPSS) version 16. All data were analyzed at 95% confidence interval and values were considered statistically significant at $p < 0.05$.

Results and Discussion

Synthesis: The result for the chemical reduction of DHA and subsequent addition of sulphur was confirmed by the nitroprusside test which gave a blue precipitate in the DDHA but not with DHA and RDHA, and lead acetate which gave a black precipitate with DDHA and not with DHA and RDHA. This confirms that the sulphur derivative was produced.

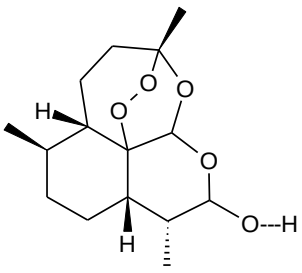
Solubility, Melting point and Thin layer chromatographic results: All the test samples DHA, RDHA and DDHA were all soluble in the solvents used except water and petroleum ether. These shows that they have similar chromophore and polarity. The melting points were; DHA 147±1.0^{o}C, RDHA 150±1.0^{o}C and DDHA 141±1.0^{o}C respectively. Thin layer chromatographic analysis also showed different R_f values for each of the products 11.9, 8.8 and 9.1 for DHA, RDHA and DDHA respectively. The differences in melting point and R_f values clearly confirms that new compounds were produced during the reduction with hydrogen and subsequent re-oxidation with sulphur [7].

GC-MS analysis: The fragmentation pattern for the test compounds is as shown;

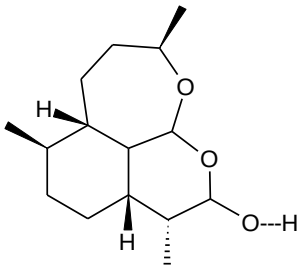
Compound **1:** Dihyroartemisinin (DHA): $C_{15}H_{24}O_5$; MS [ES+-MS] m/z (relative intensity): 284 $[M]^+$ (28.45 %), 270 $[M\text{-}CH_2]^+$ (45.24 %), 266 $[M\text{-}H_2O]^+$, 256 $[M\text{-}CO]^+$ (20.07 %), 254 $[M\text{-}(CH_3)_2]$ (2.50%)$^+$ 41 $[M\text{-}C_{13}H_{24}O_4]^+$ (100.00 %) (Base peak),

Compound **2,** Deoxydihydroartemisinin (RDHA)**:** $C_{15}H_{24}O_5$; MS [ES+-MS] m/z (relative intensity): 254 [M]+ (1.05 %), 253 [M-H]+ (2.82 %), 238 $[M\text{-}O]^+$ (1.67 %), 226 $[M\text{-}CO]^+$ (52.77 %), 222 [M-2O]+ (24.84 %), 220 $[M\text{-}H_2O_2]^+$ (21.53 %); 212$[M\text{-}C_2H_2O]^+$ (10.53 %), 184$[M\text{-}C_2H_2O_2]^+$ (21.53 %), 43$[M\text{-}C_{13}H_{23}O_2]^+$ (100.00 %, base peak).

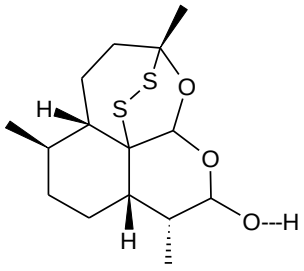
Compound **3,** Sesquiterpene lactol endodisulphide (DDHA)**:** $C_{15}H_{24}O_3S_2$; MS [ES+-MS] m/z (relative intensity): 316 [M]+ (1.35 %), 298 $[M\text{-}H_2O]$+ (62.82 %), 282 $[M\text{-}H_2S]^+$ (31.67 %), 256$[M\text{-}CH_2COOH]^+$ (21.53 %), 254$[M\text{-}H_2S - 32]^+$ (21.53 %), 248 $[M\text{-}(H_2S)_2]^+$ (12.77 %), 91$[M\text{-}C_{13}H_{23}O_2]^+$ (100.00 %, base peak).



Dihydroartemisinin



Deoxydihydroartemisinin



Sesquiterpene Lactol endodisulpide

The dihydroartemisinin, the reduced deoxydihydroxyartemisinin and the new compound; sesquiterpene lactol endosulphide were subjected to mass spectrometry (MS).The obtained MS data were matched with library data of organic compounds. The compounds were identified by comparing their diagnostic peaks with library data of these

compounds. The ES+-MS of **1** showed diagnostic peaks such as $[M]^+$ at m/z 284 $[M]^+$ (28.45 %) while 270 (45.24 %), 256 (20.07 %) and the base peak at 41 (100.00 %) represent ES the losses of - CH_2, -H_2O, and -$C_{13}H_{24}O_4$ units respectively from the $[M]^+$. Many fragmented ions also appeared in the MS of **2** but those that could readily be identified include: $[M]^+$ at m/z 254 (1.05 %) while 220 (21.53 %) indicates the loss of -H_2O_2 and $C_{13}H_{23}O_2$, the base peak. The MS fragments of **3** showed $[M]^+$ at m/z at 316 (1.35 %), loss of $–H_2S$ and S at m/z 254.
$^+$-MS data were run on Kratos MS 80

Stability with superoxide dismutase: The results of stability on incubation of test samples with (SOD) is as shown in table one. On incubation with SOD in phosphate buffer after one hour, the concentration of the pure DHA was significantly reduced from 3.17µg/ml to 0.31 µg/ml. there was no significant effect with deoxy and sulphide derivates. This suggest that DHA with the peroxide bond is not stable in SOD *in vitro* and may be the same *in vivo*.

Table 1: Incubation of pure dihydroartemisinin, deoxy and sulphur derivative with the enzyme superoxide desmutase (SOD)

Drug	**Concentration (µg/ml)**			
	Without SOD		**With SOD**	
	Before Incubation	**1.0 hour after Incubation**	**Before Incubation**	**1.0 hour after Incubation**
Pure DHA	3.17 ± 0.02	2.38 ± 0.03	3.17 ± 0.02	0.31±0.03
Sulphide DDHA	4.03 ± 0.11	3.88±0.91	4.03 ± 0.11	3.61±1.07
Deoxy RDHA	3.95 ± 0.17	3.01± 0.73	3.95 ± 0.17	3.00±1.10

Values are expressed as mean ± SEM. Significant at P< 0.05 relative to value before incubation.

Antiplasmodial Results

Table 2: Prophylactic/Repository activity

Groups Treatments	**Dose**	**Parasitaemia**	**% Chemosupression**
A. Distilled water	10ml/kg	41.02±1.38	-
B. Dihydroartermisinin	2.2mg/kg	19.10±1.22	53.43
C. Deoxydihydroartermisinin	2.4mg/kg	34.11±4.12	16.66
D. Disulphider DHA	3.1mg/kg	2.31±0.22	94.36
E. Chloroquine	5.0mg/kg	4.69±0.99	88.56

Values are expressed as means ± SEM significance relative to control p<0.5, (n=5)

The result of prophylactic treatment where the mice received the drugs before infection with *P. berghi* shows that DHA, chloroquine and the disulphide DHA prevented infection as seen in the reduction in parasitaemia to 19.10±1.22, 4.69±0.99 and 2.31±0.22 respectively. Parasitaemia was high in the negative control group and deoxydihydroartermisinin.

Table 3: Suppressive activity of dihydroartenisinin (DHA) and synthesized products deoxy (RDHA) and sulphur (DDHA) derivatives on *Plasmodium berghei berghei* infection in mice

Treatment	**Groups**	**Parasitaemia**	**% Chemosuppression**
Distilled water	A	38.83±2.71	-
Pure DHA	B	5.88±1.29	84.85
Reduced (RDHA)	C	38.32±0.98	1.31
Sulphide (DDHA)	D	5.74±0.97	85.22
Chloroquine (CQ)	E	6.62±0.88	82.95

Values are expressed as mean ± SEM, significant relative to control at *p< 0.05, (n=5).

Table 3 shows the result of suppressive activity of DHA and its reduction and sulphur derivatives relative to control. The sulphor derivative DDHA prevented infection by the parasite as seen in the reduction in parasitaemia to 5.74 giving 85.22 % chemosuppression. This is significant $p< 0.05$ relative to control. The deoxy derivative devoid of peroxide bond showed no suppressive activity.

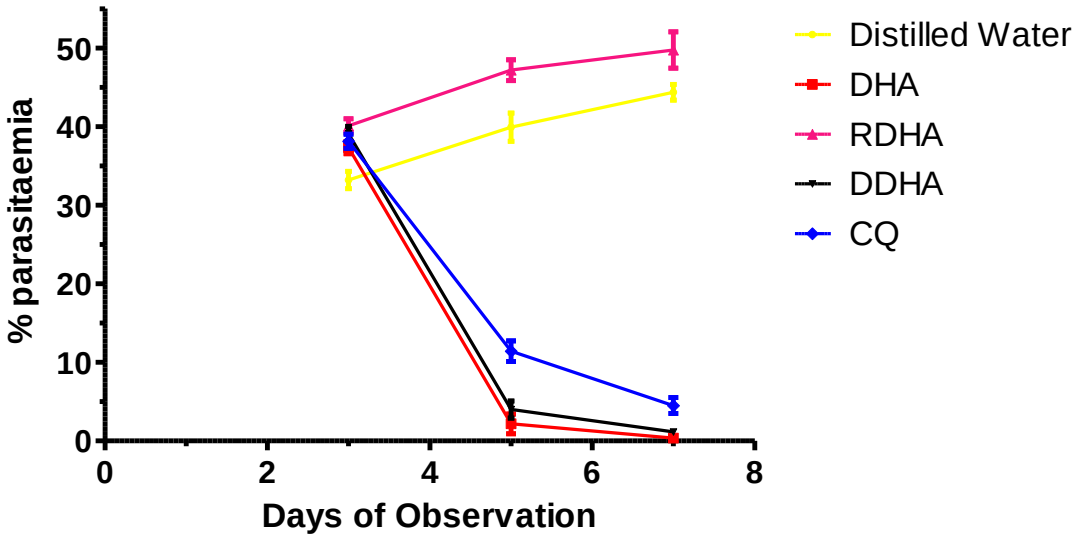


Figure 1: Effect of dihydroartemisinin (DHA), deoxy (RDHA) and sulphide (DDHA) derivative on established P. berghei infection compared with chloroquine (CQ) and distilled water (control)

Figure one shows the result of administering DHA and derivative on *P-berghei* established infection. There was a daily increase in parasitaemia in the negative control group and the RDHA derivative. There was also a daily reduction in the parasitaemia levels in group receiving pure DHA, DDHA and the positive control.

Table 4: Mean survival time of mice

Treatment	Groups	Mean Survival Time
Distilled water	A	11.21±0.76
Pure DHA	B	27.42±0.39
Reduced (RDHA)	C	13.11±0.23
Sulphide (DDHA)	D	26.39±0.87
Chloroquine (CQ)	E	26.13±1.01

Values are expressed as mean + SEM. Significant relative to control $P< 0.05$.

The result for average survival time is as shown on table 4. The mean survival time for mice treated with pure DHA and DDHA is above two weeks and comparable to that of the positive control. The negative control distilled water and RDHA had a mean survival time less than two weeks.

Discussion

The drug dihydroartemisinin a schizontocidal antimalarial is effective on asexual species of all malaria parasites [14]. Isosteric substitution of the peroxide bind produced the sulphide derivative with different physicochemical properties, stability and toxicity from the parent compound. The GC-MS expected molecular ion for the sesquiterpene lactol endodisulphide, sulphur substituted product should be 316 or 317 allowing for isotopic sulphur. The detected ion fragment results from the loss of a molecule of water M^+(-18). This ion fragment is only present in the spectrum of sulphur substituted compound. The loss of neutral molecule H_2S from molecular ion 316 gives the

fragment of mass 282. In the pure DHA with molecular ion M+284. The loss of $CH_3C^+=0$ fragment from M^+ gave the fragment 241. From above fragmentation, it was observed that all the compound follow a similar pattern in mass fragmentation.

Antiplasmodial study showed that the sulphur substituted dihydroartemisinin has a significant suppressive effect against early infection, curative effect against established infection and suppressive effect against residual infection of the parasite using 30% of LD_{50} dosage. However, the pure DHA had low prophylactic activity, probably due to its short systemic half life. With the stability of the sulphur derivative in superoxide desmutase enzyme, it implies that it should have a longer half life in the body and act as a better antimalarial.

Conclusion

The oral median lethal dose >300mg/kg body weight suggests that orally administered sulphur derivative of DHA is practically non-toxic. The synthesized sesiquiterpene lactol endodisulphide being resistant to endogenous enzyme speroxide dismutase, and less toxic with significant chemosuppression comparable to dihydroarteminin and chloroquine could be considered for treatment of resistant malaria, after necessary clinical trials.

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