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In-vivo Anti-tumor Evaluation of Dihydroartemisinin-Derived Endodisulphide on MNU-induced Liver Cancer in Sprague-Dawley Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors contributed in the design of the study and participated in conducting the experiments. Author IEU managed the literature searches. Author JSA performed the statistical analysis, wrote the protocol and the first draft of the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The study evaluated the antitumor potentials of a disulphide-substituted derivative of dihydroartemisinin (sDHA) on chemically induced cancer of the liver in Sprague-Dawley rats in comparison with its parent compound, dihydroartemisinin (DHA) and a standard anticancer drug.
Study Design: Animals were divided into seven experimental and three control groups (n=10 per group). Cancer was induced in experimental groups followed by administration of experimental agents, while control groups received either *N*-methy-*N*-nitrosourea (MNU), Tween 80 (vehicle) or distilled water alone.
Place and Duration of Study: Study was done in Faculty of Pharmacy, University of Uyo, Nigeria in 2015-2016.

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Methodology: MNU (50 mg/kg) was administered intravenously as single dose to induce cancer in experimental groups, followed by oral treatment with sDHA (37.42, 74.83 or 112.25 mg/kg/day), DHA (57.45, 114.89 or 172.34 mg/kg/day) or cyclophosphamide (0.71 mg/kg/day) for 28 days. Positive control group received only MNU, negative control group received only distilled water (0.3 ml/day), while experimental control group received only Tween 30 (0.3 ml/day). Drug treatments commenced 10 days after MNU injection and animals were observed for 52 days after drug treatments and sacrificed. Serum levels of CA-27-29, 8-OHdG and SOD were measured using ELISA method; and using immunohistochemical tissue staining techniques, Bcl-2 and Ki67 protein expressions were analyzed in hepatic cells.
Results: MNU caused elevation (P <.0001) in CA-27-29 and 8-OHdG; and reduction (P<.0001) in SOD. Hepatic cells of MNU alone treated rats demonstrated strong immunoreactivity for Bcl-2 and Ki67 (≥75%). Oral treatments of sDHA or DHA resulted in dose-dependent reductions of MNU-induced CA-27-29 and 8-OHdG elevations (P<.001) but had no effect on SOD. Additionally, MNU induced Bcl-2 and Ki67 positive immunoreactive expressions were reduced to 25-50% by sDHA and DHA; DHA showing greater effect. Cyclophosphamide reversed all the MNU induced toxic effects.
Conclusions: sDHA possesses antitumor activity against liver cancer; has lesser efficacy than DHA, but both drugs are less effective than cyclophosphamide.

Keywords: Artemisinin; Bcl-2; CA-27-29; cyclophosphamide; immunohistochemistry.

1. INTRODUCTION

Cancers are among the leading causes of morbidity and mortality globally [1]. Different treatment approaches exist for the disease, but chemotherapy (the use cytotoxic drugs) remains the first line of treatment for many cancers [2,3]. Although, standard anticancer drugs can produce good responses and survival, many unfortunately, are expensive, require frequent administration and cause serious adverse effects (especially in combination). Thus, the search for anticancer drugs that are affordable with high safety profiles while maintaining their efficacies continues.

Artemisinin derivatives are clinically used as antimalarial agents and have relative low host toxicity [4,5]. They have equally been shown to possess potent anticancer activity against a variety of human cancer models [6-8]. The presence of endoperoxide group in their molecules, have been shown to be responsible for this anti-cancer activity, but their short half-lives [5], may be a limitation in their utilization in cancer chemotherapy. It is envisaged that superoxide dismutase (SOD) enzyme, which is present in human blood, readily breaks down the pharmacophore (endoperoxide group) of artemisininsvia its anti-peroxide action and this is thought to contribute to artemisinins short half–lives [9]. In view of the above, it is hypothesized that structural modification of the endoperoxide ring of artemisinins via substitution of the peroxide oxygen atoms with suitable atoms may resist SOD effect and extend their half-lives, which has led to the synthesis of a disulphide derivative of DHA, the active metabolite of artemisinin [10]. The new drug was synthesized through structural modification of DHA by replacing its peroxide oxygen atoms with disulphide, or persulphide [10]. With the synthesis of this new compound, it would be reasonable to evaluate its pharmacokinetic, toxicity and anticancer profiles. Acute toxicity evaluation of the disulphide derivative of DHA has been done in our earlier study [11]. It has also been demonstrated to exhibit remarkable dose related anti-malaria effect in rats which compared favorably with DHA [10], but it is not yet known whether it would maintain or possess higher anticancer activity with respect to the parent drug.

This study was therefore intended to conduct an *in-vivo* evaluation of the antitumor potential of disulphide substituted derivative of DHA on *N*-Methyl-*N*-Nitrosourea (MNU) induced liver cancer in Sprague-Dawley rats in comparison with the parent drug.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

Microplate reader (Biotek, USA), pH meter (Denver Instrument, Model: 215, New York, USA), centrifuge (Zeny Inc., Model: 800-1, Salt

Lake, USA), UV/Visible spectrophotometer (Biotech, Model: 7305, Winooski, USA), rotary microtome (Leica Biosystem, Model: Leica RM 2125, Germany), wax dispenser (MH 85523B, United Kingdom), Microfield digital laboratory oven (Model: SM90234, England), automatic tissue processor (Leica TP1020, Germany), digital Olympus microscope (Model: XXT/300, Germany) and autoclave (Lifesteriware Pvt Ltd., Delhi, India) were among the equipment used for the study.

2.1.2 Drugs

Pure powder of dihydroartemisinin, DHA (UV spectral ultraviolet-visible absorption spectra absorption- 0.55, max wavelength- 270 nm; GC-MS fragmentation showed a significant molecular ion of 284; Melting point- 146-148°C); and disulphide substituted derivative of DHA, sDHA (UV spectral ultraviolet-visible absorption spectra absorption- 0.268, max wavelength, 320 nm; GC-MS fragmentation showed a significant molecular ion of 316; Melting point- 140-142°C) were obtained from the Department of Medicinal and Pharmaceutical Chemistry, Faculty of Pharmacy, University of Uyo, Nigeria.

Cyclophosphamide (Endoxan®) tablets (Zyus Biogen- Baxter, Mumbai, India), was purchased from the Pharmacy Unit of the University of Uyo Teaching Hospital, Uyo, Nigeria.

2.1.3 Reagents

CA 27-29 assay kit (Cell Biolabs Inc., San Diego, CA 92126, USA), 8-OHdG assay kit (Cell Biolabs Inc., San Diego, CA 92126, USA), SOD assay kit (Cell Biolabs Inc., San Diego, CA 92126, USA), Bcl-2 assay kit (BioVision Inc. 155 South Milpitas Blvd., Milpitas, California 95035, USA), Ki67 assay kit (BioVision Inc. California 95035, USA) and haematoxylin and Eosin stain kit (Sigma- Aldrich Inc. St. Louis, MO 63178. USA) were among reagents used for the study.

2.1.4 Chemicals

N-Methyl-*N*-Nitroso urea, MNU (Sunglong Biotech Co. Ltd, Zhejiang, China), ketamine HCl (Merck, Darmstadt, Germany), xylazine (Taj Pharmaceutical Limited, Mumbai, India), polysorbate (Tween®) 80 (Sigma-Aldrich and Merck, KGaA, Darmstadt, Germany), paraffin wax (Megha Group of Companies, Meghalaya, India), TRIS-acetate-EDTA buffer (Sigma-Aldrich Inc. St. Louis, MO 63178. USA), and phosphate buffer saline (Sigma- Aldrich Inc. St. Louis, MO 63178. USA), xylene (Taj Pharmaceutical Limited, Mumbai, India) were among chemicals used for the study.

2.1.5 Animals

Healthy, pathogen free male and non-pregnant female Sprague-Dawley rats, aged 6-8 weeks old, weighing 180-200 g were used for the study. They were housed in plastic cages (3 per cage) and maintained under standard conditions (12 h light-dark cycle, and room temperature). The rats were given free access to clean water and commercial rodent chow and allowed to acclimate for two weeks before commencement of experiment. Experimental procedures were approved by the Ethics Committee on Research, University of Port Harcourt, Nigeria. Animals were handled according to standard guidelines for care and use of laboratory animals [12].

2.2 Methods

2.2.1 Experimental design

The rats (50 females and 50 males) were randomly distributed into ten groups (labelled as groups 1-10) containing 10 animals of same sex per group. Seven groups were administered *N*-Methyl-*N*-Nitrosourea (MNU) as a single dose (50 mg/kg) through the jugular vein to induce cancer [13], and ten days afterwards treated orally with DHA (57.45, 114.89 or 172.34 mg/kg body weight), sDHA (37.42, 74.83 or 112.25 mg/kg body weight) or cyclophosphamide (0.71 mg/kg body weight) continuously for 28 days. The eighth group (positive control group) received only MNU treatment and experienced the maximal number of tumors. The ninth group (negative control) received only distilled water (0.3 ml) and generated no spontaneous tumors during the entire period of the study. The tenth group was given the vehicle of the drugs (Tween 80, 0.3 ml) and used as the experimental control group. Dosing was performed once daily between 4 and 6 pm.MNU was dissolved in physiological saline (1% w/v) buffered to pH 5.0 with 3% acetic acid, whereas the drugs, except cyclophosphamide were dissolved in Tween 80 (30%). Cyclophosphamide was dissolved in distilled water. The doses of sDHA and DHA corresponded to 10, 20 and 30% of their LD_{50} [11], while the dose of cyclophosphamide was equivalent to its therapeutic dose for cancer chemotherapy [14].

After the drug administrations, the rats were observed without treatment for additional 52 days. On the 91st day of the experiment, the animals were anesthetized with ketamine/xylazine (150/10 mg/kg, ip) and sacrificed by cervical dislocation. Blood was collected by cardiac puncture and blood serum was used to analyze specific biochemical cancer biomarkers: cancer antigen (CA-27-29), 8-hydroxydeoxyguanosine (8-OHdG) and superoxide dismutase (SOD). Livers were carefully removed and fixed in neutral buffered formalin for less than 24 h and immunohistochemically analyzed afterwards.

2.2.2 Measurement of cancer biomarkers

The cancer biomarkers analyzed were- CA 27-29, 8-OHdG and SOD. Briefly, blood serum (10 µl) and the sample dilution buffer (40 µl) was mixed in an antibody pre-coated micro ELISA strip plate specific for CA 27-29, 8-OHdG or SOD and incubated at 37°C for 30 min. The wells of the plates were washed and horseradish peroxidase (HRP)-conjugated antibody (50 µl) specific for CA 27-29, 8-OHdG or SOD was added and incubated at room temperature. TMB substrate solution- chromogenic solution A and B (50 µl each) was then added and incubated at 37°C for 15 min after proper mixing with sample solution in wells. Finally, the reaction was terminated with 0.16 M sulfuric acid (stop solution) and CA 27-29, 8-OHdG or SOD concentration was measured spectrophotometrically at 450 nm.

2.2.3 Immunohistochemical analysis (Bcl-2 and Ki67)

Immunohistochemistry was performed with the following primary and secondary antibodies: (1) Bcl-2 primary antibodies- mouse anti-Bcl-2 monoclonal antibody, C-2, 10 µg/ml (Santa Cruz Biotechnology, Santa Cruz, CA 95060). (2) Ki67 primary antibodies- mouse anti-rat Ki-67 antibody, MIB-5, 0.01 µL/ml (Dakocytomation corporation Carpinteria, CA 94010). (3) Bcl-2 and Ki67 secondary antibodies- biotinylated horse anti-mouse IgG, H+L (Vector Laboratories Inc., Burlingame, CA 94010). Tissue sections of liver (5-µm) were deparaffinised in three changes of xylene, hydrated through graded ethyl alcohol and rinsed in 3-4 changes of buffered phosphate, ending with a distilled water rinse. After removing excess water, 3% hydrogen peroxide was applied to the tissues and incubated for 5 min. The slides used were previously preheated (95°C) in a target retrieval solution for 20 min, allowed to cool for 20 min at room temperature, rinsed gently, and placed in Tris buffer bath for 5 min. Primary antibodies for Bcl-2 or Ki67were applied unto the tissue specimens and incubated for 10 min. Slides were washed in buffer and incubated with the secondary antibodies for 10 min. Sufficient freshly prepared 3,3'-diaminobenzidine (DAB) solution (the chromogen) was applied and incubated for 5 min for color detection. Tissues were lightly counterstained with Mayer's haematoxylin, blued in warm tap water, dehydrated through ascending grade of alcohol, cleared in xylene, cover-slipped and mounted with a compatible mounting medium. Positively stained cells for Bcl-2 or Ki-67 expression were identified by brown nuclear or cytoplasmic staining against light blue background, respectively. Specimens were considered immunopositive when more than 10% of cells showed clear evidence of immunostaining.

2.2.4 Statistical analysis

Analysis of data was carried out using GraphPad Prism Version 6 and all data were expressed as mean±standard error of mean (mean±SEM). Differences among treatment groups were analyzed using one way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test for intra group comparisons. Values were considered significant at $P<.05$.

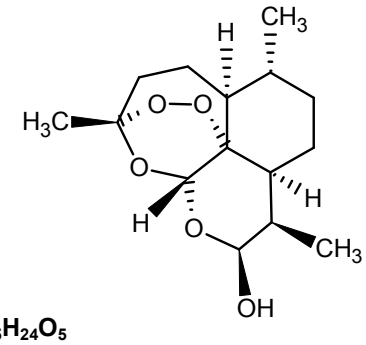
3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Serum levels of cancer biomarkers

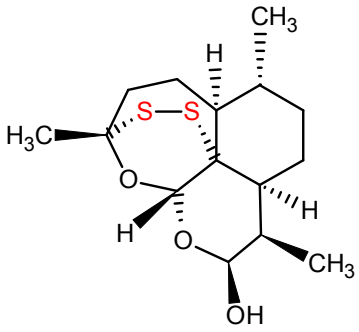
The results showed that there were no significant differences ($P>.05$) between the serum levels of CA 27-29 (cancer antigen), 8-OHdG (DNA oxidative product) and superoxide dismutase (SOD) in Tween 80 (vehicle) treated group and distilled water treated (control) group (Figs. 2-4). In addition, serum levels of CA 27-29 and 8-OHdG were significantly increased ($P< .001$) in MNU-treated group of rats, while SOD was decreased ($P<.001$) compared to control group (Figs. 2-4).

In dihydroartemisinin (DHA) or disulphide-substituted derivative of DHA (sDHA) treated groups, the serum levels of CA 27-29 and 8-OHdG were decreased ($P<.001$) compared to MNU-treated group. When compared with



$C_{15}H_{24}O_5$

Sesquiterpene lactol endoperoxide
Dihydroartemisinin



$C_{15}H_{24}S_2O_3$

Sesquiterpene lactol endodisulphide
Disulphide substituted derivative of dihydroartemisinin (sDHA)

Fig. 1. Chemical structures of dihydroartemisinin and its disulphide substituted derivative (Structures drawn by chem.draw format)

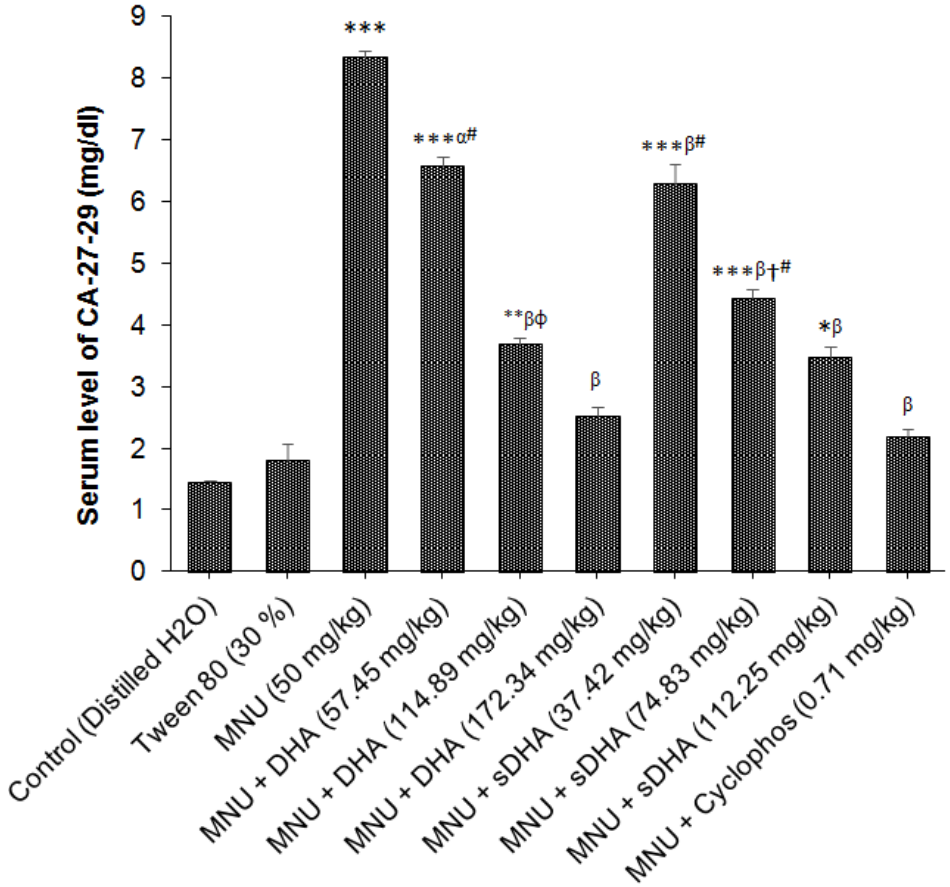


Fig. 2. Effects of 28 days administration of dihydroartemisinin (DHA), disulphide-substituted DHA (sDHA) and cyclophosphamide (cyclophos) on MNU-induced elevation of serum CA-27-29 antigen level in Sprague-Dawley rats

*Test drugs, significant from control, * P< 0.05; ** P < 0.01; *** P< 0.001, Test drugs, significant from MNU, $^{\alpha}P$ = 0.01; $^{\beta}P$< 0.001, sDHA treatment, significant from DHA, $^{\dagger}P$ = 0.01, Test drugs, significant from cyclophosphamide, $^{\phi}$ P< 0.05; $^{\#}$ P< 0.001, Mean ± S.E.M = Mean values ± Standard error of means of ten experiments*

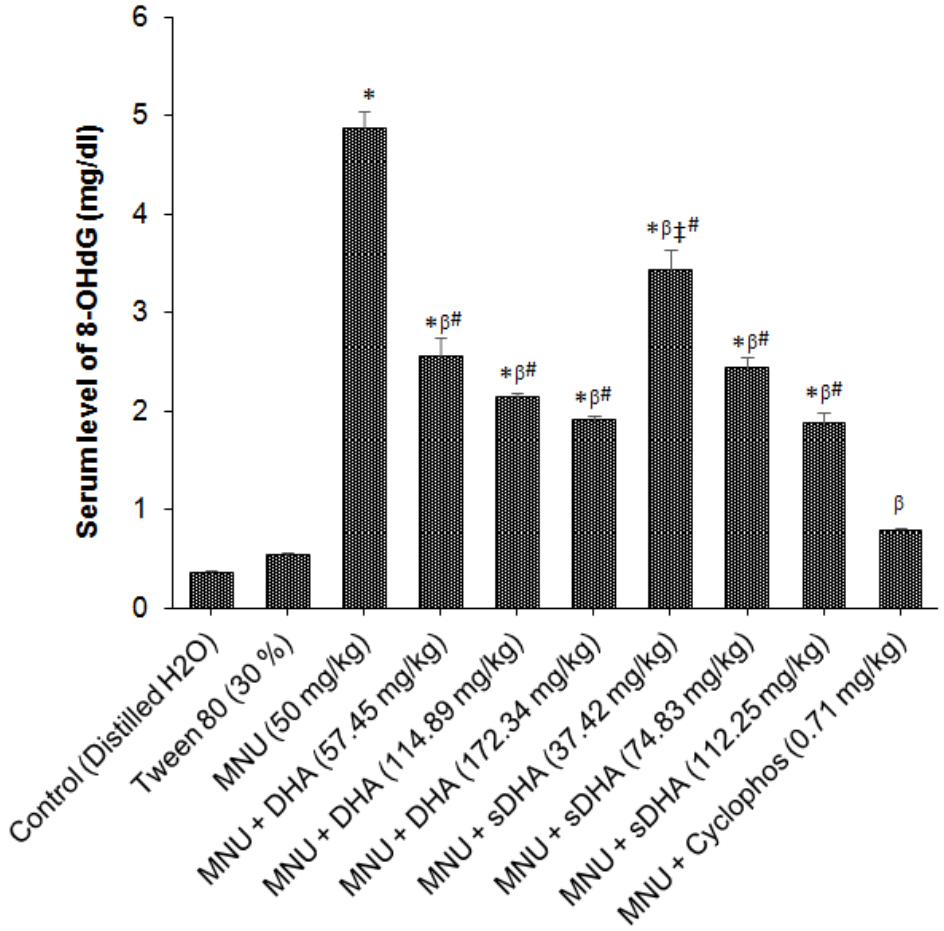


Fig. 3. Effects of 28 days administration of dihydroartemisinin (DHA), disulphide-substituted DHA (sDHA) and cyclophosphamide (cyclophos) on MNU-induced elevation of serum 8-hydroxydeoxyguanosine, 8-OHdG (cell DNA damage marker) level in Sprague-Dawley rats

*Test drugs, significant from control, * P< 0.001, Test drugs, significant from MNU, $^{\beta}$P< 0.001 sDHA treatment, significant from DHA, † P = 0.001, Test drugs, significant from cyclophosphamide, $^{\#}$ P < 0.001, Mean ± S.E.M = Mean values ± Standard error of means of ten experiments*

control, the CA 27-29 value that was obtained in high dose (172.34 mg/kg) DHA treated group was not significantly different, but all other values were higher (Figs. 2 and 3). CA 27-29 and 8-OHdG concentrations were equally reduced (*P*<.001) in cyclophosphamide treated rats compared to MNU alone treated rats, and the values were although, higher but not significantly different (*P* = .05) when compared with control (Figs. 2 and 3). The serum levels of SOD in all DHA or sDHA administered rats were lower (*P*<.001) than control (Fig. 4). The values obtained were also not significantly different from the MNU-induced level, except in the group that received high dose DHA, which had a higher (*P*< .001) level (Fig. 4). SOD level was increased in cyclophosphamide (*P* = .01) treated rats compared with MNU treated rats, but the value was lower (*P*<.001) when compared with control (Fig. 4).

Further, when DHA and sDHA effects were compared, sDHA inhibition of MNU-induced serum elevations in CA 27-29 and DNA 8-OHdG was lower, mostly at the low and mid doses, *P* = 0.01, *P*< .001 (Figs. 2 and 3). Also, only DHA inhibited MNU's effect on SOD, which was observed only in the high dose treated group (Fig. 4). However, the effects of DHA and sDHA were significantly lower (*P*<.001) when compared with cyclophosphamide (Figs. 2-4).

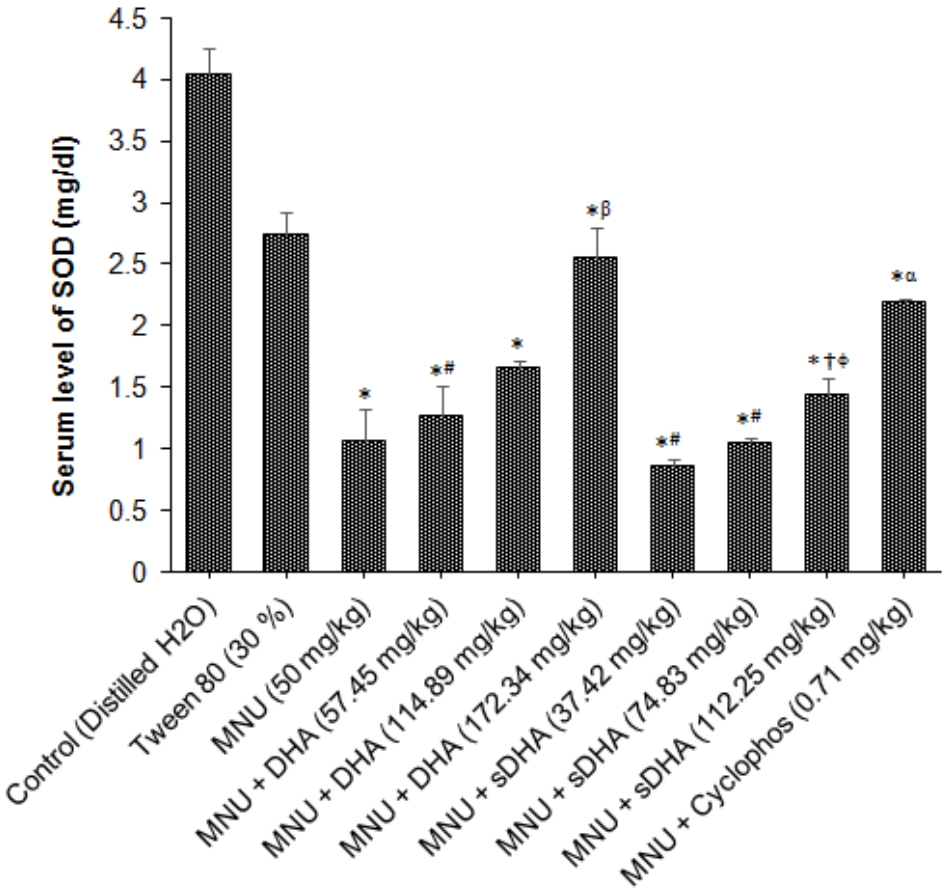


Fig. 4. Effects of 28 days administration of dihydroartemisinin (DHA), disulphide-substituted DHA (sDHA) and cyclophosphamide (cyclophos) on MNU-induced reduction of serum superoxide dismutase (SOD) enzyme level in Sprague-Dawley rats

*Test drugs, significant from control, * $P < 0.001$*
Test drugs, significant from MNU, $^{\alpha}$ $P < 0.01$; $^{\beta}$ $P < 0.001$
sDHA treatment, significant from DHA, † $P < 0.01$
Test drugs, significant from cyclophosphamide, $^{\phi}$ $P < 0.05$; $^{\#}$ $P < 0.01$
Mean ± S.E.M = Mean values ± Standard error of means of ten experiments

3.1.2 Immunohistochemistry

Liver cells of rats treated with distilled water (control) showed negative expression of Bcl-2 and Ki67 (<5% positivity), whereas MNU treatment caused strong positive immunoreactive expressions (>75% positivity) of Bcl-2 and Ki67 (Figs. 5 and 6). Liver cells of rats that received DHA or sDHA showed positive Bcl-2 expression; reactivity obtained was <75, <50 and <25% positivity, respectively (Fig. 5). Ki67 was expressed differently in DHA and sDHA treated rats: positive expression was observed in all sDHA treated rats (reactivity <60, <50 and <50% positivity, respectively), whereas it was positive only in rats that received low and mid doses of DHA, <25, <25 and <10% positivity, respectively (Fig. 6). In addition, the liver cells of cyclophosphamide treated rats showed negative Bcl-2 and Ki67 expressions (<5 or 10% positivity) as shown in Figs. 5 and 6.

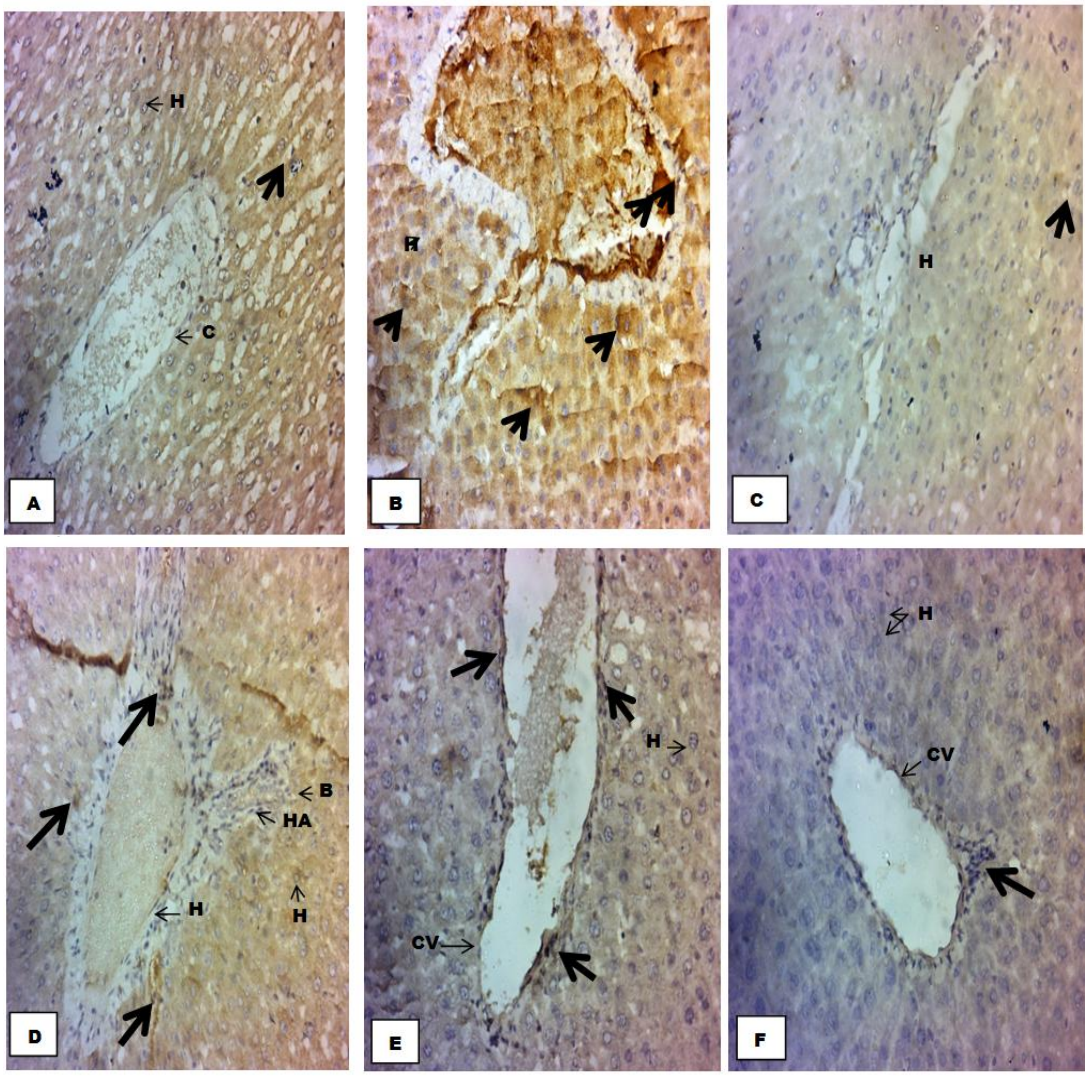
3.2 Discussion

This study reports the antitumor potential of a newly synthesized derivative of dihydroartemisinin (DHA) in comparison with the parent drug. The new compound being investigated is a sesquiterpene disulphide substituted derivative of DHA (sDHA) believed to have an extended half-life than the parent drug. Preliminary studies on the new drug

have shown that the drug exhibits an antiplasmodia effect in rats which compared favorably with the parent drug [9], but its anticancer activity was yet to be investigated prior to this study.

In this study, three doses (low, medium and high) of sDHA and DHA, equivalent to 10, 20 and 30% of their LD_{50}, respectively were used, while cyclophosphamide was used as a standard anticancer drug. Further, *N*-Methyl-*N*-Nitrosourea (MNU) was used to induce cancer in the liver of Sprague-Dawley rats in this study. This model has been used extensively to test the preventive potential of a variety of chemical compounds and interventions [12,15]. Specific biochemical and immunohistochemical cancer biomarkers were evaluated which included- CA 27-29, 8-hydroxydeoxyguanosine (8-OHdG), superoxide dismutase (SOD) enzyme, B-cell lymphoma 2 (Bcl-2) and Ki67.

From our results, MNU induced marked elevations in serum levels of CA 27-29 and 8-OHdG and reduction in SOD enzyme, indicative of cancer development in target tissues [16-19]. The immunohistochemistry results showed that the liver cells of rats that received only distilled water demonstrated negative Bcl-2 and Ki67 expressions (<5% positive); whereas there were very strong positive immunoreactive nuclear/cytoplasmic expressions of Bcl-2 and Ki67 (>75% positive) in the hepatic cells of MNU-injected rats without drug treatment. Bcl-2 is a protein that regulates cell death (apoptosis) in the body, basically by either inducing



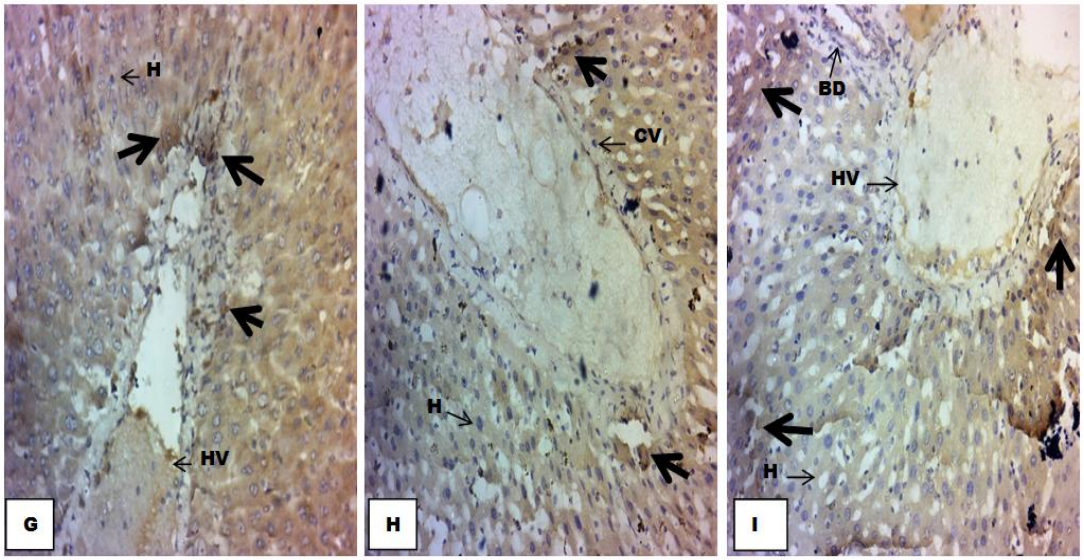


Fig. 5. Photomicrographs showing hepatic cell expression of Bcl-2 antigen in MNU treated and MNU plus DHA, sDHA or cyclophosphamide treated Sprague-Dawley rats (Bcl-2 immunohistochemical method, x400)

A: Liver of animals treated with distilled water (Control), showing negative immunoreactive nuclear expression for Bcl-2 (less than 5% positivity).

B: Liver of animals treated with MNU (50 mg/kg) alone, showing strong positive immunoreactive nuclear expression for Bcl-2 (more than 75% positivity).

C. Liver of animals treated with MNU plus cyclophosphamide (0.71 mg/kg), showing negative immunoreactive nuclear expression for Bcl-2 (less than 5% positivity).

D: Liver of animals treated with MNU plus DHA (57.45 mg/kg), showing strong positive immunoreactive nuclear expression for Bcl-2 (less than 75% positivity).

E: Liver of animals treated with MNU plus DHA (114.89 mg/kg), showing strong positive immunoreactive nuclear expression for Bcl-2 (less than 50% positivity).

F. Liver of animals treated with MNU plus DHA (172.34 mg/kg), showing positive immunoreactive nuclear expression for Bcl-2 positive (less than 25% positivity).

G: Liver of animals treated with MNU plus sDHA (37.42 mg/kg), showing strong positive immunoreactive nuclear expression for Bcl-2 (less than 75% positivity).

H: Liver of animals treated with MNU plus sDHA (74.83 mg/kg), showing strong positive immunoreactive nuclear expression for Bcl-2 (less than 50% positivity).

I. Liver of animals treated with MNU plus sDHA (112.25 mg/kg), showing positive immunoreactive nuclear expression for Bcl-2 (less than 25% positivity).

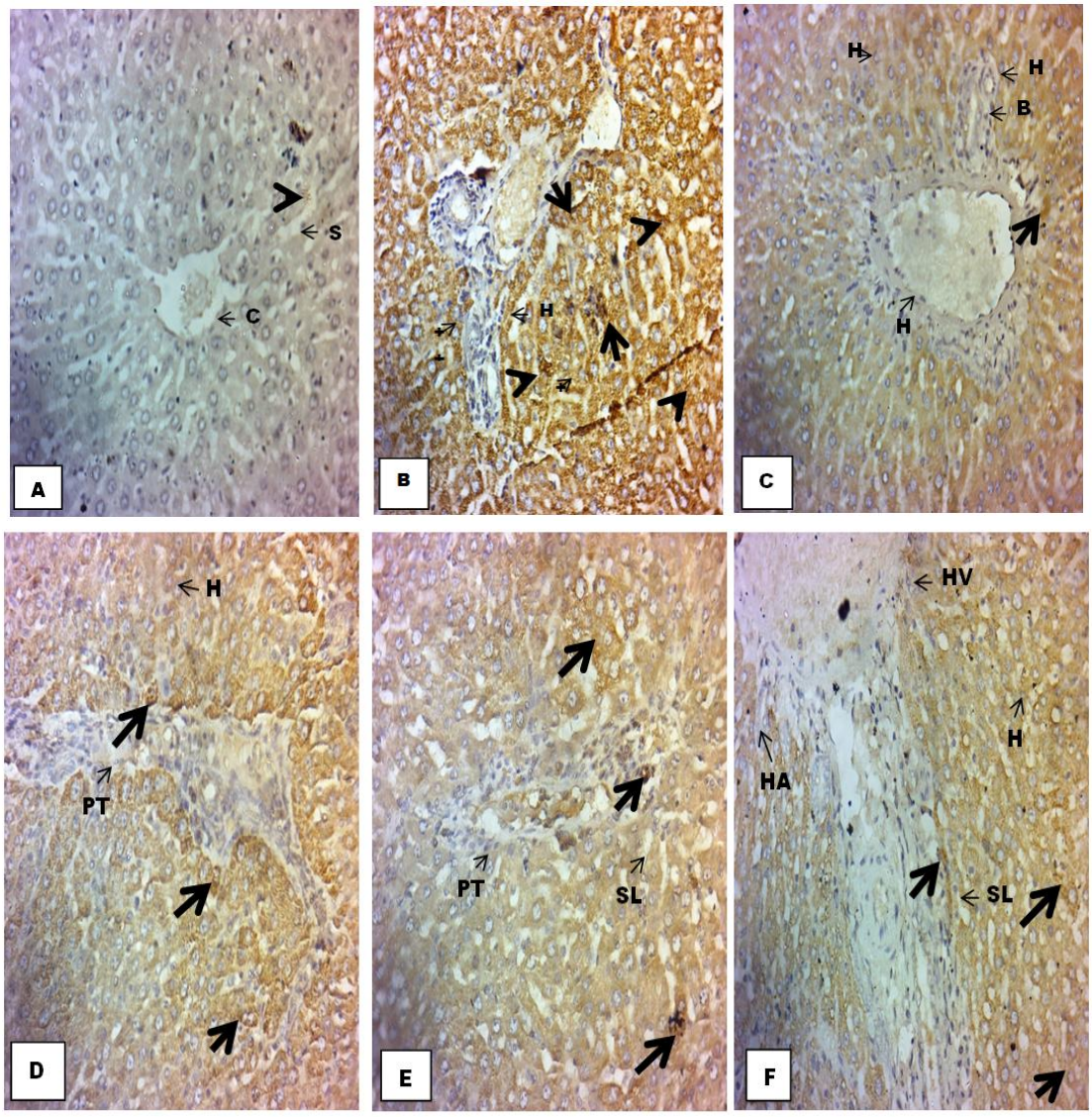
MNU: N-methyl-N-nitrosourea, DHA: Dihydroartemisinin, sDHA: Disulphide-substituted dihydroartemisinin

Key: Central vein (CV), Hepatocyte (H), Bcl-2 (→), Bile duct (BD), Hepatic Artery (HA), and Hepatic vein (HV)

(proapoptotic) or inhibiting (anti-apoptotic) apoptosis. Bcl-2 is specifically considered an important anti-apoptotic protein and is thus classified as an oncogene [20]. Ki-67 is a specific nuclear marker for cell proliferation, and overexpression is frequently seen in a variety of malignant tissues [21-23]. The strong positive expressions of Bcl-2 and Ki-67 in MNU treated rats therefore confirms the presence of cancer in livers of the treated rats. sDHA or DHA treatment decreased the MNU-induced elevations of CA 27-29 and 8-OHdG dose-dependently. However, both drugs failed to normalize levels of these cancer biomarkers, except CA 27-29 that was reduced to comparable level as control by DHA at the high dose. On the other hand, the drug treatments generally produced no effect on the MNU-induced reduction of SOD, except the high dose of DHA which was not sufficient to normalize SOD level. These findings indicate that DHA and sDHA can ameliorate cancer development, but may have little effect on cancers predominantly mediated by superoxide anion oncogenic activity, as SOD enzyme was minimally affected by both drugs. The effects of the two drugs were generally comparable, although, DHA exhibited higher effects at few doses. Cyclophosphamide, which was used as a

reference drug in this study, produced greater levels of inhibition of the MNU-induced biochemical toxicities than sDHA or DHA, and equally reversed the MNU effects. Furthermore, sDHA and DHA treatments reduced Bcl-2 and Ki67 expressions in liver in dose-dependent manners. High dose treatment of both drugs completely reversed MNU induced Ki67 expression but not Bcl-2 in liver. The results also showed that DHA produced greater inhibitory activity of Bcl-2 and Ki67 expressions in the liver and may therefore be more effective than sDHA. Cyclophosphamide treatment ameliorated Bcl-2 and Ki67 expressions better than DHA or sDHA, as it reversed MNU induced expressions of the two cancer biomarkers.

The overall results indicate that DHA and sDHA possess ability to reduce MNU-induced tumor activities in the liver, but lack recovery and may not compete favorably with cyclophosphamide in efficacy in rats. However, the results are indicative of a possibility of obtaining greater effectiveness using higher dose levels of the drugs. This is a potential area of future study. MNU has multiple organ targets, and acts as carcinogen across species [24,25]. Although, the target of MNU differs among species, its organ targets are fairly similar in rodents [25] and the carcinogenic action of MNU on liver cells in rats can be applicable for development of human disease. The present findings can therefore be used to identify therapeutic interventions.



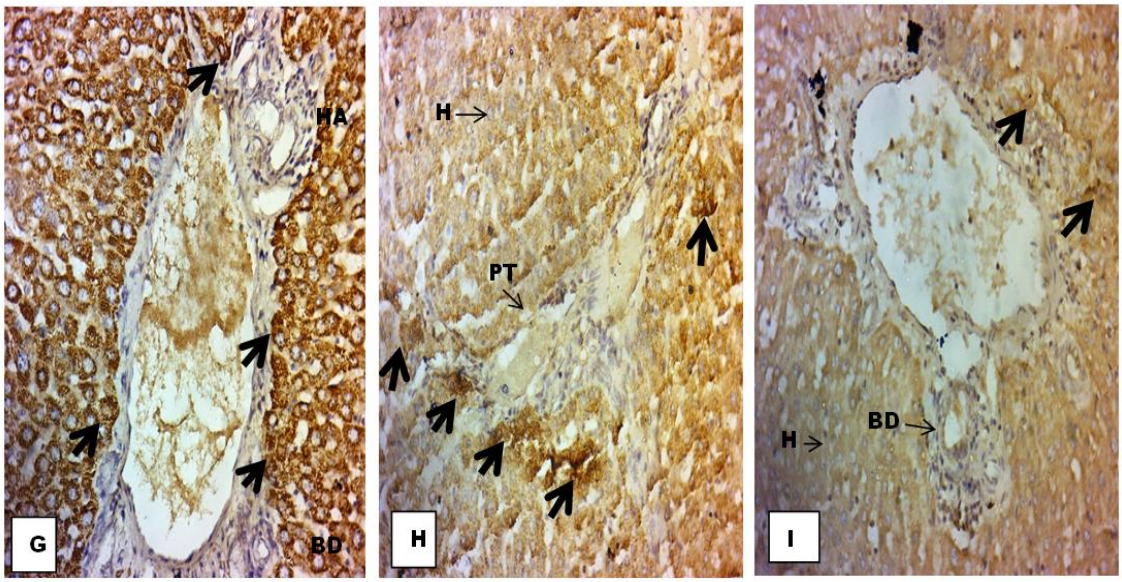


Fig. 6. Photomicrographs showing hepatic cell expression of Ki67 in MNU treated and MNU plus DHA, sDHA or cyclophosphamide treated Sprague-Dawley rats (Ki67 immunohistochemical method, x400)

A: Liver of animals treated with distilled water (Control), showing negative immunostained cytoplasmic expression for Ki67 (less than 5% positivity).

B: Liver of animals treated with MNU (50 mg/kg) alone, showing strong positive immunostained cytoplasmic expression for Ki67 (more than 75% positivity).

C. Liver of animals treated with MNU plus cyclophosphamide (0.7 mg/kg), showing negative immunostained cytoplasmic expression for Ki67 (less than 5% positivity).

D: Liver of animals treated with MNU plus DHA (54.8 mg/kg), showing positive immunostained cytoplasmic expression for Ki67 (less than 25% positivity).

E: Liver of animals treated with MNU plus DHA (109.5 mg/kg), showing positive immunostained cytoplasmic expression for Ki67 (<25% positive cells).

F. Liver of animals treated with MNU plus DHA (164.3 mg/kg), showing negative immunostained cytoplasmic expression for Ki67 (<10% positive cells).

G: Liver of animals treated with MNU plus sDHA (34.6 mg/kg), showing very strong positive immunostained cytoplasmic expression for Ki67 (<60% positive cells).

H: Liver of animals treated with MNU plus sDHA (69.3 mg/kg), showing positive immunostained cytoplasmic expression for Ki67 (<50% positivity).

I. Liver of animals treated with MNU plus sDHA (103.9 mg/kg), showing positive immunostained cytoplasmic expression for Ki67 (<50% positivity).

MNU: N-methyl-N-nitrosourea, DHA: Dihydroartemisinin, sDHA: Disulphide-substituted dihydroartemisinin

Key: Hepatocyte (H), Hepatic vein (HV), Hepatic Artery (HA), Bile duct (BD), Ki67 (→), Central vein (CV) and Sinusoidal lining (SL)

4. CONCLUSION

The study reveals that anticancer activity is not lost in disulphide-substituted dihydroartemisinin, a new drug obtained through structural modification of dihydroartemisinin. The new drug possesses potential antitumor activity against MNU-induced liver cancer. Over the dose range used, the drug has lesser efficacy than the parent drug (dihydroartemisinin), and neither the new drug nor dihydroartemisinin has comparable efficacy with cyclophosphamide. Future studies using higher doses of the new drug is recommended.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experimental procedures were approved by the Research Ethics Committee of University of Port Harcourt, Nigeria.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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