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Synthesis, Characterization and Biological Evaluation of Benzimidazole - Dihydroartemisinin Hybrids as Potential Dual Acting Antimalarial Agents

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Abstract: Malaria is a parasitic disease caused by various species of the *Plasmodium* parasite. In 2016, there were about 216 million cases resulting in 445,000 deaths, with sub-saharan Africa bearing the heaviest burden of the disease. The currently recommended treatment for malaria are combination therapies containing Artemisinin (ACT's). However, the effectiveness of the Artemisinins is being compromised by the emergence of resistance to the drug and this amplifies the need for new antimalarial drugs. The Benzimidazole scaffold is one of the privileged structures in medicinal chemistry and is associated with a number of biological activities including antimalarial activity which may be through inhibition of the Plasmodial plasmepsin II enzyme. The present study utilizes the concept of molecular hybridization to synthesize hybrid compounds that contain two pharmacophores, acting through two distinct mechanisms. The aim is to improve efficacy and possibly prevent or slow down the emergence of parasite resistance. To confirm their structures, the conjugates were purified by chromatography and characterized using Nuclear Magnetic Resonance (NMR), Mass spectrometry and Infra-red spectroscopy. Antimalarial activities of the hybrids were evaluated in-vitro against the 3D7 strain of *Plasmodium falciparum* using the parasite Lactate dehydrogenase assay. The hybrids were successfully synthesized with yields ranging from 63.48 percent to 67.60 percent and were all active against the parasite. The Mebendazole conjugate of dihydroartemisinin showed the highest activity with IC_{50} of 6.861 nM and 6.967 nM for the 5-Benzimidazolecarboxylic acid conjugate of dihydroartemisinin. All the compounds showed statistically significant ($p < 0.05$) increase in activity as compared to Dihydroartemisinin and Chloroquine alone. These hybrid compounds with improved physicochemical and pharmacological properties may serve as templates for the development of a new class of anitmalarial drugs, which possess advantages over existing drugs in terms of effectiveness and also the ability to overcome the problem of resistance during malaria chemotherapy.

Keywords: Plasmepsins, Artemisinins, Benzimidaole, Antimalarials, Hybrids.

1. Introduction

Malaria is one of the greatest scourges to confront mankind causing significant morbidity and mortality in various areas endemic to the disease, especially Sub-Saharan Africa and South-east Asia which have some of the hardest hit areas. It has been estimated that there were about 216 million new cases of malaria and 445,000 deaths annually [1]. Malaria is caused by five species of parasites belonging to the genus *Plasmodium* that affect humans (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*). Malaria caused by *P. falciparum* is the most deadly form and it predominates in West Africa [2].

During the erythrocytic stage of the life cycle, *Plasmodium* parasites consume large quantities of hemoglobin from the host cell, either to generate amino acids for its nutrition or to create space within the red blood cell for its own growth. Hemoglobin is transported to the digestive vacuole of the parasite using vesicles and in this location, the protein component of hemoglobin is digested by the sequential action of several proteolytic enzymes. Aspartate proteases (Plasmepsins I–IV) are first in the sequence followed by cysteine proteases (falcipains) and the zinc protease, falcilysin. The small peptides and free amino acids generated are then transported into the cytoplasm, leaving the heme part behind. Proteases expressed in the erythrocytic cycle of *Plasmodium* are considered promising targets [3,4] and the design of inhibitors of proteases involved in hemoglobin degradation is progressing rapidly with a view to developing a new crop of antimalarial agents. Extensive research is on-going around two groups of

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acidic endopeptidases: the aspartic endopeptidases Plasmepsins I and II (and more recently IV) and the cysteine endopeptidases Falcipain-1, –2 and –3 [3,5].

Plasmepsin inhibitors have been shown to kill *Plasmodium* parasites in both culture and animal models and are now established as viable drug targets. A variety of inhibitors with diverse structures were developed to target the *Plasmodium* Plasmepsins such as hydroxyethylamine derivatives [6–10], 1, 2- dihydroethylene [11] and diphenylurea derivatives [12,13]. Statines such as Pepstatin A and their derivatives were some of the earliest Plasmepsin inhibitors to be identified but they have the limitation of being peptidomimetic in nature and are therefore orally inactive [14,15].

Benzimidazoles are bicyclic aromatic organic compounds which have the benzene ring fused to the 4, 5 position of the imidazole nucleus. The benzimidazole family of heterocyclic compounds has received considerable attention as bioactive compounds. They are associated with a range of biological activities such as antimalarial [16], anti-microbial [17], antiviral [18], antiprotozoal [19], anti-inflammatory [20], anti-hypertensive [21] and anti-tumour [22,23] activities. They are structural isosteres of purine nucleic acids and can potentially interact with biological macromolecules such as protein, enzymes and receptors [24]. Thus, the benzimidazole nucleus is confirmed as an important pharmacophore in drug discovery.

The Artemisinins are sequiterpene lactone endoperoxide compounds with antimalarial activity and were initially isolated from the plant *Artemisia annua*. Semi-synthetic artemisinin derivatives such as artemether, arteether and artesunate have been developed and have increasingly been used for about 20 years. All are prodrugs which are metabolized to the main active compound dihydroartemisinin. The Artemisinins have been evaluated extensively and shown to be highly effective in the treatment of patients with both severe and uncomplicated falciparum malaria [25]. Due to their short plasma half-life, the Artemisinins are increasingly combined with other antimalarial drugs with longer plasma half-lives, such as mefloquine. These combinations decrease the risk of inducing resistance. In 2001, the World Health organization recommended the abolition of the use of Artemisinins as a monotherapy. The exact mechanism of antimalarial action of the Artemisinins is still a subject of debate. However, there seems to be consensus that they cause free radical damage to the malaria parasite organelles and alkylate parasite proteins.

Recent studies have detected the emergence of strains of the *Plasmodium* parasite resistant to Artemisinins; the key component of the ACT's. This phenomenon was detected in South East Asia particularly in the Greater Mekong sub-region [26–29] and also parts of sub-Saharan Africa [30]. Mutations in the *P. falciparum* K13-propeller gene is linked to Artemisinin resistance and the spread of this mutation poses a significant threat to ACT efficacy [31]. Resistance to ACT's if it becomes wide spread will be particularly be disastrous as most of the other drugs being developed to complement or replace Artemisinins are still several years away from being available in the market [32]. A possible approach to prevent this is through the concept of molecular hybridization which entails combining two or more pharmacophores into a single entity that can attack the parasite via multiple mechanisms.

Hybrid or dual drugs are defined as " a single chemical entity that consist of two drugs/active compounds/pharmacophoric units linked together covalently by a linker [33]. This aims to take advantage of the observed (or anticipated) synergistic or additive pharmacological activities of the hybrid components". A number of antimalarial hybrids have been synthesized and tested with some showing good activity against the parasite. For example, the synthesis of a novel artemisinin-quinine ester hybrid with potent antimalarial activity was described [34] in a procedure in which the Artemisinin was reduced to DHA and coupled to a carboxylic acid derivative of quinine. The novel hybrid molecule had potent activity against CQ-sensitive and CQ-resistant strains of *P. falciparum in vitro*. Artemisinin-quinoline hybrids have also been synthesized and evaluated [35–37]. The compounds had potent antimalarial activity even at low doses. Similar results were obtained with another series of Artemisinin-quinoline hybrids [38,39]. A series of artemisinin-triazine hybrids and hybrid-dimers were prepared through nucleophilic substitution, using both conventional and microwave assisted methods [40]. The hybrids were all active with the p-anisidino-substituted triazine hybrid-dimer being the most active having a 50% inhibitory concentration value in the nanomolar range. Chadwick et al. [41], synthesized a series of artemisinin–spermidine conjugates which possessed both anticancer and antimalarial activity and one of the compounds having an IC_{50} value as low as 0.21 nM against the parasite.

An extensive literature searches reveals that there are no prior reports of the antiplasmodial activity of hybrids of dihydroartemisinin linked to the Benzimidazole pharmacophore. The present study therefore reports the synthesis of novel hybrid antimalarials consisting of dihydroartemisinin covalently linked to the Benzimidazole nucleus that has the potential to target Plasmodial Plasmepsin enzyme thereby creating a single chemical entity, capable of attacking the parasite via multiple mechanisms, increasing the effectiveness of therapy and also significantly diminishing the possibility of emergence of resistance to either agents.

2. Experimental

2.1. Materials

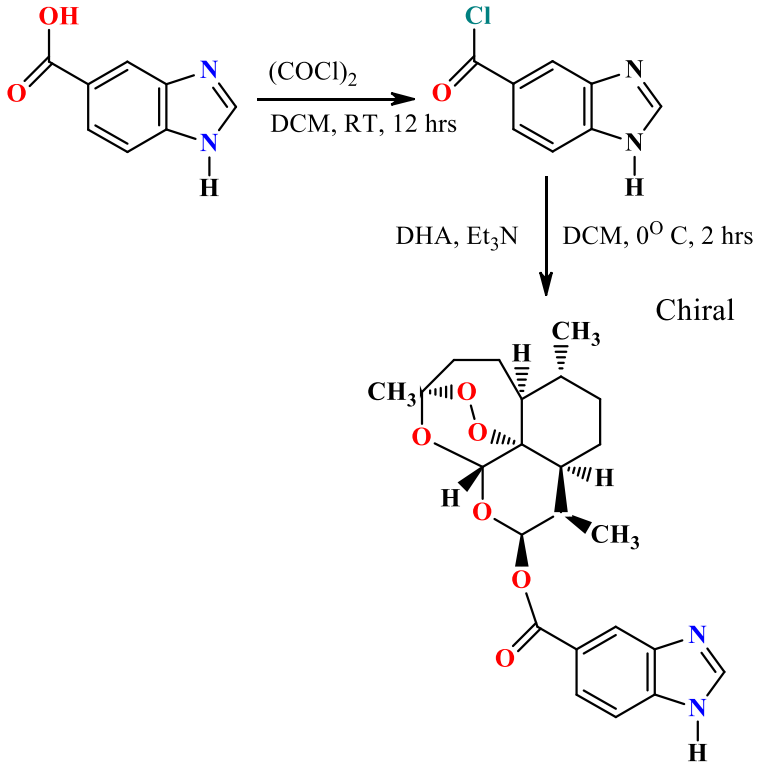
Chloroquine diphosphate was obtained from Greenfield Pharmaceutical Limited, Jiang Su, China while Mebendazole was obtained from Evans Nigeria Limited. Dihydroartemisinin (analytically pure) was purchased from Nanjing Zelang Medical Technology Company, (Nanjing, China). Boron trifluoride diethyl etherate ($BF_3.Et_2O$), Oxalyl Chloride, 2-bromoethanol, 5-Benzimidazole-2-carboxylic acid, Triethylamine, RPMI-1640 Medium, Triton X-100, Sodium L-Lactate, Trishydroxymethylamino methane (Trizma base), 3-Acetylpyridine adenine dinucleotide, Nitro Blue Tetrazolium (NBT), Phenazine Ethosulfate (PES), HEPES, Sodium hydroxide, n-Hexane, methanol, N,N-dimethylformamide (DMF), tetrahydrofuran (THF), Diethyl ether, Dichloromethane, ethyl acetate, acetic anhydride, glacial acetic acid, concentrated Hydrochloric acid, Formic Acid, Acetonitrile, Dimethylsulfoxide (DMSO), Sodium sulphate, Sodium Bicarbonate, Sodium Chloride were all obtained from Sigma-Aldrich (Germany). Albumax (Gibco, Invitrogen, USA), Dehydrated ethanol (absolute ethanol) was obtained from BDH laboratory reagents (Yorkshire, England). All the chemicals and reagents were of analytical grade and were used without further purification.

2.2. Equipment

Double Beam UV-VIS Spectrophotometer (model 1250, Shimadzu, Japan), FT-IR spectrophotometer (Cary 630 model, Agilent Technologies), Agilent LC-MS (model 1260, Infinity HPLC with Agilent 6130 single quadrupole mass spectrometer). The software used is Agilent Chemstation (for initial processing) and MassHunter (for the reporting of the results), Bruker Advance III 400MHz spectrometer with an ultrashield magnet equipped with a Bruker B-ACS-60 autosampler. The software used is Bruker Topspin/ICON NMR (with the latter controlling the autosampler) – TMS was used as internal standard. Chemical shifts were reported in parts per million. Splitting patterns are described as singlet (s), doublet (d), triplet (t), and multiplet (m). In NMR, numbering of atoms is presented as indicated in the text.

2.3. Methods

The synthetic reactions were monitored by TLC using pre-coated silica gel aluminium plates (Kieselgel 60, 254, E. Sigma-Aldrich, Germany); zones were detected visually under ultraviolet irradiation or by spraying with a solution of 0.5% vanillin in Sulphuric acid for visualization. Silica gel column chromatography was performed using Silica Gel 60 (70–230 mesh).



Scheme 1. Synthesis of 5-Benzimidazolecarboxylic Acid - Dihydroartemisinin Ester Conjugate

2.3.1. Synthesis of Conjugates

2.3.1.1. Synthesis of 5-Benzimidazolecarboxylic acid-Dihydroartemisinin Ester Conjugate [42].

(a) Synthesis of 5-Benzimidazolecarboxylic Acid Chloride:

Oxalyl chloride (0.4 mL, 4.47 mmol) was added drop wise to a solution of 5-Benzimidazolecarboxylic acid (0.99 g, 4.50 mmol) in dry CH_2Cl_2 (20 mL). The reaction mixture was stirred at room temperature (25°C) for 12 hours, and the solvent was removed in vacuo. The crude product was washed with hexane (3 x 25 mL) and dried under vacuum to give the crude acid chloride.

(b) Coupling of the acid chloride to dihydroartemisinin

Triethylamine (0.73 ml, 5.29 mmol) was added drop wise at 0°C to a solution of dihydroartemisinin (1.50 g, 5.28 mmol) and the acid chloride (1.14 g, 5.75 mmol) dissolved in dry dichloromethane (30 mL). The mixture was stirred at the same temperature for two hours, then the reaction mixture was quenched with saturated sodium bicarbonate solution (25 mL) and extracted with dichloromethane (3·x 25 mL). The organic layer was washed with 10% aqueous HCl solution (2·x 20 mL), then with water, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain 1.69 grams (74.97%) of the crude product (Scheme 1).

2.3.1.2. Synthesis of Mebendazole - Dihydroartemisinin conjugate

(a) Preparation of 10β-(2-Bromoethoxy) Dihydroartemisinin [43].

(2-(10β-Dihydroartemisinoxy) ethyl Bromide or 2-Bromo-(10 β -dihydroartemisinoxy) ethane)
2-Bromoethyl alcohol (1.42 mL, 2.49 g, 20 mmoL) and Et_2O (60 mL) were placed into a 250 mL round bottomed flask, and then $BF_3.Et_2O$ (2 mL) was added under ice bath. Dihydroartemisinin (5.0 g, 17.58 mmol) was finally added with stirring. The mixture was stirred for 2 hours in an ice bath. The reaction progress was monitored with TLC. Saturated $NaHCO_3$ was added to terminate the completed reaction. The aqueous layer was extracted with EtOAc (30 mL × 2) after liquid separation, and then the organic layers were combined. The organic layer was washed with saturated brine (40 mL) then dried with anhydrous $MgSO_4$; and the solvent was removed through rotary evaporation under reduced pressure.

(b) Coupling of the 10β-(2-Bromoethoxy) Dihydroartemisinin intermediate to Mebendazole

A solution of the bromide; 10β-(2-Bromoethoxy) Dihydroartemisinin (1.0 g, 2.46mmol) and mebendazole (amine) (1.20 g, 4.06 mmol) in DMF (60 mL) was heated in an oil bath (50-60°C) for six hours. After the reaction was complete, the mixture was evaporated to dryness under reduced pressure. The residue was extracted with CH_2Cl_2. The combined extract was washed with brine and dried to give 1.42 grams (66.70%) of the crude product (Scheme 2).

2.3.2. Purification of Conjugates
Accelerated gradient chromatography (AGC) with gradient elution was used to purify the conjugates after synthesis using appropriate solvent systems for each conjugate.

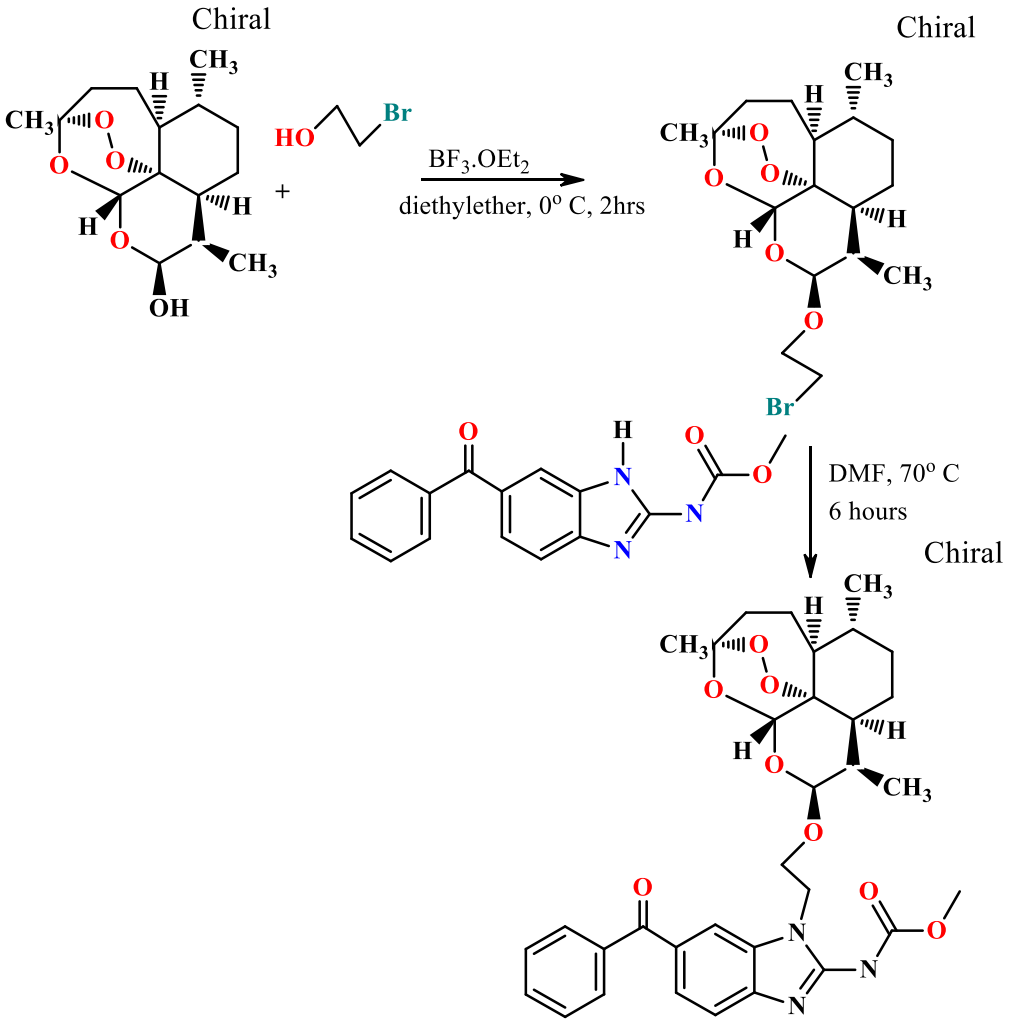
2.3.3. Characterization of Conjugates
The synthesized compounds were characterized using Ultraviolet-visible absorption spectroscopy, FT-IR spectroscopy, LC-MS, ^1H and ^{13}C NMR spectroscopic analysis.

2.3.4. Antimalarial Screening of Conjugates
2.3.4.1. Ethical consideration
Ethical clearance was sought and obtained from the Institutional Health Research Ethics Committee of the Bingham University Teaching Hospital (BHUTH), Jos, Nigeria for the research prior to commencement of the biological studies.

2.3.4.2. In vitro antimalarial screening (parasite Lactate dehydrogenase (pLDH) assay)
***Plasmodium falciparum* Culture and Maintenance.**
In vitro culture experiments were performed using the 3D7 clone of the chloroquine sensitive strain of *P. falciparum* which were grown and maintained in culture using an earlier reported method [44] with some modifications [45]. Cultures consisted of a 4% haematocrit suspension of O+ human erythrocytes in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with Gentamicin solution at 0.01 mg/mL, 25mM HEPES buffer, 25mM $NaHCO_3$, and 0.5% Albumax I. The parasites were cultured at 37°C under a low-oxygen atmosphere (5% oxygen, 5% carbon dioxide, and the 90% nitrogen). The estimation of the parasitaemia as well as parasite visualisation before incubation was done using normal light (Giemsa stain) microscopy.

Determination of *in vitro* Antiplasmodial Activity[46]

Stock solutions (1 mg/mL) of test drugs were prepared by dissolving 10 mg of drug in 1 mL of dimethyl sulfoxide (DMSO) and the volume made up to 10 mL with distilled water. All stocks were then serially diluted with distilled water to achieve the required concentrations. The 8 final concentrations obtained were 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195 ng/mL. The commercial drug standards chloroquine diphosphate (Greenfield Pharmaceutical LTD, Jiang Su, China) and Dihydroartemisinin (Nanjing Zelang Medical Technology Co Ltd, China) were used as positive controls in the biological tests. In all cases except CQ, the final solution contained less than 0.01% DMSO, which was found to be non-toxic to the parasite. (CQ stock solution was prepared in distilled water). The drug stock solutions were stored at −20° C until required. Dilutions were freshly prepared on the day of the assay.



Scheme 2. Synthesis of Mebendazole - Dihydroartemisinin Conjugate

Each sample of diluted compounds was tested in triplicate in 96-well micro-titre plates containing 100 µL of RPMI culture medium with 50 µL of *P. falciparum* culture with fresh red blood cells (pRBCs) with a haematocrit of 4% and 2% initial parasitemia and finally 100 µL of the drug solution. Wells containing parasitized red blood cells (pRBCs) in culture medium without drug were the negative control for the assay and 0.01% DMSO solution was also used as a control to confirm that the solvent did not have any effect on parasite growth.

The plates were then incubated for 72 hours at 37°C under a low-oxygen atmosphere (Incubator Memmert GmbH, Germany). The culture medium was replenished on a daily basis during the incubation period. While incubation of the plates was going on, the two reagents for detecting and measuring the LDH enzyme were prepared. The first of these was the Malstat reagent, which was made by dissolving 400 µL of Triton X-100 in 80 mL of deionized water, adding L-lactate (4.00 g), Tris buffer (1.32 g), and 0.022 g of 3-acetylpyridine adenine dinucleotide (APAD), adjusting the pH to 9 with hydrochloric acid, and bringing the volume up to 200 mL with deionized water. The second reagent was NBT/PES solution, prepared by dissolving nitro blue tetrazolium salt (0.160 g) and phenazine ethosulfate (0.008 g) in 100 mL of deionized water. The solution was stored in a foil-covered container and kept in the refrigerator until required.

When incubation was complete, plates were harvested and next, 100 µL of Malstat reagent was added to each well of a new flat bottomed 96-well microtiter plate in triplicate. Thereafter, the culture in each well was mixed and 20 µL of the culture taken from each well and added to the corresponding well of the Malstat plate and 25 µL of NTB/PES was then added to each well, initiating the lactate dehydrogenase reaction. Colour development of the LDH plate was monitored colorimetrically at 650 nm with the aid of a plate reader after an hour of incubation in the dark. (Emax-Molecular Devices Corporation, California, USA)

Analysis of test results from the LDH assay

The LDH assay [45] generates optical density (OD) values at various concentrations of the drug as raw data. OD values from negative control wells (containing parasitized but untreated RBC) represent the maximum amount of LDH (100%) that is produced by parasites. The growth value at each

concentration of the drug was obtained by expressing the OD value as a percentage of the 100% growth value and plotted against the Logarithm of the corresponding molar concentrations of the drug using GraphPad Prism Software to generate log dose–response curves from which IC_{50} values were obtained.

% Parasite Growth= (Average OD (no drug: negative control) – Average OD(test)) x 100

Average OD (no drug: negative control)

2.3.5. In vitro cytotoxicity assay

Cytotoxicity of the compounds was tested by the brine shrimp lethality assay [47] artificial sea water was prepared by dissolving 38 g of NaCl (3.8%) in 1000 ml of distilled water and was filtered to obtain a clear solution. The dried cysts of the brine shrimps were hatched in artificial seawater with strong aeration for 48 hours. The compounds were then dissolved in sea water with DMSO (<0.01%) and transferred to test tubes to obtain concentrations of 0.78125, 1.5625, 3.125, 6.25, 12.50, 25, 50, 100, 200, 300 and 400 µg/ml in 5 ml artificial sea water with 20 nauplii in each test tube. Standard drug Cyclophosphamide was used as positive control at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, 0.3906 and 0.1953µg/mL. Control test tubes were subjected to DMSO in artificial seawater at the same concentration as it was made for samples. After 24 hours' incubation at 25-30°C, the number of viable nauplii was counted using a magnifying glass. The percent (%) mortality was calculated using the following formula:

% Mortality = N_t/ N_0 x 100

Where, N_t = Number of dead nauplii after 24 hours of incubation, N_0 = Number of total nauplii transferred (n=20). The concentration-mortality data was analyzed statistically by using probit analysis for the determination of the Median lethal concentration (LC_{50}) values and linear regression for the compounds.

2.3.6. Statistical Analysis

Numerical data (IC_{50} and LC_{50} values) obtained from the assay are expressed as the mean value ± standard error of the mean. Statistical analysis was performed using the analysis of variance (ANOVA) followed by Tukey post-test with the aid of IBM Statistical Package for Social Scientist (SPSS 20) software. Statistical significance was defined at the 5% level ($p < 0.05$).

3. Results

3.1. Results of the Synthetic Reaction

The results obtained following the synthetic procedures for preparing the conjugates are presented in Table 1 below. It includes the molecular formula of the compounds, their physical appearance, melting point and the percentage yield obtained.

Table 1. Physicochemical Properties of the Synthesized Conjugates.

S/n.	Compound Code	Molecular Formula	Appearance	Melting Point (°C)	% Yield
1.	5-BICAD	$C_{23}H_{28}N_2O_6$	Brownish granules	138 – 140	67.60
2.	MEBC	$C_{33}H_{39}N_3O_8$	Dark brown oil	-	63.48

(Key: 5-BICAD = 5-Benzimidazole carboxylic acid – Dihydroartemisinin ester conjugate, MEBC = Mebendazole-Dihydroartemisinin conjugate)

3.2. Spectroscopic characteristics of the synthesized conjugates

Data extracted from the various spectroscopic analyses (UV, FTIR, NMR and MS) for the conjugates are presented below.

(5-BICAD) 3,6,9-trimethyldecahydro-12H-3,12-epoxypyrano [4,3-j] [1,2] benzodioxepin-10-yl 1H-benzimidazole-5-carboxylate

UV(λmax): 245, 320 nm

IR (Neat): 3090 cm^{-1} (C-Hstr unsaturated), 2959 cm^{-1} (C-Hstr. aliphatic), 1722 cm^{-1} (C=Ostr ester), 1599 cm^{-1} (C=Nstr unsaturated), 1274 cm^{-1} (C-Nstr)

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.25 (d, J = 7.6 Hz, 1H, 31), 8.09 – 8.03 (s, 1H, 26), 7.87 (dd, J = 7.5, 1.4 Hz, 1H, 28), 7.69 – 7.62 (dd, 1H, 27), 7.37 (d, J = 7.6 Hz, 1H, 30), 5.88 (dq, J = 7.0, 1.5 Hz, 1H, 8), 5.42 (s, 1H, 9), 2.26 (h, J = 6.8 Hz, 1H, 5), 2.10 – 1.98 (m, 1H, 3), 2.03 – 1.95 (m, 1H, 4), 1.93 – 1.43 (m, 10H, 1, 2, 7, 19, 20), 1.44 (s, 3H, 21), 0.95 (dd, J = 6.8, 1.5 Hz, 3H, 15), 0.88 (dt, J = 6.7, 1.5 Hz, 3H, 16).

^{13}C NMR (100 MHz, $CDCl_3$) δ 166.96 (C – 12), 143.98 (C – 30), 139.54 (C – 24), 138.39 (C – 25), 124.34 (C – 13), 123.72 (C – 28), 117.76 (C – 27), 114.89 (C - 26), 105.29 (C – 18), 105.28 (C – 8), 98.73 (C – 9), 81.65 (C – 6), 50.46 (C – 5), 44.64 (C – 4), 35.20 (C – 7), 35.19 (C – 1), 33.45 (C – 20), 33.44 (C – 3), 24.42 (C – 21), 24.41 (C – 2), 23.93 (C – 19), 23.92 (C – 16), 12.68 (C – 15).

HRMS (ESI): m/z $[M + H]^+$ 429.5011 (Calculated for$C_{23}H_{28}N_2O_6$: 428.4788).

10β-(2-Bromoethoxy) Dihydroartemisinin

IR (Neat): 2952 cm^{-1} (C-H str. aliphatic), 1230 cm^{-1} (C-C str), 1058 cm^{-1} (C-O str)

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.06 (s, 1H, 5), 4.85 (m, 1H, 15), 3.98 (m, 1H, 2), 3.66 (m, 2H, 3), 2.01 – 1.90 (m, 5H, 9, 9', 14, 25, 26), 1.74 - 1.51 (m, 5H, 17, 17', 18, 22, 26), 1.48 - 1.35 (m, 6H, 23, 23', 23", 19, 19', 22'), 0.97 (d, *J* = 15.2, 6.7 Hz, 3H, 6, 6', 6"), 0.95 (d, *J* = 15.2, 6.7 Hz, 3H, 24, 24', 24").
^{13}C NMR (100 MHz, $CDCl_3$) δ 104.51 (C-10), 103.20 (C-15), 89.53 (C-12), 80.93 (C-20), 67.06 (C-2), 50.53 (C-21), 44.68 (C-16), 36.78 (C-18), 36.42 (C-9), 36.28 (C-14), 32.87 (C-19), 29.96 (C-3), 25.34 (C-23), 24.23 (C-22), 23.28 (C-17), 20.21 (C-24), 13.51 (C-6).
HRMS (ESI): m/z $[M + H]^+$ 392.3011 (Calculated for$C_{17}H_{27}BrO_5$: 391.2979).

(MEBC) methyl (6-benzoyl-1-{2-[(3,6,9-trimethyl-decahydro-12H-3,12-epoxypyrano[4,3-j][1,2]benzodioxepin-10-yl)oxy]ethyl}-1H -benzimidazol-2-yl)carbamate
UV(λmax): 243, 330 nm
IR (Neat): 3096 cm^{-1} (C-Hstr unsaturated), 2926 cm^{-1} (C-Hstr. aliphatic), 1722 cm^{-1} (C=Ostr ketone), 1580 cm^{-1} (C=Nstr), 1274 cm^{-1} (C-Nstr)
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61 (s, 1H, 32), 7.85 – 7.78 (m, 3H, 28, 36, 39), 7.75 (dd, J = 7.5, 0.5 Hz, 1H, 26), 7.69 (dd, J = 7.6, 1.5 Hz, 1H, 38), 7.59 – 7.44 (m, 3H, 27, 37, 40), 5.42 (s, 1H, 9), 4.82 – 4.73 (m, 1H, 8), 4.45 – 4.27 (m, 2H, 21), 4.02 – 3.82 (m, 2H, 22), 3.62 (s, 3H, 43), 2.10 – 1.92 (m, 3H, 1', 4, 5), 1.92 – 1.43 (m, 9H, 1", 2, 3, 7, 16, 17), 1.44 (s, 3H, 18), 0.98 – 0.87 (m, 6H, 12, 13).
^{13}C NMR (100 MHz, $CDCl_3$) δ 197.86 (C – 33), 155.62 (C – 41), 144.50 (C – 25), 142.23 (C – 31), 140.03 (C – 24), 137.61 (C – 35), 133.28 (C – 38), 129.70 (C – 36, C – 39), 128.49 (C - 37, C – 40), 128.14 (C – 29), 123.24 (C – 27), 115.93 (C – 26), 110.13 (C – 28), 105.63 (C – 15), 105.57 (C – 8), 93.88 (C – 9), 81.76 (C – 6), 66.79 (C – 21), 52.44 (C – 43), 50.60 (C – 5), 44.33 (C – 4), 42.90 (C – 22), 37.95 (C – 17), 34.60 (C – 1), 34.19 (C – 7), 32.46 (C – 3), 24.14 (C – 2), 24.12 (C – 18), 21.62 (C – 16), 20.15 (C – 13), 13.61 (C – 12).
HRMS (ESI): m/z $[M + H]^+$ 606.7087 (Calculated for$C_{33}H_{39}N_3O_8$: 605.6790).

Table 2. In silico Prediction of Lipinski Drug-like Properties for the Conjugates.

Compound	Molecular Weight (g/mol)	clog*	Hydrogen Bond Acceptors (HBA)	Hydrogen Bond Donors (HBD)	Violations
5-BICAD	428.478	4.51	8	1	-
MEBC	605.678	5.87	11	1	3

* Calculated using ACD iLabs

3.3. Antimalarial activity of the conjugates

The *in vitro* antimalarial activity of the synthesised compounds against the 3D7 strain of *Plasmodium falciparum* was determined using the parasite Lactate dehydrogenase assay. The Log dose-response curves of the synthesized compounds (5-BICAD and MEBC) against the parasite strain are shown in Figures 1 and 2 respectively while those for the standard drugs (Dihydroartemisinin and Chloroquine) are showm in Figures 3 and 4 respectively. Each data point represents the mean ± SEM (n = 3). The plot shows percentage parasitemia against the log of the concentration of the compound. The half maximal inhibitory concentration (IC_{50}) values for the compounds are similarly given in Table 3 below.

Table 3. In vitro Antimalarial Activity of the Synthesized Compounds against the 3D7 Strain of *Plasmodium falciparum* Determined Using Parasite Lactate Dehydrogenase Assay.

S/n	Compound	IC_{50} (nM)
1.	5-BICAD	$6.967^{*\#}$ ± 0.687
2.	MEBC	$6.861^{*\#}$ ± 0.477
3.	Dihydroartemisinin (DHA)	9.968 ± 0.114
4.	Chloroquine (CQ)	13.003 ± 0.758

The IC_{50} values are expressed as mean ± SEM, n = 3 in each group.
* indicates significant difference compared to DHA, while # indicates significant difference compared to CQ (ANOVA followed by Tukeys post hoc test, $p < 0.05$).

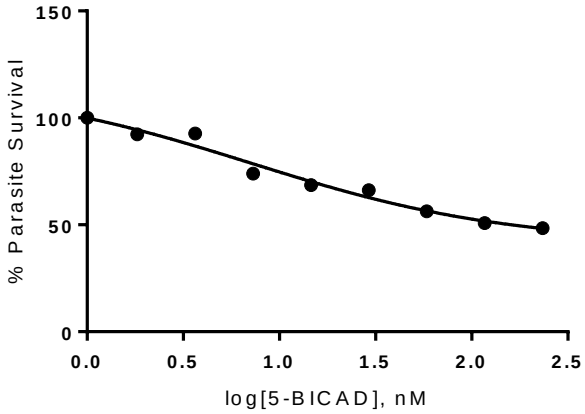


Figure 1. Log Dose Response Curve of *P falciparum* (3D7 Strain) by 5-BICAD

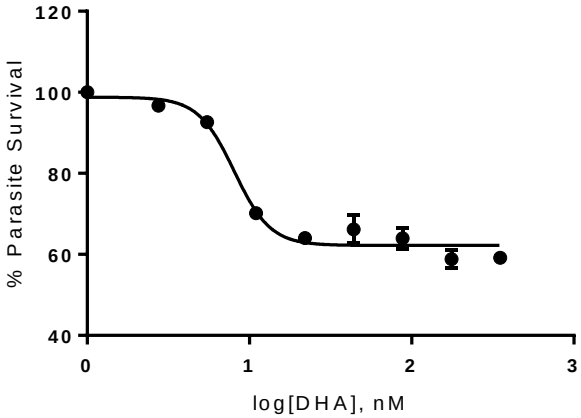


Figure 3. Log Dose Response Curve of *P falciparum* (3D7 Strain) by DHA

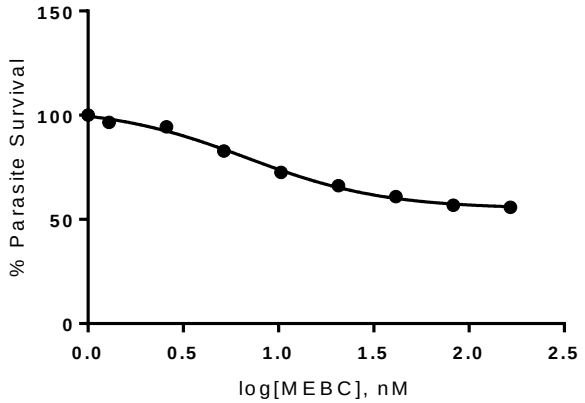


Figure 2. Log Dose Response Curve of *P falciparum* (3D7 Strain) by MEBC

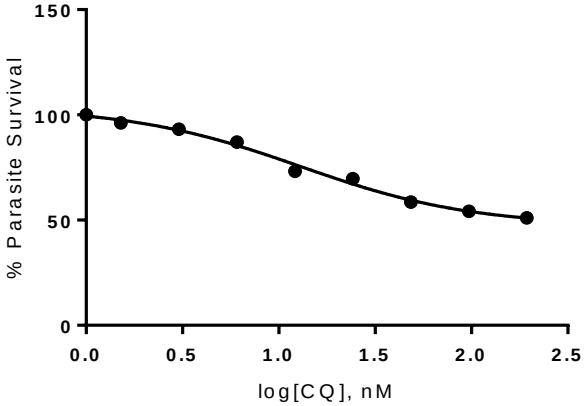


Figure 4. Log Dose Response Curve of *P falciparum* (3D7 Strain) by Chloroquine (CQ)

3.4. In vitro Brine Shrimp lethality bioassay

Cytotoxic effects as illustrated by LC_{50} values of the compounds and the standard drug Cyclophosphamide are summarized in the Table 4 below.

Table 4. *In vitro* Cytotoxicity of the Synthesized Compounds using the Brine Shrimp Lethality Assay.

S/n	Compound	LC_{50} (µg/mL)
1.	5-BICAD	361.53* ± 41.70
2.	MEBC	26.91* ± 4.99
3.	Cyclophosphamide (Standard)	1.08 ± 0.20

The LC_{50} values are expressed as mean ± SEM, n = 3 in each group.
* indicates significant difference compared to Cyclophosphamide (ANOVA followed by Tukeys post hoc test, $p < 0.05$).

4. Discussion

There is a desperate need to develop novel antimalarial therapies and strategies to combat the disease. This is because almost all previously used treatments and regimens have failed and no new drugs are on the horizon for the treatment of malaria. The artemisinin derivatives are now the only class of antimalarial agents which the world is relying on solely to manage the disease. It is even now established that the parasite has also developed resistance to this drug though the extent and spread of artemisinin resistance is presently limited to parts of Southeast Asia. This portends catastrophic danger for antimalarial control programs worldwide. It is therefore imperative to develop new drugs acting by novel mechanisms to counter artemisinin resistance and also to produce new lead compounds that can be further developed into new drugs that can serve as alternatives to the presently used antimalarials. Since no new drugs are in the pipeline, repositioning of old drugs and recombining them would serve as new sources of antimalarial agents for the future. This was the main motivation for the present study.

Presently, of all the hybrids antimalarials in development, only one compound has reached the stage of clinical trials (trioxaquine PA1103/SAR116242) and this highlights the opportunities that exist to further develop new chemical entities using known drugs or pharmacophores that are more likely to progress through the various stages of drug development and this is very appealing in this disease area associated with few resources and lack of effective treatment alternatives.

Plasmepsins are attractive targets for antimalarial therapy due to their role in the degradation of haemoglobin during erythrocytic parasite development [48]. Selective inhibitors of some of these enzymes have been designed and developed by researchers taking advantage of differences in the active site architecture between the plasmodial enzymes and their human counterparts. Inhibitors of these key classes of enzymes in the plasmodial parasite were therefore chosen and covalently linked to dihydroartemisinin to yield new classes of compounds that will have improved activity against the parasite. These inhibitors hopefully would not be too toxic to the host enzymes.

4.1. Synthesized Conjugates

The hybrids were successfully synthesized with reasonable yields of 63.48% for the Mebendazole-Dihydroartemisinin conjugate and 67.60% for the 5-Benzimidazole-Dihydroartemisinin ester conjugate. The ester was synthesized by simple nucleophilic displacement reaction between Dihydroartemisinin and the 5-Benzimidazole carboxylic acid Chloride in the presence of triethylamine. This yield is slightly higher than that obtained by Singh et al. [42] who prepared DHA ester conjugates of pharmacologically privileged substructures such as biphenyl, adamantine and fluorine and obtained their esters in yields of between 49 – 58%. It has been demonstrated that the base catalysed esterification of dihydroartemisinin with acid chlorides and anhydrides always exclusively produces α-isomers and these have the advantage of simpler chromatographic purification after synthesis [49].

4.2. Lipinski Drug-Like Properties of the Conjugates

One of the main challenges in the development of new antimalarial drugs is how to achieve a viable lead candidate with good pharmacokinetic properties. It is therefore important to ensure that the pharmaceutical and drug like properties of drugs under development are optimized alongside their efficacy. The drug likeness of the synthesized compounds was evaluated by determining the Lipinski's rule of 5 properties using ACD iLabs. The rule requires compounds to have no more than 5 and 10 hydrogen bond donors and acceptors, respectively, possess molecular weight of less than 500 amu, and also have calculated octanol-water partition coefficient (clogP) of less than 5. Specifically, Lipinski had earlier proposed that log P values in the range of 0 - 5 are acceptable while values in the range of 0 - 3 are ideal [50,51]. Generally, the aforementioned physico-chemical properties are directly related to the absorption and bioavailability of a drug molecule and directly affect the movement of a drug from the site of administration into systemic circulation. Table 2 shows the results for various parameters of Lipinski's rule of 5. It is expected that an orally active compound should not have more than one violation of these rules. In the present study, the 5-benzimidazole carboxylic acid - Dihydroartemisinin conjugate did not show any violation of Lipinski's rule of 5 indicating that it is endowed with drug-like properties and is likely to be orally bioavailable. On the other hand, the Mebendazole-Dihydroartemisinin hybrid showed 3 violations of the Lipinski rules and is unlikely to be orally bioavailable. However, there are a number of compounds which show multiple violations of the Lipinski rules but which are still orally bioavailable and this observation implies that the oral activity of the hybrid with several violations cannot be precluded. This can only be validated through experimental studies.

4.3 Spectroscopic Characteristics of the Conjugates

All new compounds were characterized by 1H-NMR, 13C-NMR, FT-IR, UV and MS analysis.

4.3.1. Benzimidazole Carboxylic Acid Ester Conjugate of DHA

The 5-Benzimidazole carboxylic acid ester conjugate of DHA (5-BICAD) showed two peaks on the UV absorption spectrum at 245 and 320 nm and these could be attributed to the conjugated pi system of the Bezimidazole nucleus. These values are also close to the reported literature values of the Benzimidaole nucleus which has been shown to have 2 peaks at 242 and 278 nm [52]. In the FTIR spectrum of the compound, the characteristic carbonyl stretching band of the ester is seen at 1722 cm^{-1} and this observation together with the disappearance of the OH stretch is indicative of the formation of the ester. Furthermore, some other bands for the compound that can be seen in the IR spectrum include the C-H stretch of unsaturated systems at 3090 cm^{-1} and the C=N stretch at 1599 cm^{-1} and the C-H stretch aliphatic at 2959 cm^{-1}. In the 1H-NMR, a signal at 9.25 ppm as a doublet belong to –NH- proton, is indicative of the presence of the Benzimidazole nucleus while the signal at 5.99 ppm can be attributed to the methine proton at position 8 of the nucleus which has experienced a downfield shift from the usual values due to the deshielding effect of the nearby oxygen atom. In the 13C-NMR a signal at 166.69 ppm belong to C=O group of the ester link proved the formation of compound. The chromatogram obtained from LCMS analysis of the compound showed one main peak with retention time of 5.097 minutes while ESI-MS of the compound also displayed the molecular ion

peak in the mass spectrum with m/z at 429.5 ($M^+ + H$) i.e. corresponding to its molecular formula $C_{23}H_{28}N_2O_6$

4.3.2. Mebendazole Conjugate of DHA

The Mebendazole conjugate of DHA (MEBC) showed two peaks in the UV absorption spectrum at 243 and 330 nm and these could be attributed to the conjugated pi system of the Bezimidazole nucleus as mentioned earlier [52]. In the FTIR spectrum of the compound, the carbonyl stretching band of the ketone can be seen at 1722 cm^{-1}. In addition, some other bands that can be observed are the C-H stretch of unsaturated systems at 3096 cm^{-1} and the C=N stretch at 1580 cm^{-1} and the C-H stretch aliphatic at 2959 cm^{-1}. Condensation of dihydroartemisinin with 2-bromoethanol produced the DHA-ethyl bromide derivative in good yield. The specteoscopic data for this intermediate was in good agreement with earlier published reports [43]. Several workers had earlier shown that hybrids synthesized by this method are usually in the 10β form [49,53,54]. In the 1H-NMR, a signal at 8.61 ppm as a singlet can be attributed to the –NH- proton at position 32 while in the 13C-NMR, a signal at 197.86 ppm belonging to C=O of the ketone is the most deshielded. Next to that is the C=O signal for the ester which resonates at 155.62 ppm. The other characteristic signals for the two pharmacophores can also be found in the spectra. The chromatogram obtained from LCMS analysis of the compound showed one main peak with retention time of 3.850 minutes while ESI-MS of the compound also displayed the molecular ion peak in the mass spectrum with m/z at 606.7 ($M^+ + H$) corresponding to its molecular formula $C_{33}H_{39}N_3O_8$

4.4. *In Vitro* Antimalarial Activity of the Conjugates

The *in vitro* antimalarial activity of the hybrid compounds were compared with that of Dihydroartemisinin and Chloroquine against a Chloroquine sensitive strain of *Plasmodium falciparum*. The activities of the compounds against the parasite were all superior to Dihydroartemisinin alone and Chloroquine alone as signified by the lower IC_{50} values which were all in the nanomolar range. In addition, all the compounds showed statistically significant difference ($p < 0.05$) in IC_{50} values as compared to the standard drugs Dihydroartemisinin alone and Chloroquine alone.

The antimalarial activity of the artemisinin–benzimidaozole conjugates were evaluated against chloroquine-sensitive 3D7 *P. falciparum* (Table 3). The compounds were found to have better activity than the control drugs Chloroquine and Dihydroartemisinin as the IC_{50} values of the two Benzimidaozle conjugates (5-BICAD and MEBC) were both about 30 % better than that of DHA alone and about 50% higher than the activity of Chloroquine alone. The values also showed statistically significant difference when compared by ANOVA. This can be attributed to several reasons. Firstly, the Benzimidazole pharmacophore is recognized as a privileged scaffold in medicinal chemistry [55] and is known to possess antimalarial activity [56]. The antiamalarial activity of compounds with this pharmacophore has been linked to their ability to inhibit *plasmodial Plasmepsin II* enzyme [57] and it is believed that synergy between this enzyme inhibitory action and artemisinin acting through its unique mechanism of free radical damage to the parasite may have been responsible for the observed improvements in activity of the conjugates compared to the reference drugs. Furthermore, *Plasmepsins* share only about 35% sequence homology with its nearest human aspartic protease, Cathepsin D [58] and this makes them good targets for malaria chemotherapy and rational drug design approaches as the chances of toxicity of the compounds to the human cells due to inhibition of Cathepsin D is minimal.

Secondly, artemisinin derivatives are believed to become activated within the iron-rich environment of the acidic parasite food vacuole and these analogues would be expected to become concentrated here by virtue of their basic nitrogen atoms as explained above. So in addition to targeting the parasites *Plasmepsin* enzyme which is involved in haemoglobin degradation, it is believed that similar accumulation of these artemisinin–benzimidazole conjugates could account for the enhanced antimalarial activity.

The results obtained for this set of conjugates are similar to that reported by Capela and co-workers [59] who developed a series of endoperoxide-dipeptidyl vinyl sulfone hybrid molecules possessing dual activity of endoperoxide activation and falcipain inhibition. In their study, the vinyl sulfone moiety was covalently linked to the endoperoxide entity via the N-terminus, using a 4-hydroxymethylbenzoic acid linker. The conjugate inhibited CQ resistant *P. falciparum* isolate (W2) in the range 2–5nM.

Presently, the recommended guideline for managing malaria is the use of drug combinations based on the Artemisinins. The current trend is to co-formulate two or more drugs into a single multicomponent tablet (e.g., P-Alaxin, Coartem) as opposed to the traditional multidrug regimen and this is believed to improve patient compliance [60]. However, based on the reported wide interest in hybrid molecules as well as the encouraging efficacy reports, the future of malaria chemotherapy may lie in the use of hybrid drugs as opposed to multicomponent ones.

4.5. In vitro Cytotoxicity Assay of the Conjugates

The brine shrimp lethality bioassay is a simple and inexpensive method for identifying potentially cytotoxic compounds and is found to have good correlation with cytotoxicity against human cells. The results for this assay are expressed as half maximal lethal concentration (LC_{50}) and shown in Table 4. The LC_{50} value for 5-BICAD was found to be 361.53 ± 41.70 µg/mL showing that it is relatively non-toxic

while MEBC is more toxic with an LC_{50} value of 26.91 ± 4.99 µg/mL. Both compounds were however found to show statistically significant differences in LC_{50} as compared to the standard drug Cyclophosphamide (1.08 ± 0.20 µg/mL).

5. Conclusions

Two new benzimidazole conjugates of dihydroartemisinin have been prepared and their antimalarial activity evaluated against the 3D7 strain of *Plasmodium falciparum*. Their toxicity against brine shrimp (Artemisia salinia) was also evaluated. The synthesis procedure was simple and efficient. From the results of the antimalarial screening, we may conclude that hybridization of the Benzimidazole nucleus with the Artemisinin pharmacophore is favourable to improved antiplasmodial activity. The study may provide a foundation for further designing and developing more potent antimalarial agents.

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