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In vivo efficacy and metabolism of the antimalarial cycleanine and improved in vitro antiplasmodial activity of novel semisynthetic analogues

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Abstract: Bisbenzylisoquinoline (BBIQ) alkaloids are a diverse group of natural products that demonstrate a range of biological activities. In this study, the *in vitro* antiplasmodial activity of three BBIQ alkaloids (cycleanine (**1**), isochondodendrine (**2**) and 2′-norcocsuline (**3**)) isolated from the *Triclisia subcordata* Oliv. medicinal plant traditionally used for the treatment of malaria in Nigeria are studied alongside two semi-synthetic analogues (**4** and **5**) of cycleanine. The antiproliferative effects against a chloroquine-resistant *Plasmodium falciparum* strain were determined using a SYBR Green 1 fluorescence assay. The *in vivo* antimalarial activity of cycleanine (**1**) is then investigated in suppressive, prophylactic and curative murine malaria models after infection with a chloroquine-sensitive *Plasmodium berghei* strain. BBIQ alkaloids (**1-5**) exerted *in vitro* antiplasmodial activities with IC_{50} at low micromolar concentrations with the two semi-synthetic cycleanine analogues showing an improved potency and selectivity than cycleanine. At oral doses of 25 and 50mg/kg body weight of infected mice, cycleanine suppressed the levels of parasitaemia, and increased mean survival times significantly compared to the control groups. The metabolites and metabolic pathways of cycleanine (**1**) were also studied using high performance liquid chromatography electrospray ionization tandem mass spectrometry. Twelve novel metabolites were detected in rats after intragastic administration of cycleanine. The metabolic pathways of cycleanine were demonstrated to involve hydroxylation, dehydrogenation, and demethylation. Overall, these *in vitro* and *in vivo* results provide a basis for the future evaluation of cycleanine and its analogues as leads for further development.

Keywords: Malaria; *Plasmodium falciparum; Plasmodium berghei*; bisbenzylisoquinoline alkaloids; cycleanine; metabolism; *in vivo* activity.

1. Introduction

In 2018, the World Health Organization (WHO) report estimated a global burden of 228 million cases accounting for 405,000 deaths (1). The majority of this burden fell on the WHO Africa Region, where malaria, particularly that caused by the most virulent etiological agent *Plasmodium falciparum*, exerts an immense economic impact. Whilst malaria cases and mortality figures continue to fall (1, 2), the development and spread of resistance to available chemotherapeutic agents poses a significant threat to malaria treatment and management (3). Natural products of plant origin have traditionally provided good sources for discovery of drug leads or novel compounds in modern drug research (4, 5). For example, artemisinin isolated from *Artemisia annua*, sweet wormwood, a traditional Chinese medicine, together with a series of its semi-synthetic derivatives, has become the first-line therapy for *P. falciparum* malaria (6, 7). However, due to the development of artemisinin drug resistance (8), novel therapies are still urgently needed.

Bisbenzylisoquinoline (BBIQ) alkaloids are a diverse group of natural products consisting of two benzylisoquinoline groups (9). BBIQ alkaloids are primarily found in the *Berberidaceae*, *Lauraceae*, *Menispermaceae*, and *Ranunculaceae* plant families. These alkaloids possess a variety of biological activities including antimalarial activities (9, 10). For example, BBIQ alkaloids isolated and identified from *Triclisia* species of the *Menispermaceae* family have antiproliferative activities (10). In Nigeria, the root of *Triclisia subcordata* Oliv. is traditionally used for the treatment of a range of diseases, including malaria (11, 12). The bioactive components of *T. subcordata* are the BBIQ alkaloids cycleanine (**1**), isochondodendrine (**2**) and 2′-norcocsuline (**3**) (Figure 1) and have previously been isolated and characterized by our group(13, 14). We have also produced synthetic analogues of cycleanine (**4** and **5**) (Figure 1) (15). The three naturally occurring BBIQ alkaloids, cycleanine (16-18), isochondodendrine (18, 19), and 2′-norcocsuline (16, 20) have been reported to possess antiplasmodial effects against chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains. Despite the promising *in vitro* biological activity of these natural BBIQ alkaloids, the *in vivo* antimalarial activity of BBIQ alkaloids has not been evaluated nor their potential *in vivo* metabolism.

Here we assess the *in vivo* antimalarial activity and metabolism of cycleanine (**1**). The effect of increasing the water solubility of cycleanine analogues (**4** and **5**) on antiplasmodial potency and selectivity will also be investigated.

This study sets out an evaluation of the *in vitro* antimalarial activities of the BBIQ alkaloids (**1**-**3**) compared to two semi-synthetic BBIQ alkaloids (**4** and **5**) derived by a modification of cycleanine at the C-5 position by introducing additional secondary or tertiary amine moieties in an attempt to increase potential solubility and potency (15). The most abundant BBIQ alkaloid in *T. subcordata* extract is cycleanine, this was therefore used to establish *in vivo* antimalarial activity in a murine malaria model. In addition, the metabolites and metabolic pathways of cycleanine were analyzed after intragastric administration in rats to help understand how cycleanine is eliminated *in vivo* to guide future optimization of cycleanine for antimalarial development.

2. Results

2.1 The semi-synthetic derivatives of cycleanine have improved in vitro antiplasmodial activity and selectivity

The *in vitro* antiplasmodial activity of the five BBIQ alkaloids (**1**-**5**) as well as a chloroquine control were performed against intraerythrocytic stages of the *P. falciparum* Dd2 chloroquine resistant strain using a Malaria SYBR Green I fluorescence assay. These data are provided in Table 1 (Figure S1) as IC_{50} values (mean ± SD for n = 3 independent biological repeats). Whilst the data for chloroquine in Dd2 are comparable to that of the W2 chloroquine resistant strain, the activities of cycleanine, isochondodendrine and 2′-norcocsuline are significantly lower in Dd2 than reported in W2, and certainly lower than that in the chloroquine sensitive strain D6. The semi-synthetic products **4** and **5** are relatively more potent than **1**-**3** in Dd2, with the most potent, **4**, some 25.2-fold more potent than its natural precursor- cycleanine (**1**).

Data from cytotoxicity studies of BBIQ alkaloids **1**-**3** in human oral epidermoid carcinoma (KB) or HCT-116 human colon carcinoma cells suggest low to moderate selectivity with SI of 14 to >133. CC_{50} data for all five compounds are available from human ovarian epithelial (HOE) cells (Table 1).

These data reinforce the findings of low selectivity, albeit improved in the semi-synthetic products **4** and **5.**

2.2 In vivo antimalarial activity of cycleanine (1)

The isolation of the abundant cycleanine (**1**) in *T. subcordata* root enabled us to investigate its efficacy and toxicity in murine malaria models after infection with *Plasmodium berghei.* The acute LD_{50} of cycleanine after 24h oral administration was determined to be 4.5 g/kg in mice, indicating a good safety profile. The malaria suppressive activity of cycleanine using two oral doses (25 and 50 mg/kg of body weight/day) following *P. berghei* infection was demonstrated through a significant suppression of parasitaemia and increased mean survival time (MST) compared to untreated controls (Table 2). In particular, the higher dose (50 mg/kg/day) showed efficacy, both in terms of suppression of parasitaemia and MST, comparable to that for chloroquine at a dose of 5 mg/kg/day. The prophylactic activity of cycleanine, with the same 25 and 50 mg/kg dosing regimen during *P. berghei* infection in mice, was also demonstrated (Table 3). At the higher dose (50 mg/kg), cycleanine showed a suppression of parasitaemia by 59.0%, only slightly less than that of 76.2% using the prophylactic pyrimethamine control at a dose of 1.2 mg/kg /day.

The curative activity and MST of mice after initial *P. berghei* infection and subsequent treatment with cycleanine (**1**) were determined. After infection of mice for three days, cycleanine were administered at both doses of 25 and 50 mg/kg and showed decreasing parasitaemia in a dose-dependent and time-dependent manner from day 3 to day 7 (Fig. 2). The speed of killing *P. berghei* parasites by chloroquine was much faster than cycleanine. Chloroquine reached 0% of parasitaemia after 5 days, while at that time cycleanine at doses of 25 and 50 mg/kg had remaining levels of 13.3 and 10.5%, respectively (Figure 2). In this curative model, the MST of mice at doses of 25 and 50 mg/kg were 21 and 25 days, respectively, which were significantly longer than the control (12 days). However, they were both shorter than that of chloroquine (30 days) (Table S1).

2.3 In vivo metabolism of cycleanine

In order to explore the *in vivo* metabolism of cycleanine, the plasma and urine of Wistar rats following an oral dose of 120 mg/kg body weight/day over a 24 hour period were analyzed for

cycleanine metabolites. Samples from urine and plasma were prepared and submitted to high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-MS/MS) analysis. The peak at the retention time of 9.7 min was cycleanine (M0) with the protonated molecular ion *m*/*z* 623.3119 $[M+H]^+$ (elemental composition $C_{18}H_{43}N_2O_6$) in the positive ion mode spectrum (Table 4, Figure 3 and S2). In MS/MS, the quasi-molecular ion loses a neutral molecular NH_2CH_3 fragment to generate an ion *m*/*z* 592.2696; also by symmetric cleavage, and breaking C-O and C-C bond to produce a fragment ion *m*/*z* 312.1594, which can also lose C_2H_6 to produce a fragment ion *m*/*z* 281.1165. After another C-O and C-C bond cleavage and subsequent loss of CH_3 and OCH_3, fragment ions *m*/*z* 204,101, 190.0857, and 159.1038 were generated. A fragment ion *m*/*z* 400.1895 was also generated by simultaneous C-O bond cleavage and C-C bond cleavage adjacent to the N atom (Figure 3, S2).

Twelve peaks on LC-MS/MS chromatograms relevant to cycleanine were detected in either urine or plasma samples (Table 4, Fig. S3). The original form of cycleanine and eleven metabolites were found from the urine of rats, which were presumed to be hydroxylation (M1, M2), demethylation and hydroxylation (M3), monodemethylation (M4), didemethylation (M5), dehydrogenation and hydroxylation (M6, M12), dehydrogenation and dihydroxylation metabolite (M7) and its isomeric metabolites (M8, M9, M11). From the cycleanine-containing plasma of rats, the original form cycleanine (M0) and five metabolites were found, which were presumed to be hydroxylation (M2, M10), dehydrogenation and hydroxylation (M6, M12), dehydrogenation and dihydroxylation (M7) metabolites. Among them, the prototype (M0), hydroxylation (M1), dehydrogenation and hydroxylation (M6, M12) metabolites were detected in both rat urine and plasma (Table 4 and Supplementary materials). Therefore, the metabolic pathway of cycleanine in rat involves hydroxylation, dehydrogenation and demethylation or their combination, which are the main means of biotransformation of cycleanine to generate a large number of metabolites (M1-M12) (Fig. S5).

142 **3. Discussion**

143 Natural products (e.g. artemisinin, quinine) have demonstrated their potential as a source of
144 antimalarial drugs. Previously, a number of BBIQ alkaloids were demonstrated to have *in vitro*
145 antiplasmodial activities (16). Cycleanine had antiplasmodial effects with IC_{50} of 70 nm (16) (or 80
146 nM (17)) against *P. falciparum* chloroquine-sensitive clone D6 (or 3D7) and IC_{50} of 4.5 μM against
147 chloroquine-resistant strain (18). Isochondodendrine showed a low IC_{50} of 0.2 μM against
148 chloroquine-resistant strain (18, 19). 2′-Norcocsuline also showed potent *in vitro* anti-plasmodial
149 activity with IC_{50} of 48 and 248 nM against chloroquine-sensitive clone D6 (3D7) and
150 chloroquine-resistant clone W2 (16, 20), respectively (Table 1). Our results against *P. falciparum*
151 chloroquine-resistant strain (Dd2) also confirmed the *in vitro* antimalarial activity of these
152 compounds but with slightly higher IC_{50} values (Table 1) compared to the corresponding values
153 reported in literature. Isochododendrine is a structurally demethylated analogue of cycleanine, and
154 showed a greater potency than cycleanine in chloroquine-resistant W2 strain and the Dd2 strain in
155 this study (Table 1). This indicated that the increase of the hydrophilicity of cycleanine could
156 improve its antiplasmodial activity. The SI values of all three BBIQ alkaloids ranged from 14 to 133
157 based on the KB or HTC-116 cells and W2 strain, which were much greater than those based on HOE
158 cells and Dd2 strain. The discrepancy might be due to the different methodologies (16) used to
159 determined IC_{50} or the different mammalian cancer cells or *P. falciparum* clones used. The
160 semi-synthetic analogues of cycleanine (**4** and **5**) produced by chemical modification of cycleanine
161 through introduction of dimethylamino- and (mono)alkynylamino- group at C-5 position exhibited
162 increase in antiplasmodial potency and SI than cyleanine. The presence of a dimethylamino group in
163 compound **4** could also increase the water solubility of the parent compound as often found in the
164 modification of other natural products such as camptothecin (21) and thymoquinone (22).
165 Compound **5** with a unique aminoalkynyl group was used as a chemical probe for exploring the
166 mechanism of action (e.g. cellular uptake) of cycleanine in cancer cells using click chemistry (15), and
167 will be also be utilized for identification of the molecular target of cycleanine in parasite-infected

168 blood cells using a chemoproteomic approach (23). By changing the amino substitution groups,
169 additional analogues of cycleanine with a variety of diverse structures will be synthesized for *in vitro*
170 antiplasmodial evaluation.

171 To further confirm and validate the efficacy of cycleanine (**1**) *in vivo*, its safety in healthy mice
172 and efficacy in murine malaria model was investigated. The LD_{50} (4.5g/kg) of cycleanine indicated
173 that cycleanine has a good safety profile, in agreement with a LD_{50} of 1.1g/kg as found previously in
174 mice (24). Using suppression, prophylactic and curative murine malaria models after infection with
175 *P. berghei* (25), cycleanine showed a similar or closer effect at an oral dose of 50mg/kg to their positive
176 controls (chloroquine (5 mg/kg) and pyrimethamine (1.2 mg/kg)). At least, a much higher dose of
177 cycleanine was needed to achieve the effects of these positive controls, indicating a mild efficacy *in*
178 *vivo*. However, its low toxicity profile could allow increase of the oral dose (e.g. 100 mg/kg), which is
179 expected to improve its efficacy. In the curative model, the slower effect of cycleanine comparing to
180 chloroquine might be due to the metabolism of cycleanine to various metabolites. The in *vivo*
181 antimalarial activity of cycleanine was consistent with its *in vitro* antiplasmodial activity. To our
182 knowledge, this is the first demonstration of the *in vivo* antimalarial efficacy of a BBIQ alkaloid,
183 cycleanine. Overall, three alkaloids (1-3) of *T. subcordata* could contribute to the anti-malarial effects
184 of this medicinal plant used in Nigeria for the treatment of malaria. BBIQ alkaloids of *Triclisia gilletii*
185 (De Wild) Staner were also reported to be attributed to its *in vitro* and *in vivo* antimalarial activity of
186 its plant extract (26).

187 Study on the metabolism of drugs can further help to understand their pharmacokinetics,
188 efficacy and safety (27). For example, metabolites of piperaquine were shown to have stronger
189 antiplasmodial activity (28). However, there were only few *in vivo* metabolism studies of BBIQ
190 alkaloids. Previously, *in vitro* metabolites of a BBIQ alkaloid, isoliensinine from the dog hepatic
191 microsomes were identified as 2'-*N*-desmethylisoliensinine, 2-*N*-desmethyl-isoliensinine, and
192 2'-*N*-6-*O*-didesmethylisoliensinine (29). The study of the pharmacokinetics and metabolism of
193 another BBIQ alkaloid, neferinein indicated that it was partially converted to liensinine,
194 desmethyl-liensinine, isoliensinine, and desmethyl-isoliensinine by CYP2D6 (30). Tetrandrine was

found to be initially biotransformed to a quinonemethide-derived metabolite mediated by CYP3A enzymes, which was then trapped by a glutathione to form a glutathione conjugate in mice (31). Metabolism of isotetrandrine by *in-vitro* rat hepatic system produced a major metabolite, N-desmethylisotetrandrine (16%), and three minor oxidized metabolites, oxo-isotetrandrine (7%), hydroxy-isotetrandrine (6%), and oxohydroxy-isotetrandrine (7%) via N-demethylation and isoquinoline ring oxidation (32).

Our identification of twelve new metabolites of cycleanine in both plasma and urine in rats using LC-MS/MS has indicated that there were various metabolic pathways of cycleanine. These metabolites of cycleanine found in rats are also likely generated in mice after the same route of oral administration, therefore they could contribute to its *in vivo* antimalarial efficacy found in the murine malarial model and its toxicity finding in healthy mice. Hydroxylation and demethylation of cycleanine were the common pathways consistent with those found in isoliensinine, neferinein and isotetrandrine described above. Preparation of these metabolites through chemical synthesis (33) or *in vitro* biotransformation using hepatic microsomes and P450 enzymes (34, 35) are possible and necessary to evaluate their potency and toxicity. Such information can be used to further guide chemical design and modification of cycleanine to improve its potency, pharmacokinetics and increasing metabolic stability (36). Further work is necessary and on-going in our laboratory to determine the *in vivo* antimalarial effects of BBIQ alkaloids (2, 3), semi-synthetic derivatives (4, 5), *in vitro* and/or *in vivo* antimalarial activity of the metabolites (M1-12) of cycleanine. Novel active drugs particularly those with a wide safety margin are required to help alleviate malaria morbidity and mortality, and to contribute to the global control of malaria and infectious diseases.

4. Materials and Methods

4.1 Chemicals

Chloroquine and pyrimethamine were sourced from Sigma-Aldrich. Cycleanine (**1**) (13), and two minor alkaloids, isochondodendrine (**2**) and 2′-norcocsuline (**3**) were isolated from *Triclisia subcordata* (14). Compound **4** and **5** (Figure 1) were previously prepared from cycleanine (**1**) (15).

4.2 *In vitro* anti-plasmodial activity

The evaluation of *in vitro* antiplasmodial activity of the alkaloids (**1**-**3**) and semisynthetic analogues (**4** and **5**) were performed on the intraerythrocytic *P. falciparum* Dd2 strain (chloroquine resistant strain) using a SYBR Green1 Fluorescence dye assay as described (22, 37, 38). Compounds **1-5** were prepared in DMSO with no greater than 1% of the total solvent concentration in any assay. Normalized fluorescence signals were measured against controls with 1% DMSO (100% growth) and after exposure to a supralethal concentration (10 μM) of chloroquine (0% growth). Determination of the 50% inhibitory concentration (IC_{50}) was performed form a Log concentration versus mean normalized fluorescence signal curve using GraphPad Prism software (v5.0). Each biological replicate consisted of three technical repeats, with three independent biological replicates performed.

4.3 Evaluation of the *in vivo* antimalarial activity of cycleanine

Malaria parasite

Chloroquine-sensitive strain of *P. berghei* were sourced from the National Institute of Medical Research (NIMER), Yaba Lagos, Nigeria and maintained by sub-passage in mice.

Parasite inoculation

Each mouse was inoculated intraperitoneally with about 1 x 10^7 *P. berghei* parasitized erythrocytes in 0.2 mL of infected blood (5 x 10^7 *P. berghei* erythrocytes/mL) according to published procedure (39).

Experimental animals

Female and male Swiss albino mice (18-25 g) were obtained from the University of Uyo's animal house. Before use mice were kept in cages and acclimatized for 10 days. All mice were kept in cross ventilated rooms at room temperature. The care and use of mice were performed in accordance with the National Institute of Health Guide for the Care and Use of laboratory Animals (NIH Publication, 1996). This investigation was approved from the University of Uyo's Animal Ethics Committee.

Determination of median lethal dose (LD_{50}) of cycleanine

The median lethal dose (LD_{50}) of cycleanine was determined using albino mice by intraperitoneal (i.p) route (40). Different doses of cycleanine (10 – 5000 mg/kg) were intraperitoneally administrated to groups of three mice each. The mice were monitored for manifestation of physical signs of toxicity including decrease of motor activity, writhing, decrease of body/limb tone, and weakness and death. The number of deaths in each group within 24 h was recorded. The LD_{50} value was calculated as geometrical means of the minimum dose producing 100% mortality and the maximum dose producing 0%.

Drug administration

Cycleanine, chloroquine and pyrimethamine were prepared in water and administered orally with the aid of a stainless metallic feeding cannula.

Suppressive activity of cycleanine

The schizontocidal activity of the cycleanine and chloroquine against early *P. berghei* infection in mice was measured according to an established protocol (25, 41, 42). On the first day, twenty-four mice were infected with the parasite and randomly separated into four groups. The mice in group 1 and 2 were given 25 and 50 mg/kg of cycleanine respectively, group 3 was given 5 mg/kg of chloroquine (positive control) and group 4 given distilled water (10 mL/kg, negative control) for four consecutive days. Thin films were made from the tail blood on the fifth day. Parasitized erythrocytes were counted in stained films (by Giemsa stain) under a microscope. The average suppression of parasitemia (%) was calculated as follows:

$$\frac{(\text{average \% parasitemia positive control} - \text{average \% parasitemia negative control})}{(\text{average \% parasitemia negative control})} * 100$$

The MST (days) of the mice in each group was determined over a period of 30 days.

Prophylactic activity of cycleanine

The prophylactic activity of cycleanine was evaluated using the method as previously described (42, 43). The mice were randomly divided into four groups of six mice per group. Groups 1 and 2 were given 25 and 50 mg/kg of cycleanine respectively, group 3 was given 1.2 mg/kg of pyrimethamine (positive control) and group 4 given 10 mL/kg of distilled water (negative control).

Administration of the cycleanine and drug continued for three consecutive days. On the fourth day, the mice were inoculated with *P. berghei*. The parasitemia level was evaluated by blood smears after 3 days. The survival time (day) of the mice were recorded over a period of 30 days and MST were calculated.

Curative activity of cycleanine

The curative activity of cycleanine was assessed according to the method described previously (42, 44). *P. berghei* was injected intraperitoneally into another twenty-four mice on the first day. Three days later, the mice were also separated into four groups of six mice per group. Groups 1 and 2 were administered different doses of cycleanine, 25 and 50 mg/kg respectively, group 3 was given 5 mg/kg chloroquine (positive control) and group 4 was given 10 mL/kg distilled water (negative control). Cycleanine and chloroquine were given once a day for 5 days. Mice tail blood samples were collected on each day, and Giemsa stained thin smears were prepared to determine the parasitemia level. The MST of the mice in each group was determined over a period of 30 days.

4.4 Metabolism of cycleanine in rats

High-performance liquid chromatography quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS/MS)

Analysis of cycleanine metabolites was performed through HPLC-Q-TOF-MS/MS system that consists of an Agilent 1260 HPLC coupled with Agilent 6530 Q-TOF mass spectrometer with dual Agilent Jet Stream electrospray ionization source (Agilent Technologies, CA, USA). The mass spectra were recorded in positive Auto MS/MS mode and the parameters were set as follows: temperature of drying and sheath gas, 300 °C and 350 °C; skimmer, 75 V; capillary voltage, 4000 V; fragmentor, 110 V; nozzle voltage, 1000 V; collision energy, 50 eV; pressure of nebulizer, 35 psi; and flow rate of the drying and sheath gas, 5 and 11 L/min, respectively. The Q-TOF mass spectra were recorded in high-resolution mode. The range of mass-to-charge ratio (*m*/*z*) scanning was set between 100 and 1200. Samples (5 μL) were loaded onto an Agilent Poroshell 120 EC-C18 column (100×2.1 mm, 2.7 μm) at 35 °C. The mobile phase consisted of water containing 0.1 % formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) at a flow rate of 0.35 mL/min. Gradient

separation was achieved by changing the proportion of the solvent B mobile phase as follows: 0- 2 min, 10% B; 2.1- 5 min, 18%- 20% B; 30- 45 min, 70%- 90% B; and 45- 50 min, 10% B. Mass hunter Workstation software (Agilent Technologies, Palo Alto, CA, USA) was utilized for the system operation and data analysis.

***In vivo* animal experiments**

In vivo animal experiments were approved by the Animal Ethics Committee of Shanghai Institute of Materia Medica, and performed according to procedures approved by the Institutional Animal Care and Use Committee of Shanghai Institute of Materia Medica, Chinese Academy of Science. Male Wistar rats were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The rats were given free access to water and standard diet under controlled humidity (45%–55%) and temperature (20 °C–24 °C), and except in the overnight fasting period before administration of cycleanine. The rats were adapted to the environment for a week.

Cycleanine (**1**) was suspended in 0.4% carboxymethyl cellulose sodium (CMC-Na) and was formulated at 12 mg/mL for intragastric administration to Wistar rats (male, 220 ± 10 g, fasted for 12 hours prior to administration) at a dose of 120 mg/kg body weight. Three rats were used for blood collection through orbital vein using cannulation at 0, 0.5, 1, 2, 4, 6, 8, 12 and 24h post dose after anaesthetization with isoflurane. The plasma samples were separated from blood by centrifugation at 12000 rpm and 4 °C for 10 min. Another three rats were placed in the metabolism cages, and urine samples were collected into tubes from 0 to 24 h after oral administration of cycleanine. All samples were stored in a -80 °C freezer before analysis. Total of 1.2 mL of plasma or urine sample was mixed with 3 times the volume of acetonitrile to precipitate proteins. After centrifugation at 14,000 rpm for 10 min, the supernatant was collected and evaporated under vacuum. The residue was reconstituted in 200 μL methanol, and 5 μL of each sample was injected into HPLC-Q-TOF-MS/MS analysis.

4.5 Statistical Analysis

Data was expressed as mean ± standard error of mean (SEM). Data was subjected to GraphPad Prism software analysis. Results were analyzed using one-way analysis of variance (ANOVA)

followed by a post Tukey-Kramer multiple comparison test. The difference between mean of the experimental and control groups was considered significant at $p < 0.05$ (ANOVA).

5. Conclusions

Three BBIQ alkaloids – cycleanine (**1**), isochondodendrine (**2**) and 2′-norcocsuline (**3**) of *T. subcordata* and two semi-synthetic analogues (**4** and **5**) of cycleanine were demonstrated to exert significant *in vitro* antiplasmodial activities against *P. falciparum*. Cycleanine (**1**) was further demonstrated to have safety and efficacy in the treatment of mice infected with *P. berghei.* Cycleanine was transformed to various metabolites in rats after oral delivery. The findings from this study support the use of *T. subcordata* as antimalarial agent in traditional medicine. BBIQ alkaloids could be exploited in novel drug development in search of antimalarial agents/drugs urgently needed to challenge resistant plasmodium species which currently present significant great threat to human life.

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Conflicts of Interest: The authors declare no conflict of interest.

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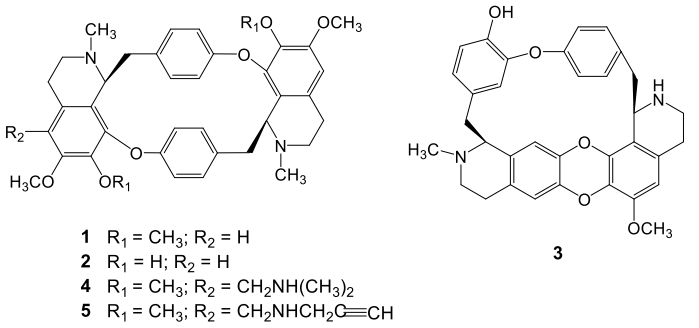

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458 **Figure 1. Chemical structure of bisbenzylisoquinoline (BBIQ) alkaloids**. Cycleanine (**1**),

459 isochondodendrine (**2**) and 2′-norcocsuline (**3**) from *T. subcordata* and two novel semi-synthetic

460 analogues (**4** and **5**) of cycleanine.

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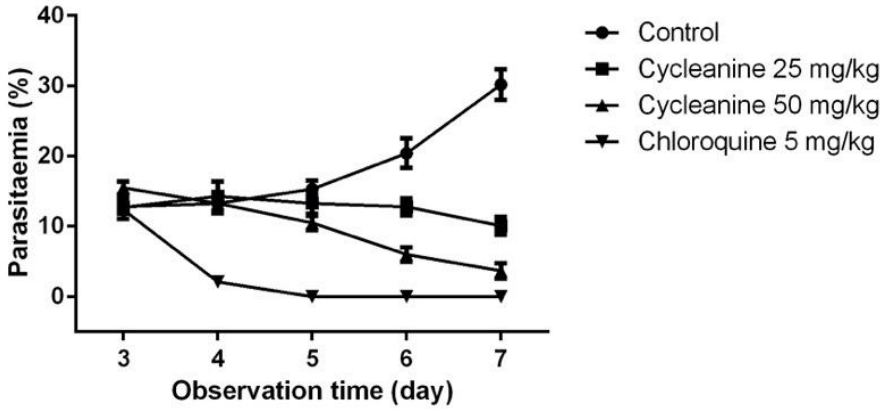

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464 **Figure 2 The curative activity of mice treated with cycleanine (1) during established *P. berghei***

465 **infection**. After infection of mice with for 3 days, cycleanine were administered at both doses of 25

466 and 50 mg/kg, while water and chloroquinine at 5mg/ml were administered as negative and positive

467 controls, respectively. The parasitaemia levels were monitored for a total duration of 4 days (from

468 day 3 to day 7).

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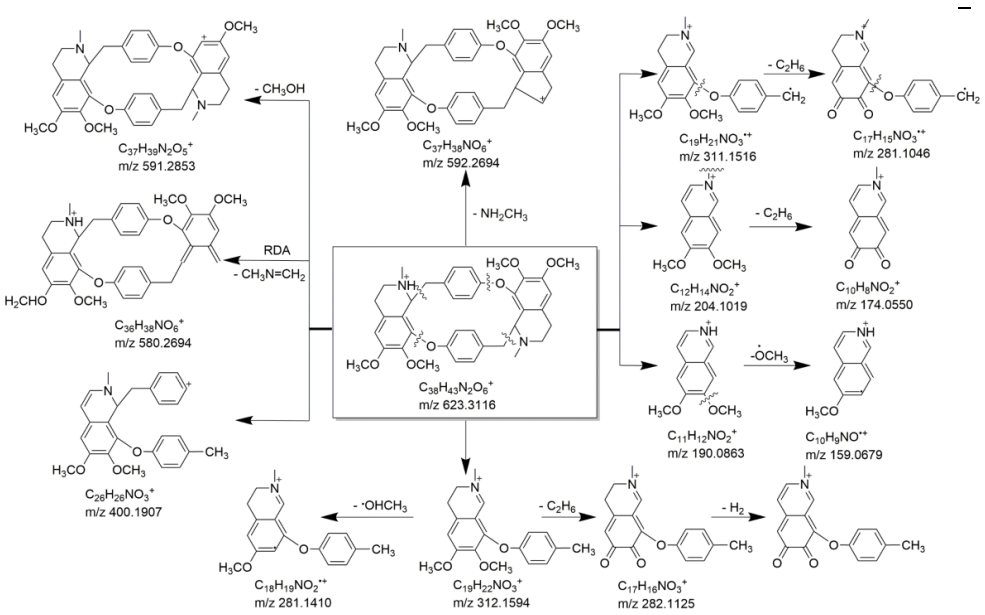

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473 **Figure 3. Possible fragmentation pattern of cycleanine**. See the analysis of fragment ions in the

474 text.

475

476

477 **Table 1.** The i*n-vitro* half maximal inhibitory concentration (IC_{50}) values of BBIQ alkaloids (**1-5**)
478 against *P. falciparum* chloroquine resistant strains (Dd2 and W2 strain), chloroquine sensitive strain
479 (D6), and the 50% cytotoxic concentration (CC_{50}) values against cancer cell lines, and selectivity
480 index (SI).
481

BBIQ alkaloids	***P. falciparum* Dd2 (μM)** [a]	***P. falciparum* W2 (μM)**[b,c]	***P. falciparum* D6 (μM)**[b,d]	**KB**[b] **or HCT**[c] **(μM)**	**HOE (μM)** [e]	**SI (KB/ W2)** [f]	**SI (HOE/Dd 2)** [f]
Cycleanine (**1**)	17.7 ± 2.0	0.25[b]; 4.5 [c]	0.07 [b]	>33.7[b]; 531 (HCT)[c]	35.0 ± 0.1	>133	2.0
Isochondodendrine (**2**)	6.1 ± 1.3	0.2 [c]	N.D. [d]	29 (HCT)[c]	10.5 ± 1.2	116	1.7
2′-Norcocsuline (**3**)	7.0 ± 1.6	0.28[b]	0.048[b]	3.8[b]	8.0 ± 0.2	14	1.1
5-[(Dimethylamino)methyl]cycleanine (**4**)	0.7 ± 0.1	N.D.	N.D.	N.D.	10.0 ± 0.2	N.D.	14.3
5-[(Propargylamino)methyl]cycleanine (**5**)	1.8 ± 0.2	N.D.	N.D.	N.D.	32.0 ± 1.6	N.D.	17.8
Chloroquine	0.18 ± 0.03	0.135[b]	0.006[b]	33.7[b]	N.D.	250	N.D.

482
483 [a] IC_{50} values are expressed as mean ± SD for n = 3 independent biological repeats.

[b] IC_{50} data against *P. falciparum* W2 and D6 strains, and CC_{50} for human oral epidermoid carcinoma (KB) cells were sourced from a previous report (16).

[c] IC_{50} data against chloroquine resistant *P. falciparum* strain, and CC_{50} for HCT-116 human colon carcinoma cells were sourced from a previous report (18).

[d] N.D., not determined.

[e] CC_{50} data for human ovarian epithelial (HOE) cells. Data in this column for **1-5** were sourced from our previous reports (13, 15).

[f] SI, this selectivity index was calculated as CC_{50} in cytotoxicity/IC_{50}*P. falciparum*.

Table 2: Suppressive activity of cycleanine during early *Plasmodium berghei* infection of mice.

Treatment	Dose (mg/kg) per day	Parasitaemia after infection for 96h (%)[a]	Suppression of parasitaemia at 96h (%)[a]	MST (days)[a]
Untreated control	-	28.3 ± 1.8	-	12.5 ± 0.3
Cycleanine	25	15.7 ± 1.8 [b]	44.7	24.7 ± 1.1 [b]
	50	3.8 ± 0.7 [b]	86.5	28.2 ± 0.9 [b]
Chloroquine	5	2.0 ± 0.8 [b]	94.0	30.0 ± 0.0 [b]

[a] values are expressed as mean ± SEM (n = 6 in each group)

[b] Significant relative to untreated control, $p < 0.001$.

Table 3: Prophylactic activity of cycleanine in *Plasmodium berghei* infection of mice.

Treatment	Dose (mg/kg) per day	Parasitaemia level after infection for 72h (%)[a]	Suppression of parasitaemia level after infection for 72h (%)[a]	MST (day)[a]
Untreated control	-	20.3 ± 0.8	-	12.7 ± 0.3
Cycleanine	25	11.5 ± 0.9 [b]	43.4	23.0 ± 0.6 [b]
	50	7.3 ± 1.0 [b]	59.0	24.5 ± 0.6 [b]
Pyrimethamine	1.2	4.8 ± 1.1 [b]	76.2	29.8 ± 0.2 [b]

[a] values are expressed as mean ± SEM (n = 6 in each group)

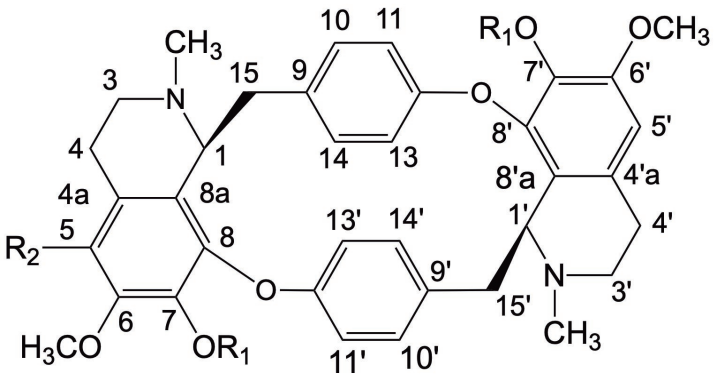
[b] Significant relative to control, $p < 0.001$.

509 Table 4. HPLC/QTOF-MS retention times, mass spectrometric data of cycleanine and its metabolites.

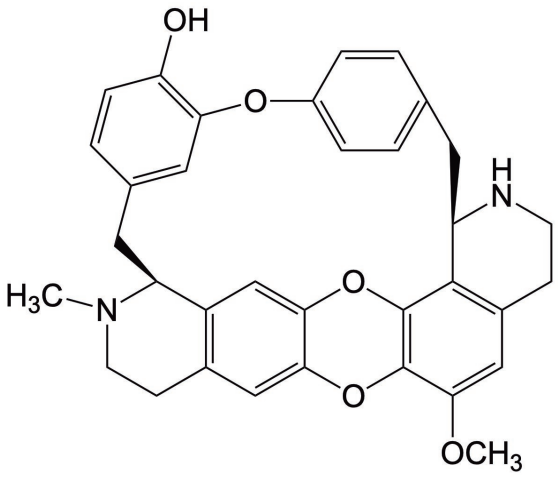
No.	t (min)	Measured $[M+H]^+$ m/z	Δppm	Formula	MS/MS fragment	Metabolic pathways	Plasma	Urine
M0	9.9	623.3125	1.41	$C_{38}H_{43}N_2O_6$	592.2696, 400.1895, 312.1583, 311.1508, 281.1165, 204.1011, 190.0857, 174.0911, 159.1038	Parent	+	+
M1	7.2	639.3075	1.36	$C_{38}H_{43}N_2O_7$	592.2472, 416.1838, 310.1422, 220.0964, 204.1046, 190.0815, 175.0955, 157.0901	hydroxylation	-	+
M2	7.9	639.3084	2.79	$C_{38}H_{43}N_2O_7$	621.2977, 416.1864, 400.1917, 327.1469, 312.1361, 220.0964, 206.0780, 175.0988	hydroxylation	+	+
M3	8.1	625.2911	0.84	$C_{37}H_{41}N_2O_7$	607.2784, 425.1379, 312.1591, 298.1434, 204.0999, 190.0854, 176.0691, 159.1033	demethylation and hydroxylation	-	+
M4	9.6	609.2956	0.96	$C_{37}H_{41}N_2O_6$	593.2750, 427.1577, 357.1449, 312.1580, 298.1435, 204.1020, 190.0850,	demethylation	-	+

					176.0704, 145.0880				
M5	10.1	595.2799	0.73	$C_{36}H_{39}N_2O_6$	578.2505, 284.1282, 176.0703, 145.0879	didemethylation		-	+
M6	10.4	637.2918	1.12	$C_{38}H_{41}N_2O_7$	328.1553, 309.1381, 202.0855, 188.0656, 157.0879	dehydrogenation and hydroxylation		+	+
M7	11.1	653.2855	0.38	$C_{38}H_{41}N_2O_8$	635.2754, 326.1384, 309.1381, 202.0855, 188.0656, 157.0879	dehydrogenation and dihydroxylation		+	-
M8	12.1	653.2868	1.23	$C_{38}H_{41}N_2O_8$	592.2459, 310.1420, 293.1154, 281.1163, 269.1169, 204.1031, 190.0884	dehydrogenation and dihydroxylation		-	+
M9	13	653.2856	0.27	$C_{38}H_{41}N_2O_8$	635.2701, 400.1881, 326.1380, 310.1427, 202.0855, 173.0820, 157.0881	dehydrogenation and dihydroxylation		-	+
M10	13.4	621.2966	0.97	$C_{38}H_{41}N_2O_6$	591.2467, 400.1893, 398.1739, 312.1572, 310.1435, 204.1013, 202.0860, 190.0863, 188.0725, 159.1028, 157.0883	dehydrogenation		+	-

M11	13.6	653.2859	0.73	$C_{38}H_{41}N_2O_8$	413.1375, 324.1595, 309.1345, 281.1158, 204.1015, 159.1021	dehydrogenation and dihydroxylation	+	+
M12	14.1	637.2919	1.61	$C_{38}H_{41}N_2O_7$	594.2486, 414.1684, 326.1381, 312.1237, 281.1159, 218.0824, 204.1013, 190.0874, 173.0830	dehydrogenation and hydroxylation	+	+



1 $R_1 = CH_3$; $R_2 = H$
2 $R_1 = H$; $R_2 = H$
4 $R_1 = CH_3$; $R_2 = CH_2NH(CH_3)_2$
5 $R_1 = CH_3$; $R_2 = CH_2NHCH_2C{\equiv}CH$



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