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

Fidelia Ijeoma Uche[1], Xiaozhen Guo[2], Jude Okokon[3], Imran Ullah[4], Paul Horrocks[4], Joshua Boateng[5], Chenggang Huang[2,*] and Wen-Wu Li[1,*]

[1] School of Pharmacy and Bioengineering, Keele University, Stoke-on-Trent, ST4 7QB, United Kingdom; fiuche@yahoo.com (FU); w.li@keele.ac.uk (WL)
[2] Shanghai Institute of Materia Medica, Chinese Academy of Science, Shanghai, 201203, China; guoxzfj@yeah.net (XG); cghuang@simm.ac.cn (CH)
[3] Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo, Uyo, Nigeria; judeefiom@yahoo.com (JO)
[4] School of Medicine, Keele University, Stoke-on-Trent, ST5 5BG, United Kingdom; Imran.Ullah@utsouthwestern.edu (IU); p.d.horrocks@keele.ac.uk (PH)
[5] School of Science, University of Greenwich, Medway Campus, ME4 4TB, United Kingdom; J.S.Boateng@greenwich.ac.uk (JB)

* Correspondence: cghuang@simm.ac.cn (CH); w.li@keele.ac.uk (WL)

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.

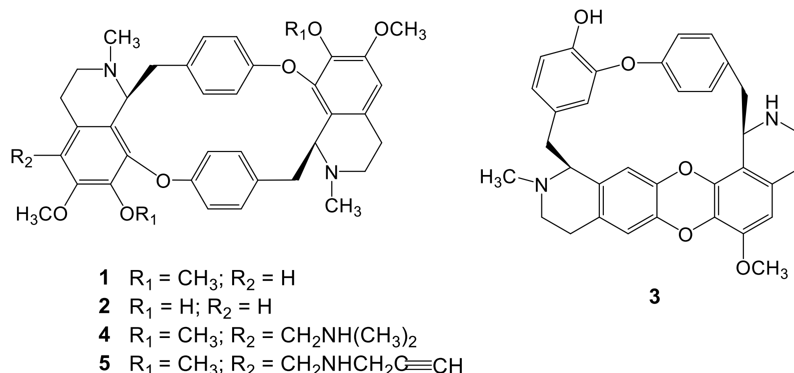


Figure 1. Chemical structure of bisbenzylisoquinoline (BBIQ) alkaloids. Cycleanine (**1**), isochondodendrine (**2**) and 2′-norcocsuline (**3**) from *T. subcordata* and two novel semi-synthetic analogues (**4** and **5**) of cycleanine.

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

83 malaria model. In addition, the metabolites and metabolic pathways of cycleanine were analyzed
84 after intragastric administration in rats to help understand how cycleanine is eliminated *in vivo* to
85 guide future optimization of cycleanine for antimalarial development.

86 **2. Results**

87 *2.1 The semi-synthetic derivatives of cycleanine have improved in vitro antiplasmodial*
88 *activity and selectivity*
89
90 The *in vitro* antiplasmodial activity of the five BBIQ alkaloids (**1**-**5**) as well as a chloroquine
91 control were performed against intraerythrocytic stages of the *P. falciparum* Dd2 chloroquine
92 resistant strain using a Malaria SYBR Green I fluorescence assay. These data are provided in Table 1
93 (Figure S1) as IC_{50} values (mean ± SD for n = 3 independent biological repeats). Whilst the data for
94 chloroquine in Dd2 are comparable to that of the W2 chloroquine resistant strain, the activities of
95 cycleanine, isochondodendrine and 2′-norcocsuline are significantly lower in Dd2 than reported in
96 W2, and certainly lower than that in the chloroquine sensitive strain D6. The semi-synthetic products
97 **4** and **5** are relatively more potent than **1**-**3** in Dd2, with the most potent, **4**, some 25.2-fold more
98 potent than its natural precursor- cycleanine (**1**).
99 Data from cytotoxicity studies of BBIQ alkaloids **1**-**3** in human oral epidermoid carcinoma (KB)
100 or HCT-116 human colon carcinoma cells suggest low to moderate selectivity with SI of 14 to >133.
101 CC_{50} data for all five compounds are available from human ovarian epithelial (HOE) cells (Table 1).
102 These data reinforce the findings of low selectivity, albeit improved in the semi-synthetic products **4**
103 and **5.**
104
105 **Table 1.** The i*n-vitro* half maximal inhibitory concentration (IC_{50}) values of BBIQ alkaloids (**1-5**)
106 against *P. falciparum* chloroquine resistant strains (Dd2 and W2 strain), chloroquine sensitive strain
107 (D6), and the 50% cytotoxic concentration (CC_{50}) values against cancer cell lines, and selectivity
108 index (SI).
109

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/Dd2)** [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.

110
111 [a] IC_{50} values are expressed as mean ± SD for n = 3 independent biological repeats.
112 [b] IC_{50} data against *P. falciparum* W2 and D6 strains, and CC_{50} for human oral epidermoid
113 carcinoma (KB) cells were sourced from a previous report [16].
114 [c] IC_{50} data against chloroquine resistant *P. falciparum* strain, and CC_{50} for HCT-116 human colon
115 carcinoma cells were sourced from a previous report [18].

116 [d] N.D., not determined.
117 [e] CC_{50} data for human ovarian epithelial (HOE) cells. Data in this column for **1-5** were sourced
118 from our previous reports [13,15].
119 [f] SI, this selectivity index was calculated as CC_{50} in cytotoxicity/IC_{50}*P. falciparum*.
120

121 ### *2.2 In vivo* **antimalarial activity of cycleanine (1)**

122 The isolation of the abundant cycleanine (**1**) in *T. subcordata* root enabled us to investigate its
123 efficacy and toxicity in murine malaria models after infection with *Plasmodium berghei.* The acute
124 LD_{50} of cycleanine after 24h oral administration was determined to be 4.5 g/kg in mice, indicating a
125 good safety profile. The malaria suppressive activity of cycleanine using two oral doses (25 and 50
126 mg/kg of body weight/day) following *P. berghei* infection was demonstrated through a significant
127 suppression of parasitaemia and increased mean survival time (MST) compared to untreated
128 controls (Table 2). In particular, the higher dose (50 mg/kg/day) showed efficacy, both in terms of
129 suppression of parasitaemia and MST, comparable to that for chloroquine at a dose of 5 mg/kg/day.
130 The prophylactic activity of cycleanine, with the same 25 and 50 mg/kg dosing regimen during *P.*
131 *berghei* infection in mice, was also demonstrated (Table 3). At the higher dose (50 mg/kg), cycleanine
132 showed a suppression of parasitaemia by 59.0%, only slightly less than that of 76.2% using the
133 prophylactic pyrimethamine control at a dose of 1.2 mg/kg /day.
134

135 **Table 2:** Suppressive activity of cycleanine during early *Plasmodium berghei* infection of mice.
136

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]

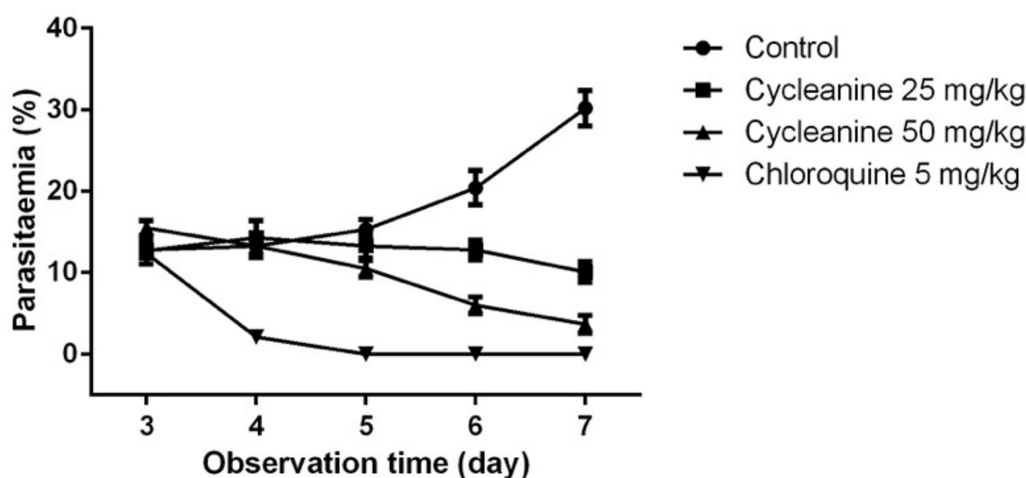
137
138 [a] values are expressed as mean ± SEM (n = 6 in each group)
139 [b] Significant relative to untreated control, $p < 0.001$.
140

141 **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]

142
143 [a] values are expressed as mean ± SEM (n = 6 in each group)
144 [b] Significant relative to control, $p < 0.001$.
145

146 The curative activity and MST of mice after initial *P. berghei* infection and subsequent treatment
147 with cycleanine (**1**) were determined. After infection of mice for three days, cycleanine were
148 administered at both doses of 25 and 50 mg/kg and showed decreasing parasitaemia in a
149 dose-dependent and time-dependent manner from day 3 to day 7 (Fig. 2). The speed of killing *P.*
150 *berghei* parasites by chloroquine was much faster than cycleanine. Chloroquine reached 0% of
151 parasitaemia after 5 days, while at that time cycleanine at doses of 25 and 50 mg/kg had remaining
152 levels of 13.3 and 10.5%, respectively (Figure 2). In this curative model, the MST of mice at doses of
153 25 and 50 mg/kg were 21 and 25 days, respectively, which were significantly longer than the control
154 (12 days). However, they were both shorter than that of chloroquine (30 days) (Table S1).
155



156
157
158 **Figure 2 The curative activity of mice treated with cycleanine (1) during established *P. berghei***
159 **infection**. After infection of mice with for 3 days, cycleanine were administered at both doses of 25
160 and 50 mg/kg, while water and chloroquinine at 5mg/ml were administered as negative and positive
161 controls, respectively. The parasitaemia levels were monitored for a total duration of 4 days (from
162 day 3 to day 7).
163

164 **2.3 *In vivo* metabolism of cycleanine**

165
166 In order to explore the *in vivo* metabolism of cycleanine, the plasma and urine of Wistar rats
167 following an oral dose of 120 mg/kg body weight/day over a 24 hour period were analyzed for
168 cycleanine metabolites. Samples from urine and plasma were prepared and submitted to high
169 performance liquid chromatography electrospray ionization tandem mass spectrometry
170 (HPLC-MS/MS) analysis. The peak at the retention time of 9.7 min was cycleanine (M0) with the
171 protonated molecular ion *m/z* 623.3119 $[M+H]^+$ (elemental composition $C_{18}H_{43}N_2O_6$) in the positive
172 ion mode spectrum (Table 4, Figure 3 and S2). In MS/MS, the quasi-molecular ion loses a neutral
173 molecular NH_2CH_3 fragment to generate an ion *m/z* 592.2696; also by symmetric cleavage, and
174 breaking C-O and C-C bond to produce a fragment ion *m/z* 312.1594, which can also lose C_2H_6 to
175 produce a fragment ion *m/z* 281.1165. After another C-O and C-C bond cleavage and subsequent loss
176 of CH_3 and OCH_3, fragment ions *m/z* 204,101, 190.0857, and 159.1038 were generated. A fragment ion
177 *m/z* 400.1895 was also generated by simultaneous C-O bond cleavage and C-C bond cleavage
178 adjacent to the N atom (Figure 3, S2).
179

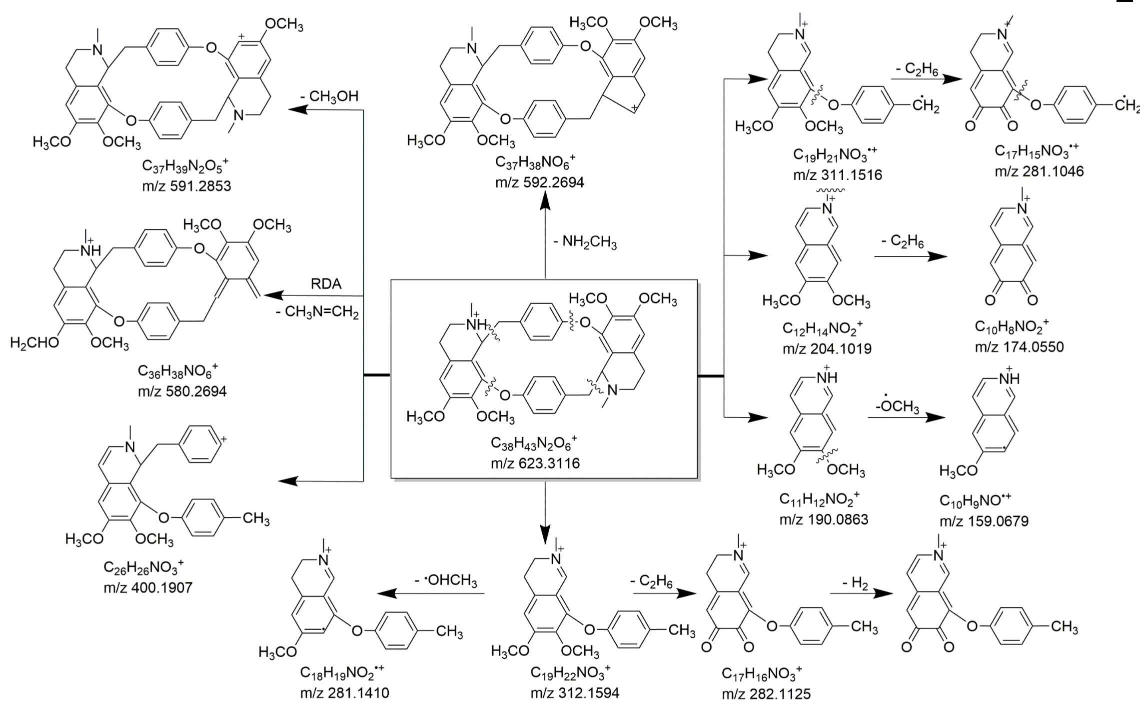


Figure 3. Possible fragmentation pattern of cycleanine. See the analysis of fragment ions in the text.

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).

200 Table 4. HPLC/QTOF-MS retention times, mass spectrometric data of cycleanine and its metabolites.
201

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, 176.0704, 145.0880	demethylation	-	+
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	-	+

202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223

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	+	+

224 **3. Discussion**

225 Natural products (e.g. artemisinin, quinine) have demonstrated their potential as a source of
226 antimalarial drugs. Previously, a number of BBIQ alkaloids were demonstrated to have *in vitro*
227 antiplasmodial activities [16]. Cycleanine had antiplasmodial effects with IC_{50} of 70 nm [16] (or 80
228 nM [17]) against *P. falciparum* chloroquine-sensitive clone D6 (or 3D7) and IC_{50} of 4.5 μM against
229 chloroquine-resistant strain [18]. Isochondodendrine showed a low IC_{50} of 0.2 μM against
230 chloroquine-resistant strain [18,19]. 2′-Norcocsuline also showed potent *in vitro* anti-plasmodial
231 activity with IC_{50} of 48 and 248 nM against chloroquine-sensitive clone D6 (3D7) and
232 chloroquine-resistant clone W2 [16,20], respectively (Table 1). Our results against *P. falciparum*
233 chloroquine-resistant strain (Dd2) also confirmed the *in vitro* antimalarial activity of these
234 compounds but with slightly higher IC_{50} values (Table 1) compared to the corresponding values
235 reported in literature. Isochododendrine is a structurally demethylated analogue of cycleanine, and
236 showed a greater potency than cycleanine in chloroquine-resistant W2 strain and the Dd2 strain in
237 this study (Table 1). This indicated that the increase of the hydrophilicity of cycleanine could
238 improve its antiplasmodial activity. The SI values of all three BBIQ alkaloids ranged from 14 to 133
239 based on the KB or HTC-116 cells and W2 strain, which were much greater than those based on HOE
240 cells and Dd2 strain. The discrepancy might be due to the different methodologies [16] used to
241 determined IC_{50} or the different mammalian cancer cells or *P. falciparum* clones used. The
242 semi-synthetic analogues of cycleanine (**4** and **5**) produced by chemical modification of cycleanine
243 through introduction of dimethylamino- and (mono)alkynylamino- group at C-5 position exhibited
244 increase in antiplasmodial potency and SI than cyleanine. The presence of a dimethylamino group in
245 compound **4** could also increase the water solubility of the parent compound as often found in the
246 modification of other natural products such as camptothecin [21] and thymoquinone [22].
247 Compound **5** with a unique aminoalkynyl group was used as a chemical probe for exploring the
248 mechanism of action (e.g. cellular uptake) of cycleanine in cancer cells using click chemistry [15], and
249 will be also be utilized for identification of the molecular target of cycleanine in parasite-infected
250 blood cells using a chemoproteomic approach [23]. By changing the amino substitution groups,
251 additional analogues of cycleanine with a variety of diverse structures will be synthesized for *in vitro*
252 antiplasmodial evaluation.

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

269 Study on the metabolism of drugs can further help to understand their pharmacokinetics,
270 efficacy and safety [27]. For example, metabolites of piperaquine were shown to have stronger
271 antiplasmodial activity [28]. However, there were only few *in vivo* metabolism studies of BBIQ
272 alkaloids. Previously, *in vitro* metabolites of a BBIQ alkaloid, isoliensinine from the dog hepatic
273 microsomes were identified as 2'-*N*-desmethylisoliensinine, 2-*N*-desmethyl-isoliensinine, and
274 2'-*N*-6-*O*-didesmethylisoliensinine [29]. The study of the pharmacokinetics and metabolism of

another BBIQ alkaloid, neferinein indicated that it was partially converted to liensinine, 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

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

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

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

339 **Drug administration**

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

342 **Suppressive activity of cycleanine**

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

351 $$\frac{(\text{average \% parasitemia positive control} - \text{average \% parasitemia negative control})}{(\text{average \% parasitemia negative control})} * 100$$

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

353 **Prophylactic activity of cycleanine**

354 The prophylactic activity of cycleanine was evaluated using the method as previously described
355 [42,43]. The mice were randomly divided into four groups of six mice per group. Groups 1 and 2
356 were given 25 and 50 mg/kg of cycleanine respectively, group 3 was given 1.2 mg/kg of
357 pyrimethamine (positive control) and group 4 given 10 mL/kg of distilled water (negative control).
358 Administration of the cycleanine and drug continued for three consecutive days. On the fourth day,
359 the mice were inoculated with *P. berghei*. The parasitemia level was evaluated by blood smears after
360 3 days. The survival time (day) of the mice were recorded over a period of 30 days and MST were
361 calculated.

362 **Curative activity of cycleanine**

363 The curative activity of cycleanine was assessed according to the method described previously
364 [42,44]. *P. berghei* was injected intraperitoneally into another twenty-four mice on the first day. Three
365 days later, the mice were also separated into four groups of six mice per group. Groups 1 and 2 were
366 administered different doses of cycleanine, 25 and 50 mg/kg respectively, group 3 was given 5
367 mg/kg chloroquine (positive control) and group 4 was given 10 mL/kg distilled water (negative
368 control). Cycleanine and chloroquine were given once a day for 5 days. Mice tail blood samples were
369 collected on each day, and Giemsa stained thin smears were prepared to determine the parasitemia
370 level. The MST of the mice in each group was determined over a period of 30 days.
371

372 **4.4 Metabolism of cycleanine in rats**

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

375 Analysis of cycleanine metabolites was performed through HPLC-Q-TOF-MS/MS system that
376 consists of an Agilent 1260 HPLC coupled with Agilent 6530 Q-TOF mass spectrometer with dual
377 Agilent Jet Stream electrospray ionization source (Agilent Technologies, CA, USA). The mass spectra
378 were recorded in positive Auto MS/MS mode and the parameters were set as follows: temperature of
379 drying and sheath gas, 300 °C and 350 °C; skimmer, 75 V; capillary voltage, 4000 V; fragmentor, 110
380 V; nozzle voltage, 1000 V; collision energy, 50 eV; pressure of nebulizer, 35 psi; and flow rate of the
381 drying and sheath gas, 5 and 11 L/min, respectively. The Q-TOF mass spectra were recorded in
382 high-resolution mode. The range of mass-to-charge ratio (*m/z*) scanning was set between 100 and
383 1200. Samples (5 μL) were loaded onto an Agilent Poroshell 120 EC-C18 column (100×2.1 mm, 2.7
384 μm) at 35 °C. The mobile phase consisted of water containing 0.1 % formic acid (solvent A) and
385 acetonitrile containing 0.1% formic acid (solvent B) at a flow rate of 0.35 mL/min. Gradient
386 separation was achieved by changing the proportion of the solvent B mobile phase as follows: 0- 2
387 min, 10% B; 2.1- 5 min, 18%- 20% B; 30- 45 min, 70%- 90% B; and 45- 50 min, 10% B. Mass hunter
388 Workstation software (Agilent Technologies, Palo Alto, CA, USA) was utilized for the system
389 operation and data analysis.

390 ***In vivo* animal experiments**

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

398 Cycleanine (**1**) was suspended in 0.4% carboxymethyl cellulose sodium (CMC-Na) and was
399 formulated at 12 mg/mL for intragastric administration to Wistar rats (male, 220 ± 10 g, fasted for 12
400 hours prior to administration) at a dose of 120 mg/kg body weight. Three rats were used for blood
401 collection through orbital vein using cannulation at 0, 0.5, 1, 2, 4, 6, 8, 12 and 24h post dose after
402 anaesthetization with isoflurane. The plasma samples were separated from blood by centrifugation
403 at 12000 rpm and 4 °C for 10 min. Another three rats were placed in the metabolism cages, and urine
404 samples were collected into tubes from 0 to 24 h after oral administration of cycleanine. All samples
405 were stored in a -80 °C freezer before analysis. Total of 1.2 mL of plasma or urine sample was mixed
406 with 3 times the volume of acetonitrile to precipitate proteins. After centrifugation at 14,000 rpm for
407 10 min, the supernatant was collected and evaporated under vacuum. The residue was reconstituted
408 in 200 μL methanol, and 5 μL of each sample was injected into HPLC-Q-TOF-MS/MS analysis.

409 **4.5 Statistical Analysis**

410 Data was expressed as mean ± standard error of mean (SEM). Data was subjected to GraphPad
411 Prism software analysis. Results were analyzed using one-way analysis of variance (ANOVA)
412 followed by a post Tukey-Kramer multiple comparison test. The difference between mean of the
413 experimental and control groups was considered significant at $p < 0.05$ (ANOVA).

414 **5. Conclusions**

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

423 **Supplemental Materials:** Figure S1: Dose-response antiplasmodial curves of BBIQ alkaloids (**1-5**); Figure S2:
424 HPLC ESI-MS/MS spectrum of cycleanine; Figure S3: Extracted ion chromatograms of cycleanine (M0), its 5

425 metabolites (M2, M6, M7, M10 and M12) in plasma, 11 metabolites (M1-6, M7-9, M11-12) in urine of rat. M0 and
426 M1, M6, and M12 were present in both urine and plasma. Figure S4: MS/MS spectra of cycleanine metabolites
427 (M1-12). Figure S5: Possible metabolic pathway of cycleanine in rats. Table S1: Curative activity and mean
428 survival time (MST) of mice treated with cycleanine (1) during established *P. berghei* infection.

429 **Funding:** This research was funded by Nigerian ETF and NDDC, and the APC was funded by Keele University.
430 **Conflicts of Interest:** The authors declare no conflict of interest.

431 **References**

432 1. WHO. *World malaria report 2019*; Geneva: World Health Organization: 2019.
433 2. Bhatt, S.; Weiss, D.J.; Cameron, E.; Bisanzio, D.; Mappin, B.; Dalrymple, U.; Battle, K.; Moyes, C.L.;
434 Henry, A.; Eckhoff, P.A., et al. The effect of malaria control on Plasmodium falciparum in Africa
435 between 2000 and 2015. *Nature* **2015**, *526*, 207-211, doi:10.1038/nature15535.
436 3. Spring, M.D.; Lin, J.T.; Manning, J.E.; Vanachayangkul, P.; Somethy, S.; Bun, R.; Se, Y.; Chann, S.;
437 Ittiverakul, M.; Sia-ngam, P., et al. Dihydroartemisinin-piperaquine failure associated with a triple
438 mutant including kelch13 C580Y in Cambodia: an observational cohort study. *Lancet Infect Dis* **2015**,
439 *15*, 683-691, doi:10.1016/S1473-3099(15)70049-6.
440 4. Cragg, G.M.; Newman, D.J. Natural products: a continuing source of novel drug leads. *Biochim*
441 *Biophys Acta* **2013**, *1830*, 3670-3695, doi:10.1016/j.bbagen.2013.02.008.
442 5. Fernandez-Alvaro, E.; Hong, W.D.; Nixon, G.L.; O'Neill, P.M.; Calderon, F. Antimalarial
443 Chemotherapy: Natural Product Inspired Development of Preclinical and Clinical Candidates with
444 Diverse Mechanisms of Action. *J Med Chem* **2016**, *59*, 5587-5603, doi:10.1021/acs.jmedchem.5b01485.
445 6. Bridgford, J.L.; Xie, S.C.; Cobbold, S.A.; Pasaje, C.F.A.; Herrmann, S.; Yang, T.; Gillett, D.L.; Dick, L.R.;
446 Ralph, S.A.; Dogovski, C., et al. Artemisinin kills malaria parasites by damaging proteins and
447 inhibiting the proteasome. *Nat Commun* **2018**, *9*, 3801, doi:10.1038/s41467-018-06221-1.
448 7. Tu, Y. Artemisinin-A Gift from Traditional Chinese Medicine to the World (Nobel Lecture). *Angew*
449 *Chem Int Ed Engl* **2016**, *55*, 10210-10226, doi:10.1002/anie.201601967.
450 8. Lu, F.; Culleton, R.; Zhang, M.; Ramaprasad, A.; von Seidlein, L.; Zhou, H.; Zhu, G.; Tang, J.; Liu, Y.;
451 Wang, W., et al. Emergence of Indigenous Artemisinin-Resistant Plasmodium falciparum in Africa. *N*
452 *Engl J Med* **2017**, *376*, 991-993, doi:10.1056/NEJMc1612765.
453 9. Schiff Jr, P.L. Bisbenzylisoquinoline Alkaloids. *J Nat Prod.* **1997**, *60*, 934-953.
454 10. Weber, C.; Opatz, T. Bisbenzylisoquinoline Alkaloids. *Alkaloids Chem Biol* **2019**, *81*, 1-114,
455 doi:10.1016/bs.alkal.2018.07.001.
456 11. Sofowora, L.A. *medicinal plants and traditional medicine in Africa*; John Wiley and Sons Limited:
457 Chichester, 1982.
458 12. Asuzu, I.U.; Anaga, A.O. The antiulcer effect of the methanolic extract of Triclisia subcordata Leaves
459 in rats. *J Herbs Spices & Med. Plants* **1996**, 45-53.
460 13. Uche, F.I.; Drijfhout, F.P.; McCullagh, J.; Richardson, A.; Li, W.W. Cytotoxicity effects and apoptosis
461 induction by bisbenzylisoquinoline alkaloids from Triclisia subcordata. *Phytother Res* **2016**, *30*,
462 1533-1539, doi:10.1002/ptr.5660.
463 14. Uche, F.I.; Abed, M.N.; Abdullah, M.I.; Drijfhout, F.P.; McCullagh, J.; Claridge, T.W.D.; Richardson,
464 A.; Li, W.W. Isochondodendrine and 2′-norcocsuline: additional alkaloids from Triclisia subcordata,
465 induced cytotoxicity and apoptosis in ovarian cancer cell lines. *RSC Adv.*, **2017**, *7*, 44154-44161.
466 15. Uche, F.I.; McCullagh, J.; Claridge, T.W.D.; Richardson, A.; Li, W.W. Synthesis of
467 (aminoalkyl)cycleanine analogues: cytotoxicity, cellular uptake, and apoptosis induction in ovarian
468 cancer cells. *Bioorg Med Chem Lett* **2018**, *28*, 1652-1656, doi:10.1016/j.bmcl.2018.03.038.

469 16. Angerhofer, C.K.; Guinaudeau, H.; Wongpanich, V.; Pezzuto, J.M.; Cordell, G.A. Antiplasmodial and
470 cytotoxic activity of natural bisbenzylisoquinoline alkaloids. *J Nat Prod* **1999**, *62*, 59-66,
471 doi:10.1021/np980144f.
472 17. Fadaeinasab, M.; Taha, H.; Fauzi, P.N.; Ali, H.M.; Widyawaruyanti, A. Anti-malarial activity of
473 isoquinoline alkaloids from the stem bark of Actinodaphne macrophylla. *Nat Prod Commun* **2015**, *10*,
474 1541-1542.
475 18. Otshudi, A.L.; Apers, S.; Pieters, L.; Claeys, M.; Pannecouque, C.; De Clercq, E.; Van Zeebroeck, A.;
476 Lauwers, S.; Frederich, M.; Foriers, A. Biologically active bisbenzylisoquinoline alkaloids from the
477 root bark of Epinetrum villosum. *J Ethnopharmacol* **2005**, *102*, 89-94, doi:10.1016/j.jep.2005.05.021.
478 19. Mambu, L.; Martin, M.T.; Razafimahefa, D.; Ramanitrahasimbola, D.; Rasoanaivo, P.; Frappier, F.
479 Spectral characterisation and antiplasmodial activity of bisbenzylisoquinolines from Isolona
480 ghesquiereina. *Planta Med* **2000**, *66*, 537-540, doi:10.1055/s-2000-8610.
481 20. Guinaudeau, H.; Bohlke, M.; Lin, L.Z.; Angerhofer, C.K.; Cordell, G.A.; RuangrungsiN.
482 (+)-Angchibangkine, a new type of bisbenzylisoquinoline alkaloid, and other dimers from Pachygone
483 dasycarpa. *J Nat Prod* **1997**, *60*, 258-260, doi:10.1021/np960568e.
484 21. Kingsbury, W.D.; Boehm, J.C.; Jakas, D.R.; Holden, K.G.; Hecht, S.M.; Gallagher, G.; Caranfa, M.J.;
485 McCabe, F.L.; Faucette, L.F.; Johnson, R.K., et al. Synthesis of water-soluble (aminoalkyl)camptothecin
486 analogues: inhibition of topoisomerase I and antitumor activity. *J Med Chem* **1991**, *34*, 98-107,
487 doi:10.1021/jm00105a017.
488 22. Johnson-Ajinwo, O.R.; Ullah, I.; Mbye, H.; Richardson, A.; Horrocks, P.; Li, W.W. The synthesis and
489 evaluation of thymoquinone analogues as anti-ovarian cancer and antimalarial agents. *Bioorg Med*
490 *Chem Lett* **2018**, *28*, 1219-1222, doi:10.1016/j.bmcl.2018.02.051.
491 23. Drewes, G.; Knapp, S. Chemoproteomics and Chemical Probes for Target Discovery. *Trends Biotechnol*
492 **2018**, *36*, 1275-1286, doi:10.1016/j.tibtech.2018.06.008.
493 24. Wambebe, C.; Gamaniel, K.; Akah, P.; Kapu, S.; Samson, A.; Orisadipe, A.; Okogun, J. Central and
494 uterotonic effects of cycleanine. *Indian J Pharmcol* **1997**, *29*, 366-372.
495 25. Fidock, D.A.; Rosenthal, P.J.; Croft, S.L.; Brun, R.; Nwaka, S. Antimalarial drug discovery: efficacy
496 models for compound screening. *Nat Rev Drug Discov* **2004**, *3*, 509-520, doi:10.1038/nrd1416.
497 26. Kikueta, C.M.; Kambu, O.K.; Mbenza, A.P.; Mavinga, S.T.; Mbamu, B.M.; Cos, P.; Maes, L.; Apers, S.;
498 Pieters, L.; Cimanga, R.K. In vitro and in vivo antimalarial activity and cytotoxicity of extracts and
499 fractions from the leaves, root-bark and stem-bark of Triclisia gilletii. *J Ethnopharmacol* **2013**, *149*,
500 438-442, doi:10.1016/j.jep.2013.06.043.
501 27. Yisimayili, Z.; Guo, X.; Liu, H.; Xu, Z.; Abdulla, R.; Akber Aisa, H.; Huang, C. Metabolic profiling
502 analysis of corilagin in vivo and in vitro using high-performance liquid chromatography quadrupole
503 time-of-flight mass spectrometry. *J Pharm Biomed Anal* **2019**, *165*, 251-260,
504 doi:10.1016/j.jpba.2018.12.013.
505 28. Liu, H.; Zhou, H.; Cai, T.; Yang, A.; Zang, M.; Xing, J. Metabolism of piperaquine to its antiplasmodial
506 metabolites and their pharmacokinetic profiles in healthy volunteers. *Antimicrob Agents Chemother*
507 **2018**, *62*, doi:10.1128/AAC.00260-18.
508 29. Zhou, H.; Li, L.; Jiang, H.; Zeng, S. Identification of three new N-demethylated and O-demethylated
509 bisbenzylisoquinoline alkaloid metabolites of isoliensinine from dog hepatic microsomes. *Molecules*
510 **2012**, *17*, 11712-11720, doi:10.3390/molecules171011712.

511 30. Huang, Y.; Bai, Y.; Zhao, L.; Hu, T.; Hu, B.; Wang, J.; Xiang, J. Pharmacokinetics and metabolism of
512 neferine in rats after a single oral administration. *Biopharm Drug Dispos* **2007**, *28*, 361-372,
513 doi:10.1002/bdd.556.
514 31. Tian, Y.; Shen, S.; Jiang, Y.; Shen, Q.; Zeng, S.; Zheng, J. CYP3A5 mediates bioactivation and
515 cytotoxicity of tetrandrine. *Arch Toxicol* **2016**, *90*, 1737-1748, doi:10.1007/s00204-015-1584-8.
516 32. Wu, W.N.; McKown, L.A.; Gopaul, V.S. In-vitro metabolism of isotetrandrine, a bisbenzylisoquinoline
517 alkaloid, in rat hepatic S9 fraction by high-performance liquid chromatography-atmospheric pressure
518 ionization mass spectrometry. *J Pharm Pharmacol* **2004**, *56*, 749-755, doi:10.1211/0022357023547.
519 33. Cernak, T.; Dykstra, K.D.; Tyagarajan, S.; Vachal, P.; Krska, S.W. The medicinal chemist's toolbox for
520 late stage functionalization of drug-like molecules. *Chem Soc Rev* **2016**, *45*, 546-576,
521 doi:10.1039/c5cs00628g.
522 34. Ren, X.; Yorke, J.A.; Taylor, E.; Zhang, T.; Zhou, W.; Wong, L.L. Drug oxidation by cytochrome
523 P450BM3 : metabolite synthesis and discovering new P450 reaction types. *Chemistry* **2015**, *21*,
524 15039-15047, doi:10.1002/chem.201502020.
525 35. Cusack, K.P.; Koolman, H.F.; Lange, U.E.; Peltier, H.M.; Piel, I.; Vasudevan, A. Emerging technologies
526 for metabolite generation and structural diversification. *Bioorg Med Chem Lett* **2013**, *23*, 5471-5483,
527 doi:10.1016/j.bmcl.2013.08.003.
528 36. Thompson, T.N. Optimization of metabolic stability as a goal of modern drug design. *Med Res Rev*
529 **2001**, *21*, 412-449, doi:10.1002/med.1017.
530 37. Smilkstein, M.; Sriwilaijaroen, N.; Kelly, J.X.; Wilairat, P.; Riscoe, M. Simple and inexpensive
531 fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob Agents*
532 *Chemother* **2004**, *48*, 1803-1806.
533 38. Aldulaimi, O.; Uche, F.I.; Hameed, H.; Mbye, H.; Ullah, I.; Drijfhout, F.; Claridge, T.D.W.; Horrocks,
534 P.; Li, W.W. A characterization of the antimalarial activity of the bark of Cylicodiscus gabunensis
535 Harms. *J Ethnopharmacol* **2017**, *198*, 221-225, doi:10.1016/j.jep.2017.01.014.
536 39. Odetola, A.; Basir, O. Evaluation of Antimalarial Properties of Some Nigerian Medicinal Plants. In
537 *Proceedings of African Bioscience Network, Federal Ministry of Science and Technology, Nigerian Society of*
538 *Phrmacology and Drug Research and Production unit, University of Ife organized Workshop.*, Sofowora, A.,
539 Ed. Ife, Nigeria, 1980; pp. 275 – 283.
540 40. Lorke, D. A new approach to practical acute toxicity testing. *Arch Toxicol* **1983**, *54*, 275-287.
541 41. Knight, D.J.; Peters, W. The antimalarial activity of N-benzyloxydihydrotriazines. I. The activity of
542 clociguanil (BRL 50216) against rodent malaria, and studies on its mode of action. *Ann Trop Med*
543 *Parasitol* **1980**, *74*, 393-404.
544 42. Okokon, J.E.; Antia, B.S.; Mohanakrishnan, D.; Sahal, D. Antimalarial and antiplasmodial activity of
545 husk extract and fractions of Zea mays. *Pharm Biol* **2017**, *55*, 1394-1400,
546 doi:10.1080/13880209.2017.1302966.
547 43. Peters, W. Drug resistance in Plasmodium berghei Vincke and Lips, 1948. I. Chloroquine resistance.
548 *Exp Parasitol* **1965**, *17*, 80-89, doi:10.1016/0014-4894(65)90012-3.
549 44. Ryley, J.F.; Peters, W. The antimalarial activity of some quinolone esters. *Ann Trop Med Parasitol* **1970**,
550 *84*, 209-222.

551