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By Gloria Serra

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Synthesis, Profiling and In Vivo Evaluation of Cyclopeptides Containing N-Methyl Amino Acids as Antiplasmodial Agents

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KEYWORDS antiplasmodial, cyclopeptides, synthesis, physicochemical properties

ABSTRACT: Malaria remains a major infectious disease where important needs are to mitigate symptoms and to prevent the establishment of infection. Cyclopeptides containing N-methyl amino acids with *in vitro* activity against erythrocytic forms as well as liver stage are presented. The synthesis, parasitological characterization, physicochemical properties, *in vivo* evaluation and mice pharmacokinetics are described.

Malaria is a life-threatening illness caused by *Plasmodium spp.* parasites that are transmitted to human through the bite of an infected female *Anopheles* mosquito. *P. falciparum* is the most deadly of the six species that cause malaria in humans. According to the World Health Organization (WHO) 2017 report, 216 million cases of malaria occurred worldwide in 2016, principally in the sub-Saharan region.[1] Though the burden of malaria has been reduced since 2000, the number of estimated deaths was 445,000 in 2016, with the majority children under 5 years of age.

The emergence of malaria parasite resistance to the available drugs including artemisinin and its derivatives,[2] and the *Anopheles* vector resistance to the currently used insecticides, threaten malaria control efforts. Therefore, novel, safe and effective drugs to treat this disease are urgently needed. Recent efforts in antimalarial drug discovery have focused on new targets, nonclassical chemical scaffolds, and vaccines.[3] A recent review by Burrows *et al* lists changes in anti-malarial target candidate (TCP) and target product profiles (TPP) over the last years.[4] The investigations for the development of new drugs have been directed to compounds that not only treat disease symptoms but also contribute toward the elimination and eradication of malaria. To achieve eradication, new drugs should inhibit multiple parasite stages. However, the majority of the antimalarials only treat the symptom-causing erythrocytic stages of the parasite.[5] Few classes of these drugs, including cytochrome bc1 inhibitors (such as atovaquone) and antifolate drugs (such as pyrimethamine), are active against liver stage as well as erythrocytic forms. Therefore, these compounds are able to prevent the establishment of infection (prophylactic activity) and to mitigate disease symptoms.
During the last years, an important number of bioactive macrocycles has been reported.[6] These compounds possess desirable properties that make them promising candidates for the discovery of novel drug molecules. In general, they present structural features to favor bioactive conformations, selectivity to the receptors and metabolic stability.[7] In addition, it was demonstrated that multiple backbone N-methylation of cyclic peptides remarkably improves their cell permeability and therefore can be utilized in the design of orally available drugs.[8]

In the last few years, our group has reported the synthesis of cyclopeptides and their evaluation as antiplasmodial candidates.[9] Based on the potential for macrocycles as drugs and on our previous work, we decided to prepare analogs of cyclopeptides, containing N-methyl amino acids (NMe-AA) to evaluate how N-methylation affects the bioactivity of these antimalarial compounds. In this work we disclose, the synthesis of nine cyclopeptides, their physicochemical properties and their intraerythrocytic antiplasmodial activity and selectivity. In addition, two compounds were evaluated against the liver stage of the parasite and two cyclopeptides were selected to assess the *in vivo* activity in mice infected with *P. berghei*. Moreover, animal pharmacokinetic for one of these compounds was determined.

Recently, our research team reported the synthesis and antiplasmodial activity of compound **1** (EC_{50}= 28 nM, Figure 1).[9b] In this study, we concluded that the presence of three Gly, one Thr and two Cys in the cyclohexapeptide, had a great impact in the biological activities. Based on this structure, we prepared three analogs, **2**, **3** and **4** containing one, two or three N-MeGly, respectively, to investigate the impact of these changes on the inhibitory activity of this series. In addition, Thr(O^{t}Bu) in **4** was replaced by Ser(O^{t}Bu) to obtain **5**. Cyclohexapeptide **6** and cyclopentapeptide **7**, were obtained by substitution, by Phe, or elimination of one N-MeGly in **5**, respectively. Compounds **8**- **10** are close analogs of **2**-**4**, re-

spectively, which were obtained by replacements of Thr(O^{t}Bu) with Glu.

Compounds **2-7**, were synthesized using SPPS and solution macrocyclization (Scheme 1). The 2-chlorotrityl resin (2-CTC) was employed to minimize the formation of diketopiperazines,[10] and HBTU and DIPEA were used as coupling reagent in most cases. HCTU and DIPEA resulted in a more effective reagent for the coupling of the next amino acid to NMe-Gly. A combination of DIC and Cl-HOBt was used when the next amino acid was Fmoc-L-Cys(Trt)-OH, to decrease the possibility of racemization, a common problem when amide bonds are formed from a Cys carboxyl.

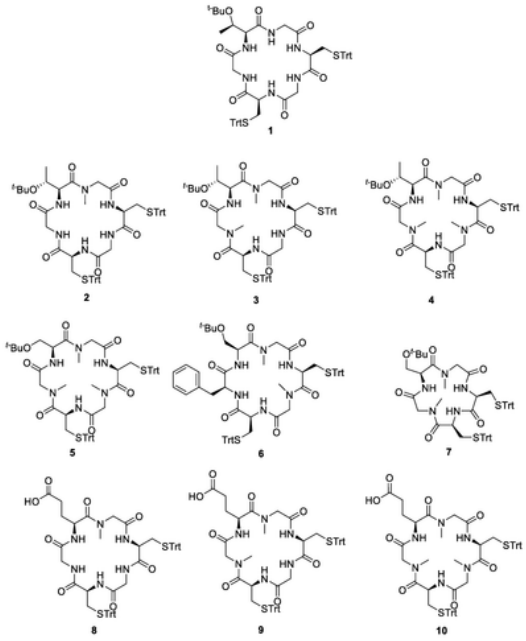
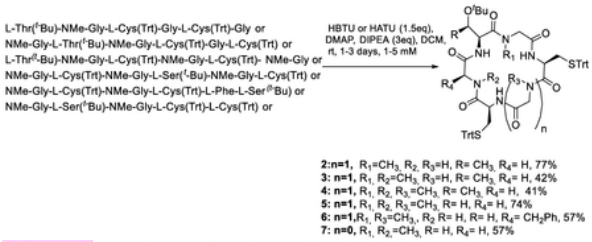


Figure 1. Cyclopeptides analogs synthesized in this work (**2-10**) and lead compound **1**.[9b]

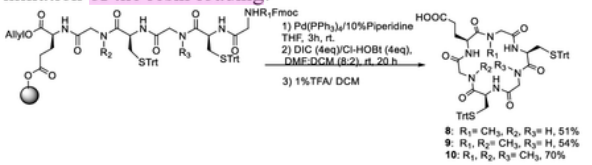
To minimize racemization, and thereby the formation of diastereomers during ring closure,[11] we started peptide sequence precursor of **2**, with Glycine at the *C-terminus*, which also minimizes steric hindrance during the macrocyclization process. The first attempts to prepare peptide precursors of **3**, **6** and **7** using NMe-Gly as the first or second amino acid attached to the resin, resulted in low yields probably due to the formation of diketopiperazine favored by the turn-inducing NMe amino acid. For this reason, to obtain these precursors in high yield, we selected sequences where NMe-Gly was not the first or second amino acid. However, the peptide precursor of **4** and **5** were obtained in very good yields, using NMe-Gly as first or second amino acid attached, respectively. In addition, the macrocyclizations proceeded in good yields and no racemization was observed by the HPLC analyses and the NMR spectra.



Scheme 1. Synthesis of cyclopeptides by solution macrocyclization

Concerning the macrocyclization reaction, the longest reaction time is for the synthesis of **2,** which contains only one turn-inducing NMe-Gly. Cyclopeptides **2** and **5** were obtained in very good yields (77 and 74%) and the rest of the cyclopeptides (**3**, **4**, **6** and **7**) in good yields (41-57 %).

Compounds **8**, **9** and **10** were synthesized using on-resin macrocyclization, Scheme 2.[9b] Fmoc-L-Glu-OAll was loaded onto 2-CTC resin with an excess of DIPEA and Fmoc SPPS protocol was followed. Then, the removal of the allyl ester and Fmoc was performed using [Pd(PPh$_3$)$_4$] in a solution of 10% piperidine in THF and macrocyclization was realized using DIC and Cl-HOBt. No racemization was detected by analysis of the HPLC chromatograms and the NMR spectra of the crude cyclopeptide. Consequently, the desired compounds were obtained in 51 to 70% overall yields, based on the determination of the resin loading.



Scheme 2. Synthesis of cyclopeptides by on resin cyclization

The obtained compounds were evaluated in vitro against chloroquine resistant *P. falciparum* K1 (24 h incubation, ^{3}H-hypoxanthine incorporation readout),[12] and drug sensitive *P. falciparum* 3D7 (SYBR Green assay),[13] Table 1. The results for compounds **2** and **3**, which present the same sequences that compound **1,** allowed us to conclude that the activity against both parasite strains is improved with the growing number of N-methyl Gly. However, cyclopeptide **4**, which presents three N-methyl Gly is less active than **3**. The substitution of Thr(^{t}Bu) in **4** by Ser(^{t}Bu) in **5**, diminishes the activity against both strains. Moreover, the EC_{50} is higher for cyclopentapeptide **7** where one N-Methyl Gly in **5** was eliminated. Cyclohexapeptide **6** which presents a Phe instead of N-Me Gly in **5**, is less active against the two strains. Compounds **8** and **9**, analogs to **2** and **3** (Glu by Thr(^{t}Bu)) are inactive against *P. falciparum* 3D7. However, cyclopeptide **10**, analog to **4,** showed subnanomolar EC_{50} against K1 and 12 nM against 3D7 strain. It seems that when a Glu, with the free carboxyl group, is present, three NMe-Gly would be required to maintain the activity. Moreover, all active compounds are not toxic against murine macrophage or HepG2 cells, showing excellent selectivity (SI).

Cyclop.	EC_{50} / EC_{90} *P. falciparum* K1(nM)[a]	EC_{50} *P. falciparum* 3D7(nM)[b]	SI IC_{50}[c]/ EC_{50} *Pf*K1	SI IC_{50}[d]/ EC_{50} *Pf*3D7
2	8.0 /39	3.8 ± 0.1	>12500	$>6.6x10^5$
3	0.008 /1.0	0.25 ± 0.02	$>1.3x10^7$	$>1.0x10^6$
4	0.040 / 1.0	1.0 ± 0.3	$>2.5x10^6$	$>2.5x10^6$
5	0.13 / 4.0	1.4 ± 0.6	$>7.7x10^5$	$>1.8x10^5$
6	9.0 / 59	1.8 ± 0.1	$>1.1x10^4$	$>1.4x10^5$
7	150 / 900	nd	nd	nd
8	nd	5400 ± 100	nd	>19
9	nd	210 ± 10	nd	>477
10	0.20 / 4.0	12 ± 1	nd	>20833

[a] Control: chloroquine: EC_{50} = 0.47 µM, Artemisinin: EC_{50}= 20 nM, Artesunate: EC_{50}= 3 nM; [b] Control: Pyrimethamine: EC_{50}= 35 nM, Artesunate: EC_{50}= 5 nM; [c] Cytotoxicity against murine macrophages J774; [d] Cytotoxicity against HepG2 cells; nt: not tested

Table 1. Parasite grow inhibition expressed as EC_{50} in nM and selectivity index.

Then, compounds **4** and **6** were assessed in an *in vitro* assay where the capability of drug treated parasite to undergo new invasions is used as surrogate of parasite viability.[14] A double-colorimetric FACTS analysis is used to quantify new invasions of pre-stained hRBCs by drug treated parasites. This assay uses 2 time points drug treatment to distinguish rapidly parasiticidal compounds from moderate or slow acting antimalarials. Figure 2 shows the *in vitro* results for **4**, **6** and four standard antimalarial drugs (chloroquine, atovaquone, pyrimethamine and artesunate) which were included to validate the results produced and allow a comparative classification of the killing behavior of the compounds tested. Both compounds displayed estimated effects over parasite viability comparable to slow agents: atovaquone and pyrimethamine which finally impair pyrimidine biosynthesis. These results point to an antimalarial mode of action that not produces a rapid effect on parasite viability.

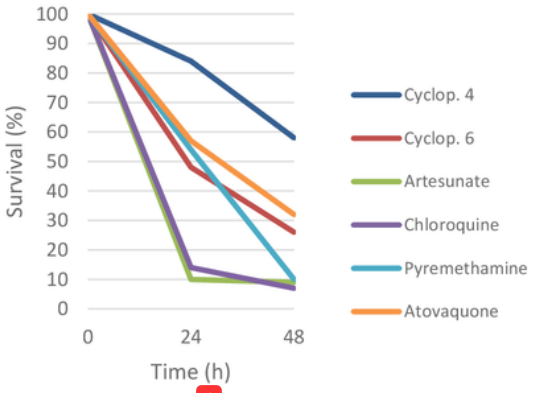


Figure 2. Killing profile against *P. falciparum* 3D7A strain for compound **4**, **6**, artesunate, chloroquine, atovaquone and pyrimethamine tested at 10 times the EC_{50}.

To assess the prophylactic potential of this series, we used the luciferase-based phenotypic screen of malaria exoerythrocytic-stage. This assay uses the exoerythrocytic-stage of the rodent malaria parasite, *Plasmodium berghei,* and a human hepatoma cell line.[15] Cyclopeptides **4** and **6** were actives in the liver stage of the parasite showing low and submicromolar EC_{50} values (0.018 and 0.335 µM, respectively).

Next, determination of physicochemical properties, Log D, solubility and permeability was performed, Table 2. Taking into account the three properties, compounds **2**, **3** and **10** could be selected as the more promising to intend the *in vivo* evaluation. However, as **3** and **10** had lower EC_{50} values against the two *P. falciparum* strains than **2** (Table 1) they were chosen as representative compounds to assess the *in vivo* activity of this series.

A suppressive growth test was used with *P. berghei* NK65 strain infected mice as described previously.[16] Briefly, Swiss outbred mice were inoculated with infected red blood cells. On day 2, 3 and 4, administration of a single oral dose of each compound (50 mg · kg-1) was carried out. Two control groups were used, one treated with chloroquine and the other with vehicle. Blood smears from the mouse tails were prepared on days 5, 7 and 9 post infection, then, the parasitemia was evaluated and the percent inhibition of parasite growth calculated (Figure 3).

Cyclop.	Log D, pH 7.0[a]	Solubility PBS, pH 7.4/ pH 1.0 (µM)	PAMPA ($Pe x10^{-6}$ cm/s)
2	5.8	0.9/ 1.2	0.6
3	6.0	0.4 / 3.0	1.0
4	3.7	**nd**	**nd**
5	6.3	0.2/ 0.7	0.3
6	8.0	0 /0	0.2
7	7.7	0/ 0	2.0
8	3.1	nd	..[b]
9	3.7	nd	..[b]
10	3.9	436/ 28	0.2

[a] measured (see Supporting Information); [b] the compound was not detected in the acceptor compartment.

Table 2. Physicochemical properties determined for the cyclopeptides

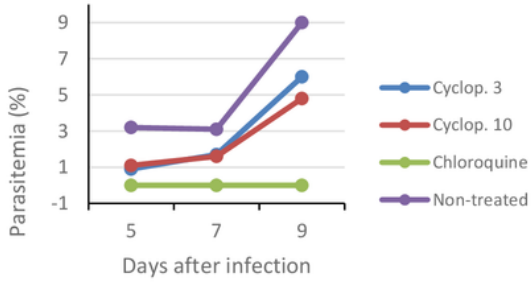


Figure 3. *In vivo* evaluation of cyclopeptides **3** and **10**

Cyclopeptides **3** and **10** were actives against *P. berghei* parasites reducing 71 and 66% the parasitemia on day 5, respectively. Even though, it was not possible to obtain a 100% reduction during this study, the parasitemia was reduced until day 9. In order to evaluate the oral bioavailability, plasma pharmacokinetic of compound **3** in male Swiss Albino mice, following a single oral administration was investigated.[17] Animals were administered with solution formulation of **3** *via* oral route at 50 mg/kg. The blood samples were collected at 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 hr. Plasma concentrations of **3** were quantifiable up to 12 hr (lower limit of quantitation 5.16 ng/mL). The determinate pharmacokinetic parameters are showed in Table 3.

T_{max} (hr)	C_{max} (ng/mL)	AUC_{last} (hr*ng/mL)	AUC_{inf} (hr*ng/mL)	$T_{1/2}$ (hr)
2.00	61.1	314.8	390.7	4.93

Table 3. Pharmacokinetic parameters of cyclopeptide **3**

The results demonstrate that compound **3** reaches maximum concentration at 2 h, with $T_{1/2}$ =4.93 h. It is worth noting that the mean plasma concentrations between 0.5 to 12 h, were higher than 9 ng/mL (see Supporting Information).

In conclusion, a novel class of antimalarial cyclopeptides containing N-methyl Gly with enhanced antiplasmodial activity has been reported. The compounds were rapidly accessed using SPPS and solution or on-resin macrocyclization. The parasitological profiling demonstrated improved activity of cyclopeptides containing N-methyl Gly, compared with **1**, with EC_{50} in a low nanomolar or subnanomolar range against *P. falciparum* K1 and 3D7. Cyclopeptides **4** and **6** showed parasite viability comparable to atovaquone and pyrimethamine which i[illegible]r pyrimidine biosynthesis. In addition, these compounds are active against the liver stage of the parasite showing submicromolar EC_{50}. Cyclopeptides **3** and **10** have confirmed *in vivo* efficacy and **3** presents a considerable half-life of 4.95 h. Investigations toward [illegible]cidating the mechanism of action underlying this series are in progress and will be reported in the due course.

ASSOCIATED CONTENT

All animal experiments were performed according to national animal welfare regulations after authorization by the local authorities (Universidade Federal de São Paulo, CEUA N [illegible]0080816).

Supporting Information

Synt[illegible]sis and analytical data of the obtained cyclopeptides, details for *P. falciparum in vitro* [illegible] *in vivo* assays, physicochemical and mouse pharmacokinetic studies.

This material is available free of charge via the Internet at http://pubs.acs.org

Supporting Information (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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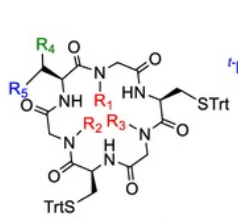
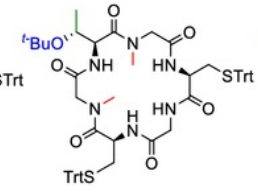
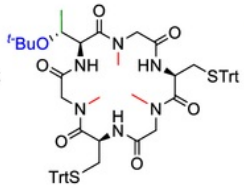


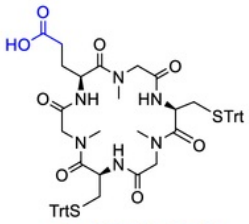
R_1, R_2, R_3= CH_3 or H
R_4= H or CH_3,
R_5= O^{t}Bu or CH_2COOH,



EC_{50}: *P.falc* K1= 0.008 nM
EC_{50}: *P.falc* = 0.25 nM
SI>1.0x10^6
In vivo efficacy
$t_{1/2}$= 4.95 h



EC_{50}: *P.falc* K1= 0.04 nM
EC_{50}: *P.falc* = 1.0 nM
SI>1.0x10^6
EC_{50}: *P. b.* (liver st.)= 18 nM



EC_{50}: *P.falc* K1= 0.2 nM
EC_{50}: *P.falc* = 12 nM
SI>2.0x10^4
In vivo efficacy

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