Tropical Journal of Pharmaceutical Research January 2024; 23 (1): 85-90
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v23i1.11

Original Research Article

Potential role of CI-679 against artemisinin-resistant *Plasmodium falciparum*

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Sent for review: 1 March 2023 *Revised accepted: 4 January 2024*

Abstract

Purpose: *To investigate the role of nitroquine (CI-679) against artemisinin-resistant Plasmodium falciparum (P. falciparum) C580Y strain.*
Methods: *Antimalarial activity of CI-679 against blood stages in Plasmodium yoelii (P. yoelii) - infected BALB/c mice model was first identified. Thereafter, in vitro assays were performed to investigate the inhibitory activity against blood stages of artemisinin-sensitive P. falciparum 3D7 strain. Finally, the potential effect of CI-679 was also investigated on artemisinin-resistant P. falciparum, which was constructed by introducing C580Y mutation in K13 of the 3D7 using the CRISPR-CAS9 technology.*
Results: *CI-679 significantly suppressed the growth of rodent malaria parasite, P. yoelii BY265, in a dose-dependent manner, and also inhibited the development of the parasites in mice ($p < 0.05$). Furthermore, CI-679 efficiently inhibited the growth of artemisinin-sensitive P. falciparum 3D7 in vitro, with more sensitivity against late phase of blood stages ($p < 0.05$). Also, CI-679 suppressed the development of artemisinin-resistant P. falciparum C580Y strain, and the inhibitory effect was comparable to that of artemisinin-sensitive 3D7 strain.*
Conclusion: *CI-679 exhibits potent antimalarial activity against blood stages of P. yoelii BY265 in vivo, and both artemisinin-sensitive P. falciparum 3D7 and artemisinin-resistant P. falciparum C580Y in vitro. Further pharmacokinetic properties, tolerability and safety of the compound need to be investigated to support this claim.*

Keywords: *CI-679, Nitroquine, Blood stage, Artemisinin-resistant, Plasmodium falciparum*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Malaria is the most important parasitic disease affecting human beings. It affected about 3 billion people and caused 247 million cases and 619,000 deaths in 2021, the majority being children under 5 years old living in Sub-Saharan Africa [1]. Five species of the genus *Plasmodium* cause all malarial infections in man. *Plasmodium vivax* is the most widespread species, whereas *P. falciparum* is responsible for most deaths [1]. Deployment of first-line artemisinin-based combination therapies (ACTs) has resulted in

real progress in reducing malaria burden [1]. However, the development of resistance to ACTs and its partners raises the worrying possibility of treatment failure. After years of efforts, kelch 13 (*Pf*k13), which resides on chromosome 13 of the *P. falciparum* genome, was identified as the artemisinin resistance molecular marker [2].

Over 20 mutations in *Pf*k13 have been identified after treatment with artemisinin derivatives across 15 locations in Southeast Asia [3], among which, at least 10 kelch13 mutations have been validated as molecular markers for artemisinin resistance according to WHO criteria [4], notably C580Y [2,5]. Emergencies and spread of resistance to artemisinin have posed a major hindrance to the implementation of effective strategies for malaria control and elimination [1,5]. Thus, there is an urgent need for new antimalarial drugs with different mechanisms, especially those that act against currently artemisinin-resistant strains.

In the early 1960s, a collaborative program of synthesis of folic acid analogs in which the pteridine ring was replaced by a quinazoline moiety was launched by the Departments of Chemistry of Parke Davis and Company, Ann Arbor, Michigan and Hounslow, Middlesex, England [6]. The interest of such research soon focused on 2,4-diamino-6-substituted derivatives for their promising properties that inhibit activities of dihydrofolate reductases and thymidylate synthetases of mammals and bacteria [7,8]. Representative 2,4-diamino-6-substituted quinazolines were further tested for antiparasitic activities and CI-679 (2,4-diamino-6-[(3,4-dichlorobenzyl)-nitrosoamino]-quinazoline) attracted special attention [9]. However, the research and development of CI-679 were retarded by the successes achieved with artemisinin. With the development and spread of artemisinin resistance, interest in CI-679 has resurged in times past.

Thus, this study aimed at investigating the *in vitro* antimalarial activity of CI-679 against *P. yoelii* in murine model and artemisinin-sensitive *P. falciparum* 3D7 and artemisinin-resistant *P. falciparum* C580Y strains.

EXPERIMENTAL

Parasite and mice

Female BALB/c mice (6 weeks old) were purchased from Beijing Huafukang Bioscience Company Limited, Beijing, China. *Plasmodium yoelii* strain BY265 was maintained in mice by serial passages in the laboratory at the Department of Pathogenic Biology, Army Medical University, Chongqing, China. Artemisinin-sensitive *P. falciparum* strain 3D7 was maintained and artemisinin-resistant *P. falciparum* C580Y was constructed in the Research Center for Translational Medicine, Key Laboratory of Arrhythmias of the Ministry of Education of China, East Hospital, Tongji University School of Medicine, Shanghai, China. All studies were conducted according to appropriate legislation and institutional policies on the care and use of animals. All animal studies were reviewed and approved by the Animal Ethics Committee of the Army Medical University Institute of Medical Research, Chongqing, China (approval no. AMUWEC20192132).

Study design

Efficacy was evaluated against blood stages of *P. yoelii in vivo,* and both *P. falciparum* 3D7 and *P. falciparum* C580Y *in vitro*. *In vivo* studies were designed to evaluate the antimalarial activity of CI-679 against blood stages in murine model. *In vitro* studies were designed to evaluate the efficacy against blood stages of artemisinin-sensitive *P. falciparum* 3D7 and artemisinin-resistant C580Y.

Parasite culture and establishment of *P. falciparum* C580Y

Plasmodium falciparum 3D7 blood stages were cultured as described previously with 5 % O_2 and CO_2 at 37 ^oC [10]. Synchronous cultures were obtained by sorbitol treatment for the ring stage or purified on a 40/70 % discontinuous gradient for schizont stage.

Artemisinin-resistant *P. falciparum* C580Y strain was obtained through mutation of C580Y in K13 of 3D7 using Crispr-Cas9 technology [11]. The K13_C580Y mutation (shield mutation) and the Sir2B shield mutation were introduced into the Cas9i system using plasmid *pL6cs-K13-Sir2B*. For transfection, fresh human-type O erythrocytes were electroporated in cytomix (120 mM KCl, 10 mM KH_2PO_4, 25 mM HEPES, 2 mM EGTA, 0.15 mM $CaCl_2$, and 5 mM $MgCl_2$, pH 7.6) with 100 µg of each plasmid under standard electroporation parameters and enriched late schizonts were immediately mixed with the electroporated erythrocytes.

Positive selection drugs were applied 72 h post-transfection, and media and drugs were renewed every day for the first 7 days. Final concentrations of drugs were 2.5 µg/mL for BSD (Sigma), 2.5 nM for WR99210 (Sigma), and 1.5

µM for DSM1. The desired transgenic 3D7 strain *K13:Sir2B-mut*^{Cas9i} was obtained in about 20 days. The correct introduction of K13_C580Y mutation and shield mutations was verified as reported [11].

In vivo efficacy of CI-679 in *P. yoelii*-infected BALB/c mice

To investigate the antimalarial activity of CI-679 against blood stages, BALB/c mice were infected with 1 × 10^6 parasitized erythrocytes of *P. yoelii* strain BY265, followed by treatment with intragastric injection (ig) of 2.5, 5.0 and 10.0 mg/kg of CI-679 daily for 3 days. To investigate the therapeutic effect of CI-679, mice were treated with 2.5 mg/kg of CI-679 five (5) days post-infection (dpi) or 3 dpi daily for 3 days.

Determination of parasitemia and mice survival rate

Blood smears were made daily from the tail veins of mice and stained with Giemsa. Parasitemia was counted from at least 5,000 red blood cells (RBCs) using microscopy. Blood smears and mortality data were recorded daily till the control was void of parasitemia at which time all treated mice survived and negative blood film result was considered cured.

In vitro blood-stage parasite inhibition assay

A total of 0 - 3 h ring-stage survival assay ($RSA^{0-3\ h}$) was performed to evaluate the activity of CI-679 against *P. falciparum* sensitive 3D7 and artemisinin-resistant C580Y strains [12]. Parasites were synchronized several times to acquire the accurate 0-3 h post-invasion rings, followed by the $RSA^{0-3\ h}$. Similarly, the trophozoite-stage survival assay ($TSA^{24-27\ h}$) was done with 24 - 27 h post-invasion trophozoites and the schizont-stage survival assay ($SSA^{36-39\ h}$) with 36 - 39 h post-invasion schizonts. In each assay, accurately synchronized parasites were exposed to 0 to 640 ng/mL discontinuous gradient (0, 0.01, 0.04, 0.16, 0.64, 2.5, 10, 40, 160, 640 ng/mL) of CI-679 or 0.1 % dimethyl sulphoxide (DMSO) as solvent for 6 h, washed with incomplete medium, and cultured for another 66, 42 and 30 h, respectively. After drug removal blood films were developed to assess the survival rates of the strains microscopically. Each assay was repeated 3 times independently. Forty thousand erythrocytes were counted in each technical repetition. For EC_{50} determinations, data were fitted into the log(inhibitor) versus response–variable slope (4-parameter) model in GraphPad Prism 9.0.

Statistical analysis

Graphical representation and statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Inc.). Unless otherwise stated, the results were presented as mean ± standard error of the mean (SEM). Two-way ANOVA was used to compare parasitemia between CI-679 treatments and control group mice. The two-tailed Student's *t*-test was used to test differences in *in vitro* proliferation. The log-rank (Matel–Cox) test was used to compare the survival distributions of the two groups. For parasite EC_{50} determination, data were fitted into the specified model using nonlinear least squares analysis. $P < 0.05$ was considered statistically significant. Biological replicates (n) indicated in Figure legends refer to the number of mice or number of independent cultures.

RESULTS

CI-679 suppresses the growth of *P. yoelii* in a dose-dependent manner

A detailed analysis of the intragastric dose-dependent profile was undertaken in the *P. yoelii* BALB/c mouse 3-day dosing model. The results showed that a dose of 2.5 mg/kg conferred full protection (Figure 1 A) and all the mice survived (Figure 1 B) in groups treated with CI-679.

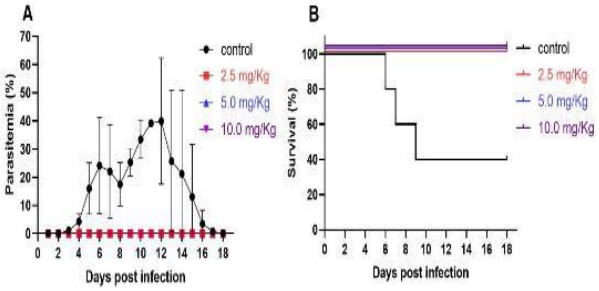

Figure 1: *In vivo* dose-dependent efficacy of CI-679 in the *P. yoelii*-infected BALB/c mice. (**A**) Blood parasitemia versus days after infection for administered doses (mg/kg). (**B**) Survival of parasitized BALB/c mice treated with different doses of CI-679 and control group mice. Results are shown as mean ± SEM (n=5) in triplicates

CI-679 inhibits the development of *P. yoelii*

The role of CI-679 was investigated at the peak of parasitemia (reached between 5 to 7 days post-infection (dpi) in control group), and the results demonstrated that all mice treated with CI-679 were clear of parasitemia soon after administration (7 dpi) (Figure 2 A) and also survived (Figure 2 B). Furthermore, with rising parasitemia (3-5 dpi), all mice treated with CI-679

were clear of parasitemia after administration (6 dpi) (Figure 2 C) and also survived (Figure 2 D).

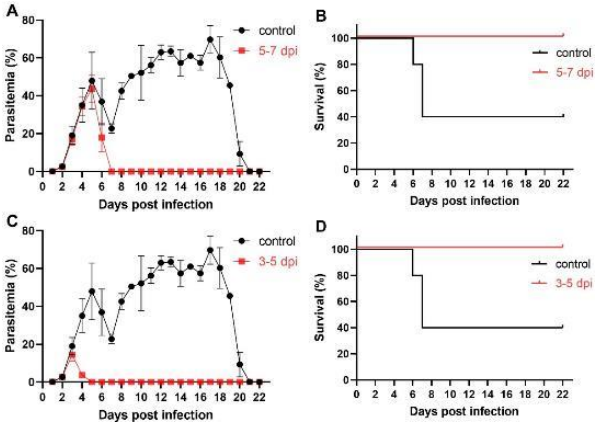

Figure 2: Efficacy of CI-679 on peak and rising stages of parasitemia in *P. yoelii*-infected BALB/c mice. (**A**) Blood parasitemia versus days after infection for administered doses at the peak of parasitemia (5 - 7 dpi). (**B**) Survival of parasitized BALB/c mice treated with CI-679 (5-7 dpi) and the control group mice. (**C**) Blood parasitemia versus days after infection for administered doses at the rising stage of parasitemia (3 - 5 dpi). (**D**) Survival of parasitized BALB/c mice treated with CI-679 (3 - 5 dpi) and the control group mice. Results were shown as means ± SEM; n = 5 in triplicate. $P < 0.0001$ versus control

CI-679 inhibits the development of artemisinin-sensitive *P. falciparum* strain *in vitro*

The $RSA^{0\text{-}3\ h}$, $TSA^{24\text{-}27\ h}$ and $SSA^{36\text{-}39\ h}$ were performed to evaluate the role of CI-679 on the ring, trophozoite and schizont stages (Figure 3 A). Further measurements of parasitemia at 72 dpi indicated that the development of all blood stages has been inhibited, with lower proliferation ratios in $TSA^{24\text{-}27\ h}$ and $SSA^{36\text{-}39\ h}$ (Figure 3 B). The result suggested that the mechanism of CI-679 may be associated with the development of ring stages, trophozoites and schizonts in red blood cells.

CI-679 inhibits the development of artemisinin-resistant *P. falciparum* strain

The K13_C580Y mutation was introduced into the Cas9i system by plasmid *pL6cs-K13-Sir2B* (Figure 4 A) and the results showed that there was no significant difference between the antimalarial activities of CI-679 against both *P. falciparum* 3D7 and C580Y parasites (Figure 4 B). These results suggested that CI-679 exhibited a potent inhibitory effect against artemisinin-resistant malaria parasites.

DISCUSSION

Drug research and development is time- and capital-intensive. An alternative strategy is to evaluate the antimalarial activity of molecules that are already licensed for new medical indications, an approach known as drug re-purposing which has been successfully applied in oncology [13]. Since the drugs are already in routine clinical use for other purposes, early-phase clinical trials could be bypassed, saving time and money. Thus, this strategy offers a promising alternative to traditional drug development pipelines. Patients with *P. falciparum* hyperparasitemia are at increased risk of treatment failure, severe malaria and death [14]. Parasitemia level serves as a predictor for severe malaria [15]. Herein, the results showed that CI-679 suppressed the development of *P. yoelii* BY265 blood stages. Furthermore, CI-679 has a wide therapeutic window, and also rapidly inhibited the development of parasites. Thus, severe malaria and death become less likely.

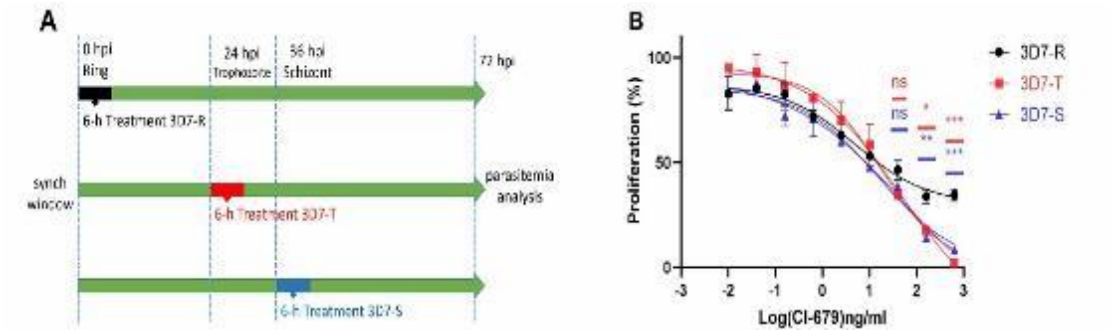

Figure 3: *In vitro P. falciparum* 3D7 growth inhibition. (**A**) Schematic overview of the experiment design. (**B**) Fitted concentrations for 50 % of maximal effects (EC_{50}) were 5.04, 17.98, and 15.74 ng/mL for 3D7-R, 3D7-T, and 3D7-S, respectively. Experiments were done in triplicates per concentration and 27-30 data points per fit; results were presented in mean ± SEM. *$P < 0.05$ 3D7-R versus 3D7-T (160 ng/mL), **$p < 0.01$ 3D7-R versus 3D7-S (160 ng/mL), ***$p < 0.001$ 3D7-R versus 3D7-S (640 ng/mL) and 3D7-R versus 3D7-T (640 ng/mL)

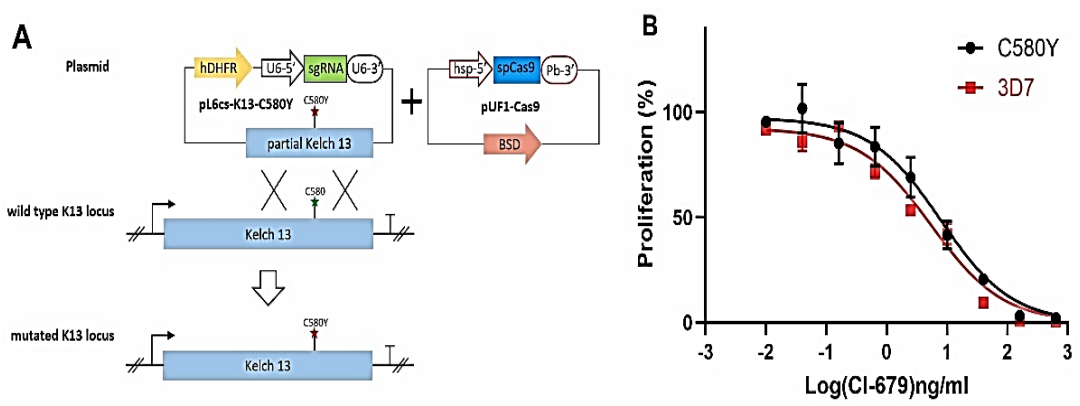

Figure 4. *In vitro P. falciparum* C580Y growth inhibition. (**A**) Sketch of *pL6cs-K13-C580Y* and *pUF1-Cas9*. (**B**) Fitted EC_{50} were 5,471, and 7.886 ng/mL for 3D7 and C580Y, respectively. Experiments were done in triplicates per concentration and 18-20 data points per fit; the plot presented as mean ± SEM, $p > 0.05$

During the blood stage, *Plasmodium* parasites replicate rapidly and exponentially to > 10^{12} parasites per patient. This rapid growth requires sustained pools of nucleotides for the synthesis of DNA and RNA [16].

Folate is essential for the survival and growth of the malaria parasite and DNA synthesis and the folate metabolic pathway constitutes an antimalarial target [17]. Also, CI-679 exhibited potent activity in inhibiting the development of blood stages of artemisinin-sensitive *P. falciparum* 3D7 *in vitro*, with more sensitivity against late phase of the blood stage. This is very likely because CI-679 is a product of the synthesis of folic acid analogs. Development of parasites in the late phase of the blood stage might be impeded by insufficient DNA synthesis due to the administration of CI-679.

The *P. falciparum* parasites have independently developed partial resistance to artemisinin in several foci in the Greater Mekong subregion (GMS), Africa, Oceania and Latin America [1]. As a consequence, the increasing prevalence of artemisinin resistance and the emergence of resistance to partner drugs in ACTs may well reverse the recent substantial gains in malaria control [5]. For eradication to be effective, the concept of a drug combination of Single Encounter Radical Cure and Prophylaxis (SERCaP) was developed [18]. Thus, clinical candidates will necessarily require combination with mechanistically distinct drug partner(s) to alleviate the potential for drug resistance. CI-679 exhibited potent antimalarial activity against blood stages of the artemisinin-resistant *P. falciparum* C580Y strain, suggesting that CI-679 may be explored as an alternative antimalarial agent against artemisinin-resistant *P. falciparum* and a potential combination partner for ACTs.

CONCLUSION

It has been established that CI-679 exhibits potent antimalarial activity against the blood stages of *P. yoelii* BY265 *in vivo*, as well as the artemisinin-sensitive *P. falciparum* 3D7 and artemisinin-resistant *P. falciparum* C580Y *in vitro*. Further pharmacokinetic properties, tolerability and safety of the compound need to be investigated to support its preclinical development and advancement to human clinical trials. In addition, with the rising concern of resistance to antifolates, possible drug resistance mechanisms based on *in vitro* selection should also be investigated.

DECLARATIONS

Acknowledgements

This work was supported by grants from the State Key Program of the National Natural Science Foundation of China (no. 81830067), the National Natural Science Foundation of China (no. 81772226) and the Natural Science Foundation of Chongqing, China (nos. cstc2018jcyjAX0443 and cstc2018jcyjAX0609).

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. WYX, QFZ, JZ and NT conceived and designed the experiments. NT, YMZ, YD, YF and HXL performed the experiments. NT and YMZ analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

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