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Mechanisms of artemether toxicity on single cardiomyocytes and protective effect of nanoencapsulation

Ana Carolina Moreira Souza\textsuperscript{1,2} Andrea Grabe-Guimarães\textsuperscript{1} Jader dos Santos Cruz\textsuperscript{3} Artur Santos-Miranda\textsuperscript{3} Charlotte Farah\textsuperscript{2}
Liliam Teixeira Oliveira\textsuperscript{1,2} Alexandre Lucas\textsuperscript{4} Franck Aimond\textsuperscript{2}
Pierre Sicard\textsuperscript{2} Vanessa Carla Furtado Mosqueira\textsuperscript{1} Sylvain Richard\textsuperscript{2}

\textsuperscript{1}Pharmaceutical Sciences Graduate Program (CiPharma), Pharmacy School, Federal University of Ouro Preto, Ouro Preto, Minas Gerais, Brazil
\textsuperscript{2}Physiologie et Médecine Expérimentale du Cœur et des Muscles (PhyMedExp), Université de Montpellier, CNRS, Inserm, Montpellier, France
\textsuperscript{3}Department of Immunology and Biochemistry, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
\textsuperscript{4}Institut des Maladies Métaboliques et Cardiovasculaires (I2MC), Inserm/Université Paul Sabatier UMR1048, Toulouse, France

Correspondence
Dr. Sylvain Richard, Physiologie et Médecine Expérimentale du Cœur et des Muscles (PhyMedExp), Inserm U1046, CNRS UMR 9214, Université de Montpellier, CHU Arnaud de Villeneuve, 371, Avenue du Doyen Gaston Giraud, 34295 Montpellier Cedex 05, France.
Email: sylvain.richard@inserm.fr

Background and Purpose: The artemisinin derivative, artemether, has antimalarial activity with potential neurotoxic and cardiotoxic effects. Artemether in nanocapsules (NC-ATM) is more efficient than free artemether for reducing parasitaemia and increasing survival of \textit{Plasmodium berghei}-infected mice. NCs also prevent prolongation of the QT interval of the ECG. Here, we assessed cellular cardiotoxicity of artemether and how this toxicity was prevented by nanoencapsulation.

Experimental Approach: Mice were treated with NC-ATM orally (120 mg·kg\textsuperscript{-1} twice daily) for 4 days. Other mice received free artemether, blank NCs, and vehicle for comparison. We measured single-cell contraction, intracellular Ca\textsuperscript{2+} transient using fluorescent Indo-1AM Ca\textsuperscript{2+} dye, and electrical activity using the patch-clamp technique in freshly isolated left ventricular myocytes. The acute effect of free artemether was also tested on cardiomyocytes of untreated animals.

Key Results: Artemether prolonged action potentials (AP) upon acute exposure (at 0.1, 1, and 10 µM) of cardiomyocytes from untreated mice or after in vivo treatment. This prolongation was unrelated to blockade of K\textsuperscript{+} currents, increased Ca\textsuperscript{2+} currents or promotion of a sustained Na\textsuperscript{+} current. AP lengthening was abolished by the NCX inhibitor SEA-0400. Artemether promoted irregular Ca\textsuperscript{2+} transients during pacing and spontaneous Ca\textsuperscript{2+} events during resting periods. NC-ATM prevented all effects. Blank NCs had no effects compared with vehicle.

Conclusion and Implications: Artemether induced NCX-dependent AP lengthening (explaining QTc prolongation) and disrupted Ca\textsuperscript{2+} handling, both effects increasing pro-arrhythmogenic risks. NCs prevented these adverse effects, providing a safe alternative to the use of artemether alone, especially to treat malaria.

**KEYWORDS**
Action potential prolongation, CamKII phosphorylation, enhanced Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, pro-arrhythmogenic risks, spontaneous Ca\textsuperscript{2+} waves

Abbreviations: ACT, artemisinin-based combination therapies; AP, action potential; DLS, dynamic light scattering technique; EADs, early afterdepolarizations; LV, left ventricular; NCs, nanocapsules; NCX, membrane-bound Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger; PCL, poly-ε-caprolactone; PDI, polydispersity index; PLB, phospholamban; SERCA, sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase; SL, sarcomere length; SR, sarcoplasmic reticulum; VA, ventricular arrhythmia; WHO, World Health Organization.
1 | INTRODUCTION

Malaria is a mosquito-borne infectious disease caused by protozoa of genus *Plasmodium* sp., which represents a major health problem. According to the World Health Organization (WHO), malaria is endemic in nearly 100 countries and territories, particularly in underprivileged areas of Africa, Asia, and Latin America. In 2018, there were 228 million cases of malaria and an estimated 405,000 malaria deaths (WHO, 2018). In absence of an effective malaria vaccine, chemotherapy is still an unavoidable strategy to fight the disease. Unfortunately, the resistance of *Plasmodium falciparum* to several antimalarial drugs, such as chloroquine and mefloquine, is increasing dramatically (Lehane, McDevitt, Kirk, & Fidock, 2012; Price et al., 2004). Combinations of drugs are the most successful strategy used in malaria chemotherapy (Oguche et al., 2014; Sowunmi et al., 2017) Artemether–lumefantrine, artesunate–amodiaquine, dihydroartemisinin–piperaquine, artesunate–mefloquine, and artesunate–sulfadoxine–pyrimethamine are first-line treatment protocols used in countries where malaria is endemic (WHO, 2018). They rely mainly on the use of artesinisin derivatives such as artemether, a semisynthetic sesquiterpene lactone. Despite antimalarial effectiveness on schizonticide and gametocide activities (Brossi et al., 1988), artemether presents several drawbacks including a short half-life, cardiotoxicity, neurotoxicity, haematological toxicity and immunotoxicity (Gu, Cui, Wu, Shi, & Teng, 1989; Silamut et al., 2003; Yin et al., 2014). Regarding cardiotoxicity, several experimental studies have shown that rat and dog treatment with artemether (Yin et al., 2014), artesunato (Yin et al., 2014), or arteether by intramuscular or oral route prolongs the QT interval of the ECG. We have recently shown that oral administration of artemether at doses of 40, 80, and 120 mg·kg⁻¹ caused QT and QTc interval prolongation on both uninfected and *Plasmodium berghei*-infected mice (Souza et al., 2018). QT interval prolongation is an electrocardiographic surrogate marker of drug toxicity. It predisposes to torsades de pointes and is frequently associated with occurrence of malignant ventricular arrhythmia (VA), potentially leading to sudden death (Chan, Isbister, Kirkpatrick, & Dufful, 2007; Isbister & Page, 2013). Different cellular mechanisms can be involved in drug-induced QT interval prolongation, including blocking of outward *I*_k,V* and *I*_Ks currents; alteration in Ca²⁺ cycling; and activation of persistent inward *I*_Na current (Haverkamp, Breithardt, Camm, & Janse, 2000). It is therefore important to perform in vitro studies to identify the molecular effects of artemether, understand any potential cardiotoxicity and find alternatives to reduce those effects.

Polymeric nanocapsules (NCs) have been developed to reduce adverse effects and toxicity of different drugs (Branquinho, Roy, et al., 2017; Leite et al., 2007; Mosquera et al., 2004). NCs are nanocarriers with an oily core in which lipophilic drugs are transported (Legrand, Barratt, Mosquera, Fessi, & Devissaguet, 1999). NCs are able to modify drug release profile by altering biodistribution and bioavailability (Branquinho, Roy, et al., 2017). Recently, we demonstrated the potential of NCs to reduce cardiotoxic effects of oral administration of free artemether at doses of 40, 80, and 120 mg·kg⁻¹ in both uninfected and *P. berghei*-infected mice. In addition, NCs containing artemether (NC-ATM) were effective in combating malaria, achieving both cure and increasing the survival rate of mice by oral route (Souza et al., 2018). Given that, it was necessary to understand the effect of free artemether on cardiomyocytes. We focused on the excitation–contraction coupling of single cardiomyocytes from mice, and we investigated the mechanisms involved. Additionally, we tested whether the use of NCs is a valuable strategy for preventing potential adverse effects after in vivo repeated-dose treatment of mice, in conditions similar to those of clinical therapy.

2 | METHODS

2.1 | NCs preparation and artemether in vitro release kinetics

We prepared PCL blank-NCs, or loaded with artemether (NC-ATM), by the polymer deposition method followed by solvent displacement as described before (Fessi, Puisieux, Devissaguet, Ammoury, & Benita, 1989). Polymer (PCL, 24 mg), soy lecithin (Epiekuron™, 170, 30 mg), and 100 µl of artemether oil solution (80 mg·ml⁻¹) were dissolved briefly in 2.0 ml of acetone. The aqueous phase was prepared with complete dissolution of 30 mg of poloxamer 188 in 8 ml of MilliQ water. Organic phase was then poured into aqueous
phase using a syringe; the mixture was maintained under agitation for 10 min; and solvents were evaporated under reduced pressure (Heidolph Rotary Evaporator Instruments, Germany) until reaching 2-ml final volume with 4 mg·mL⁻¹ of artemether in colloid suspension of NCs. Blank-NCs were prepared as described above in the absence of artemether and using 100 µL of Miglyol 812 as the NCs oily core. The mean hydrodynamic diameter, polydispersity index (PDI), and ζ potential of NCs were determined using Zetasizer Nano ZS equipment (Malvern Instruments, UK), dynamic light scattering technique (DLS), and DLS coupled with microelectrophoresis for size and ζ potential measurements, respectively. NC-ATM were developed previously and characterized in detail by our group, and more than 90% of artemether was encapsulated in NCs (Vidal-Diniz, 2014). Free artemether was prepared as suspension mixing 0.09 g of artemether with 0.3 g of carboxymethylcellulose and 2.0 mL of sorbitol dispersed in 15 mL of MilliQ water. Vehicle (control solution) was prepared similarly, but in the absence of artemether. We maintained the formulations at controlled temperature, protected from light, and we prepared them freshly on the day of treatment.

In vitro studies of dissolution kinetics of free artemether and release kinetics of artemether from NCs were conducted using the inverted dialysis method in PBS (pH 7.4) under sink conditions (20% of saturation solubility) previously determined as 14 µg·mL⁻¹ at 37°C. Free form artemether crystals (1.4 mg) or 350 µL of ATM-PCL NCs (concentration of 4 mg·mL⁻¹) were placed at time 0 in 500 mL of PBS release media at 37°C in a thermostatic shaker bath containing five dialysis sacks (SpectraPor 12,000-14,000 MWCO) with 1 mL of PBS. At each time interval (0, 5, 30, 60, and 120 min), a dialysis sack was withdrawn simultaneously with a sample of released media (500 µL). The samples were diluted 1:1 with acetonitrile, then vortex-mixed, centrifuged at 500 x g for 5 min, and supernatant assayed by HPLC-UV to determine artemether concentrations using a validated method (da Cesar & Pianetti, 2009). Three independent experiments were conducted, with each one tested in triplicate.

2.2 | Animals

All animal care and experimental procedures conformed to Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010, for animal protection. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the British Journal of Pharmacology. The project was approved by the French Ministère de la Recherche et de l’Enseignement Supérieur (No. 02571.01) and the local institutional animal research committee (Languedoc Roussillon, CEEA-LR-1069). The experiments also complied with the guidelines established by the Brazilian College of Animal Experimentation (COBEA).

Here, we used 6- to 8-week-old male C57BL/6J mice weighing 20–22 g (Janvier Labs, Saint Berthevin, France) for all experiments. Animals were bred and housed (four to five mice in each cage) under pathogen-free conditions in a temperature-regulated room (22 ± 2°C; 12-h day/12-h night cycle) and an environment to meet their physical needs (standard rodent chow diet and ad libitum access to water) and behavioural needs (wood bedding material, cardboard tunnel, nesting material, and wooden gnawing sticks). The animals were killed humanely by cervical dislocation according to Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, on the Protection of Animals Used for Scientific Purposes. This method allows rapid death without injection of any substance that might interfere with cell electrophysiology for cell studies. In order to reduce the number of animals (3Rs), we made efforts to use cells isolated from one animal for different protocols performed in parallel (e.g., patch-clamp, different recording conditions, and Ca²⁺ fluorescence imaging).

Mouse models of malaria using P. berghei are well established, usable and cost-effective for studying different aspects of human infections including parasite–host interaction, antimalarial treatments, drug resistance, T cell mediated immunity, antigenic variation, and erythrocyte invasion pathways (Burget et al., 2020; Hernandez-Valdareas, Rihet, & Iraqi, 2014; Kurup, Butler, & Harty, 2019; Patel, Simpson, Batty, & Zalounis, 2015). Here, we tested only male mice in order to follow up our previous studies showing the efficacy of NC-ATM in P. berghei-infected male mice (Souza et al., 2018). We sought to compromise between a reasonable number of animals (Sneddon, Halsey, & Bury, 2017) and used the results necessary to answer our main questions (artemether toxicity and protective effect of nanocapsulation).

2.3 | In vitro treatment protocol

We first studied the acute cellular effects of artemether in vitro following the application of 0.1, 1.0, and 10 µM of the free artemether on single cardiomyocytes obtained from untreated animals (n = 16) and tested in different experimental conditions. Effects in the artemether group were compared to the control group exposed to DMSO:Tween (as the vehicle to artemether used acutely) diluted appropriately in the buffer medium to obtain the same in vitro concentrations. For repeated-dose treatments, the mice were blindly randomized to the four different groups: vehicle (n = 10), blank-NCs (n = 11), free-ATM (n = 11), and NC-ATM (n = 10 mice). Unrelated animals were randomized individually to cages, then cages were randomized to treatment groups housed in the same environment (same shelves) before starting the protocol. We administered treatments by oral route at 120 mg·kg⁻¹, twice daily (12/12 h) for 4 days (eight oral administrations). Treatment administration was not blinded to avoid complexity and errors due to the handling of animals. We could use only two animals (from two different groups) per day for single-cell recordings (Ca²⁺ imaging and cellular electrophysiology) requiring prior enzymic isolation of the cardiomyocytes. The start of the protocol was therefore shifted sequentially for each “pair” of animals in order to meet this requirement. The animals were treated identically with administrations by the same experimenter and at the same time.
times of the day throughout the experiment. Animals were assigned randomly to Lonoptix, patch-clamp experiments, biochemistry, and echocardiography.

2.4 | Single cardiomyocyte isolation

Cardiomyocytes were isolated from mice with no treatment and from mice treated with artemether formulations (free form or NCs) or with control solutions (vehicle or blank-NCs). The cell isolation procedure was initiated 2 h after administration of the last dose (day 4, eighth dose). The heart was perfused by means of the Langendorff system for enzymatic treatment and the use of 0.1 g·ml⁻¹ liberase (high research grade; Roche, Basel, Switzerland) circulated at a flow rate of 5-10 ml·min⁻¹ for 6-8 min at 37°C as described before (Branquinho, Roy, et al., 2017).

2.5 | Sarcomere contraction and measurements of intracellular Ca²⁺

Sarcomere length (SL) shortening, an index of contraction, and intracellular Ca²⁺ transients of left ventricular (LV) myocytes loaded with fluorescent ratiometric Ca²⁺ indicator Indo-1AM (2 mM, Invitrogen Grand Island, NY, USA) were monitored with an IonOptix system (Milton, MA, USA) as described before (Branquinho, Roy, et al., 2017). Cells were field-stimulated with 1-ms current pulses delivered via two platinum electrodes at 1.0 Hz and illuminated at 365 nm by means of a Xenon arc bulb light. Cytosolic Ca²⁺ was determined by Indo-1AM fluorescence, which emits at 405 and 480 nm concurrently (ratio of 405 nm/480 nm). To assess occurrence of abnormal macroscopic Ca²⁺ events, cells were stimulated for a period of 30 s, followed by rest period of 30 s.

2.6 | Cellular electrophysiological recordings

Whole-cell voltage- and current-clamp experiments (HEKA, Harvard Bioscience, Inc., Holliston, MA, USA) were performed (22–24°C) on freshly isolated LV cells. For action potential (AP) measurements, a 1-nA pulse lasting 2–4 ms was applied, at a frequency of 1 Hz for 5 min. Pipette solution was filled with (in mM) 20 KCl; 10 HEPES; 130 KOH; 130 aspartic acid; 2 MgCl₂; and 5 NaCl (pH 7.4). The cell bath solution contained (in mM) NaCl 130; KCl 4; MgCl₂ 1.8; CaCl₂ 1.8; HEPES 10; and glucose 11 (pH 7.4). To evaluate the acute effects of free artemether (10 μM) on the AP waveform, freshly isolated myocytes were incubated for 15 min before recording. To assess the contribution of the Na⁺/Ca²⁺ exchanger (NCX) and the late Na⁺ current (I₅Na,Late) in the effect of artemether on AP, cells were exposed to 1-μM SEA-0400 or 20-μM ranolazine, respectively, in presence of artemether. Currents were digitally sampled at 10 kHz, and their amplitude was normalized to whole-cell membrane capacitance (current densities pA·pF⁻¹). In all whole-cell experiments, cells were kept at rest for 2 min prior to any experimental protocol in order to allow proper equilibration between cell media and intracellular pipette solution. Moreover, cells with total series resistance over 8 MΩ were not considered in the analysis to prevent voltage-clamp errors.

2.7 | Biochemistry and western blot analysis

For biochemistry, perfused isolated hearts from mice were acutely exposed to artemether (10 μM) for 15 min using the Langendorff technique. Simple Western (Jess, ProteinSimple, CA) was based on capillary electrophoresis (CE) and offers a size assay that combines CE-SDS with immunodetection to separate proteins by MW (Schiafaretta et al., 2019). The separated proteins were attached to the capillary wall by a proprietary photo-activated chemical crosslink. Subsequent immunodetection was achieved automatically by incubating and washing the capillary with primary and secondary antibodies conjugated with HRP and detected via chemiluminescence. Protein expression was measured by peak area with Compass software (Bio-Technie, ProteinSimple). Diluted protein lysate was mixed briefly with fluorescent master mix and heated at 37°C for 10 min. Protein mix (3 μl), total protein normalization reagent, blocking reagent, wash buffer, target primary antibody, secondary HRP (ready to use DM-001, "detection module"), and chemiluminescent substrate were dispensed into designated wells in a manufacturer-provided 66–440 kDa of matrix microplate (SMW 004, ProteinSimple). We used Anti-phospholamban antibody (ab85146; Abcam, France), phospho-Ser⁰¹⁵-PLB and phospho-Thr⁰¹⁷-PLB (Badrilla, A010-12 and A010-13, respectively; UK), and home-made phospho-Ser²⁰⁰⁸_RyR2 and phospho-Ser²⁰¹⁴_RyR2 antibodies. The Immuno-related procedures used comply with the recommendations made by the British Journal of Pharmacology.

2.8 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). For in vitro studies, cells were chosen randomly. Data analyses were performed blinded for experimenters as much as possible. Reported values are means ± SEM, except for artemether in vitro kinetic study (mean ± SD). D’Agostino and Pearson omnibus and Shapiro–WilK normality tests were used. Comparisons between groups of more than two samples were made using one-way ANOVA. Where ANOVA was used, post hoc tests were conducted only where F was significant (P < 0.05) and there was no variance inhomogeneity. Tukey post-test was used to compare all pairs of columns using GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, CA, USA). Unpaired two-sample t tests were used when appropriate. Only those studies where each group size was at least five animals were analysed statistically and are indicated by symbols in the graphs. N indicates the number of mice, and n indicates the number of independent cardiomyocytes isolated from these mice. Differences
were considered significant when \( P < 0.05 \). Data on the number of arrhythmic cells were analysed using Fisher’s exact test. \( P < 0.05 \) was determined as significant. The data that support the findings of this study are available from the corresponding author upon reasonable request.

## 2.1 Materials

We purchased artemether (dihydroartemisinin methyl ether), poly-
\( \varepsilon \)-caprolactone (PCL), poloxamer 188 (Pluronic\textsuperscript{\textregistered} F68), carboxymethyl-
cellulose, and HPLC grade acetone from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). The solution of artemether dissolved in oil was produced by the National Malaria Control Program, Ministry of Health, Brazil. Soy lecithin with approximately 75% of phosphatidyl-
choline (EpiKuron™ 170) was a generous gift from Cargill (Germany). We purchased Miglyol® 812N from Sasol Germany GmbH. MilliQ water was purified using a Symplicity\textsuperscript{®} System (Millipore, Bedford, USA). We protected free artemether and NC-ATM from direct light exposure throughout the experiments.

## 2.2 Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Fabbro et al., 2019; Alexander, Kelly et al., 2019; Alexander, Mathie et al., 2019).

## 3 RESULTS

### 3.1 NCs preparation and release kinetics of artemether in vitro

The NC-ATM formulations used in the present work have been char-
acterized previously (Souza et al., 2018). The blank NCs and ATM-NCs were monodispersed formulations (PDI < 0.3) of colloidal particles that presented mean hydrodynamic sizes of 197 and 254 nm, and negative \( \zeta \) potential of ~56 and ~50 mV, respectively, which account for their colloidal stability due to electric repulsion between particles. The polymeric NCs were able to encapsulate artemether in high concen-
trations (4 mg·mL\(^{-1}\)), which could be due to the high lipophilicity of artemether (log \( P_{\text{sw}} \) 3.53). NCs maintain their colloidal stability and artemether content under storage for at least 3 months at 4°C (Souza et al., 2018). In vitro release of artemether from the NCs device was evaluated. Results showed low artemether release after incubation for 30 min (~15%) and 2 h (~45%) compared to faster dissolution of free artemether (~32% and ~95%, respectively) (Figure 1). NCs acts as a device to prolong artemether release, and direct comparison of free artemether and ATM-NCs may be influenced by the difference in release kinetics. We thus used the free drug to identify the major mechanisms of artemether toxicity on cardiomyocytes.

### 3.2 Acute in vitro effect of artemether on cell contraction and Ca\(^{2+}\) cycling

We examined the cellular and molecular effects of 0.1-, 1.0-, and 10-\(\mu\)M free artemether on SL shortening and intracellular Ca\(^{2+}\) of LV cardiomyocytes freshly isolated from control animals and investigated within 1 to 4 h after cell isolation. The acute effects of artemether are illustrated in Figure 2a,b. Artemether had no significant effects on SL shortening, or on either the amplitude or decay of the Ca\(^{2+}\) transient (Figure 2c,d,f). However, free artemether, at all concentrations, caused a reduction in diastolic Ca\(^{2+}\) (Figure 2e). Free artemether also promoted irregular Ca\(^{2+}\) transients during pacing and abnormal spontaneous Ca\(^{2+}\) waves during resting periods, which were not seen in untreated cells or cells exposed to vehicle (Figure 3a–e). The number of cells exhibiting these aberrant Ca\(^{2+}\) events increased dose-dependently with maximal effects seen at 1 and 10 \(\mu\)M (Figure 3f).

### 3.3 Acute post-translational effects of artemether on phospholamban and ryanodine receptors

As we did not expect changes of protein expression during acute effects of artemether, we explored post-translational modifications of proteins involved in altered Ca\(^{2+}\) handling (Figure 4). Cardiac sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a) activity ensures the uptake of Ca\(^{2+}\) in the sarcoplasmic reticulum (SR) during the relaxation and diastolic period. SERCA2a activity is critically regulated by the inhibitory protein phospholamban (PLB) in various physiopathological situations (Minamisawa et al., 1999). Phosphorylation at Serine16 (Ser\(^{16}\)-PLB) by cAMP-dependent protein kinase (PKA), or Threonine17 (Thr\(^{17}\)-PLB) by calmodulin-dependent protein kinase II
**Figure 2** Acute effect of free artemether on sarcomere length (SL) shortening and Ca$^{2+}$ transient in left ventricular myocytes isolated from untreated mice. Freshly isolated cells were exposed to free artemether (ATM) for 15 min at concentrations of 0.1 μM \((n = 13, N = 5)\), 1.0 μM \((n = 15, N = 5)\), and 10 μM \((n = 15, N = 5)\), or to vehicle (control; \(n = 9, N = 5\)).

Contraction and Ca$^{2+}$ transients were evoked by electrical pacing at 1.0 Hz. (a, b) Typical records of the effect of ATM (10 μM) or vehicle on sarcomere length (SL) shortening (a) and Ca$^{2+}$ transient (b). (c–f) Averaged effects of ATM (0.1, 1.0, and 10 μM) on SL shortening (c), Ca$^{2+}$ transient (d), Ca$^{2+}$ diastolic (e), and Ca$^{2+}$ transient decay (τ) (f). Data shown are means ± SEM; \(n\) values as indicated above. \(P < 0.05\), significantly different from control at all concentrations; one-way ANOVA followed by Tukey’s post hoc test.

(CaMKII), relieves this inhibition and enhances the rate of Ca$^{2+}$ uptake (Hagemann & Xiao, 2002). Acute application of free artemether (10 μM) promoted phosphorylation at the CaMKII-specific Thr$^{17}$-PLB site but not at the PKA-specific Ser$^{16}$-PLB (Figure 4a–c). Post-translational modifications of the cardiac ryanodine receptor RyR2 protein, which controls SR Ca$^{2+}$ release during excitation-contraction coupling, are also identified as one of the molecular mechanisms responsible for Ca$^{2+}$ handling alterations (Dennis, Duhunty, & Beard, 2018; Niggli et al., 2013). Free artemether promoted RyR2 phosphorylation at Serine(S)2814 but not at S2808 (Figure 4d–g).

### 3.4 Acute effect of artemether on cardiomyocyte AP

Myocyte contraction and intracellular Ca$^{2+}$ dynamics are conditioned by cell excitability. Typical APs of LV myocytes isolated from untreated animals were recorded (Figure 5a). Free artemether acutely applied in vitro markedly prolonged the AP (Figure 5a, bottom panels; Figure 5b,d). We investigated the potential involvement of the repolarizing voltage-gated outward K$^+$ currents in this effect. The different components, namely, I$_{Na,L}$ (fast component of the transient outward current), I$_{K,slow}$ (slow inactivating outward K$^+$ current), and I$_{s}$ (sustained outward K$^+$ current), in AP repolarization were recorded as described in the Supporting Information. Free artemether had no effect on the global K$^+$ current (Figure S1). Detailed analysis confirmed that the drug affected neither the I$_{Na,L}$ (responsible for the early AP repolarization) nor I$_{K,slow}$ (slow inactivating outward K$^+$ current) involved in late AP repolarization (Figure S1).

Any increase in an inward current could also account for the marked delayed repolarization of the AP. The I$_{Na,L}$ current was a good candidate to test. We used ranolazine, a selective inhibitor of I$_{Na,L}$ reported to be 38-fold more potent in inhibiting I$_{Na,L}$ than peak (transient) I$_{Na,L}$ (Belardinelli, Shryock, & Fraser, 2006; Undrovinas, Belardinelli, Undrovinas, & Sabbah, 2006). Figure 5a (left panel) shows that ranolazine (20 μM) had no effect on the AP, both in control conditions and after application of artemether (10 μM). Therefore, the results showed that ranolazine did not prevent the effect of artemether on AP repolarization (Figure 5a, left panel; Figure 5b,c), suggesting that promotion of I$_{Na,L}$ by artemether does not contribute to the effect of artemether on AP duration.
FIGURE 3  Acute exposure to free artemether promotes irregular Ca$^{2+}$ transients during pacing and spontaneous diastolic Ca$^{2+}$ events in left ventricular myocytes, isolated from untreated mice. Freshly isolated cells (n = 15 isolated from N = 5 mice in each group) were exposed to free artemether (Free-ATM) for 15 min at concentrations of 0.1 (a), 1.0 (b), and 10 μM (c) or to vehicle (control). Ca$^{2+}$ transients were evoked by electrical pacing at 1.0 Hz (horizontal bars, white colour), followed by a resting period (horizontal bars, black colour) (a–e). ATM promoted aberrant Ca$^{2+}$ events (indicated by arrows) at all concentrations tested during pacing and resting periods. (f) Summary data. Number of cells exhibiting abnormal Ca$^{2+}$ events at 0.1-, 1.0-, and 10-μM Free-ATM versus control (e) and untreated cells (d). Data shown are means ± SEM; n values as indicated above. *P < 0.05, significantly different from control; Fisher’s exact test

FIGURE 4  Activation of CaMKII-dependent phosphorylation of PLB and RyR2 after acute exposure of isolated hearts to artemether. Isolated hearts were exposed acutely to artemether (Free ATM; 10 μM; n = 6 mice) or vehicle (control; N = 6 mice) for 15 min using the Langendorff perfusion technique. Representative western blots of the effects of artemether on (a) PLB phosphorylation at Serine16 (Ser$^{16}$-PLB) by cAMP-dependent protein kinase (PKA) and Threonine17 (Thr$^{17}$-PLB) by calmodulin-dependent protein kinase II (CaMKII), and (b) phosphorylation of RyR2 at Serine-(S)2814 by CaMKII, and at Serine-(S)2808 as possible phosphorylation sites for PKA. (c) Relative level of phospho-Ser$^{16}$-PLB/pentamer PLB. (d) Relative level of phospho-Thr$^{17}$-PLB/pentamer PLB. (e) Relative level of phospho-Ser$^{2808}$RyR2/total protein. (f) Relative level of phospho-Ser$^{2814}$RyR2/total protein. (g) Relative level of phospho-Ser$^{2814}$RyR2/phospho-Ser$^{2808}$RyR2. In panels (c)–(g), data shown are means ± SEM; *P < 0.05, significantly different from control; unpaired t test.

To explore further the mechanism involved in the effect of ATM, we investigated the role of the membrane bound NCX. The NCX is a bidirectional regulator of cytosolic Ca$^{2+}$ that plays a key role in Ca$^{2+}$ homeostasis during excitation–contraction coupling of cardiomyocytes (Shattuck et al., 2015). The NCX is particularly essential for removing intracellular Ca$^{2+}$ during relaxation. The NCX
forward mode enables Ca\(^{2+}\) removal and generates a depolarizing inward Na\(^{+}\) current that can be pro-arrhythmogenic. SEA-0400 selectively inhibits both forward and reverse modes of the NCX (Ozdemir et al., 2008; Tanaka et al., 2002). SEA-0400 is expected to reduce Ca\(^{2+}\) overload due to the reverse mode and reduce NCX-related Na\(^{+}\) entry (against Ca\(^{2+}\) extrusion) in the forward mode. Results showed that SEA-0400 (1 μM) prevented the lengthening effect of free *artemether* on AP duration (Figure 5a, right panel; Figure 5d,e). We also noted an intrinsic influence of SEA-0400 on AP duration and a hyperpolarizing action on the resting membrane potential (which is not voltage-clamped) in control. This may have been because the intrapipette solution used was free of Ca\(^{2+}\) buffer EGTA, thereby enabling access to compartmentalized signals including Na\(^{+}\)/Ca\(^{2+}\) modulation of the NCX. Overall, this observation is consistent with a role of NCX current in controlling cardiomyocyte resting membrane potential.

### 3.5 Effects of repeated doses of *artemether* and NC-ATM administered in vivo on cell contraction and Ca\(^{2+}\) cycling

We investigated the effects of repeated-dose treatment administered to mice of free *artemether* (120 mg·kg\(^{-1}\)) on parameters of the
excitation–contraction coupling in paced single intact LV myocytes. We measured SL shortening (cell contraction) and intracellular Ca$^{2+}$. Free 
artemether decreased cell contraction (Figure 6a,c) and Ca$^{2+}$
transient amplitude (Figure 6b,d) but had no effect on Ca$^{2+}$ transient decay (Figure 6e). Diastolic Ca$^{2+}$ was decreased (Figure 6b,e). Irregular Ca$^{2+}$ transients during pacing and spontaneous Ca$^{2+}$ waves during resting periods occurred (Figure 7a,e). We did not observe these effects in other experimental conditions (Figure 7b–e). To summarize, any of the effects associated with free 
artemether treatment on contraction and Ca$^{2+}$ handling were observed in the control groups (vehicle and blank-NCs). Importantly, encapsulation of 
artemether (NC-ATM) prevented the effects of the free form of the drug (Figures 6 and 7).

3.6 | Effects of repeated dose of free 
artemether and NC-ATM administered in vivo on cardiomyocyte AP and L-type calcium current

We investigated the effect of free 
artemether treatment on cellular electrical activity and Ca$^{2+}$ entry, two major determinants of the Ca$^{2+}$ transient and associated cellular contraction. Figure 8a shows typical

AP recorded in all experimental conditions. Treatment with free-
artemether markedly delayed AP repolarization (Figure 8a). Indeed, 
APD$_{90}$ was increased but not APD$_{50}$ (Figure 8b,c). In contrast, no 
effect was observed in the control groups (vehicle and blank-NCs). The prolonging effect of free 
artemether on AP duration was fully prevented by its nanoencapsulation (NC-ATM). The other parameters such as resting membrane potential (Figure 8d), AP amplitude (Figure 8e), and the maximum rate of depolarization (dV/dt$_{max}$) were not changed significantly.

The contraction of cardiac cells is largely determined by the rise of intracellular Ca$^{2+}$ initiated by the transmembrane Ca$^{2+}$ entry carried out by inward L-type Ca$^{2+}$ current (I$_{Ca.L}$). Repeated treatment of mice with free 
artemether, as well as with vehicle, blank-NCs, or NC-ATM, had no effect on the I$_{Ca.L}$ waveform (Figure S2A). There was no change in the peak current amplitude (reported as density) or decay kinetics. Evaluation of the current-to-voltage relationship (Figure S2B), steady-state activation (Figure S2C), and voltage-dependent availability for activation (Figure S2D) also showed no difference. Therefore, the reduction of the Ca$^{2+}$ transient was neither related to a decrease nor a prolongation of AP duration related to a modification in the amplitude or properties of I$_{Ca.L}$.

**FIGURE 6** Nanoencapsulation prevents the effects of repeated-dose treatment of mice with 
artemether on the contraction and the Ca$^{2+}$ transient in freshly isolated single LV myocytes. Mice were treated with vehicle (control), free 
artemether (Free-ATM, 120 mg·kg$^{-1}$), blank-NCs, or NC-ATM (120 mg·kg$^{-1}$). (a, b) Representative contraction and Ca$^{2+}$ transient evoked by electrical field stimulation at 1.0 Hz in single myocytes from the ATM and NC-ATM groups after 4 days of treatment; (a) contraction as measured from sarcomere length (SL) shortening; (b) Ca$^{2+}$ transient expressed as the fluorescence ratio F405/F480. (c–f) Averaged effects in single myocytes isolated from the control (n = 12, N = 4), ATM (n = 11, N = 4), blank-NCs (n = 13, N = 4), and NC-ATM groups (n = 13, N = 4); (c) SL shortening (%); (d) Ca$^{2+}$ transient; (e) diastolic Ca$^{2+}$; and (f) Ca$^{2+}$ transient decay (Tau). In panels (c)–(f), data shown are means ± SEM; n values as indicated above.
3.7 | Effects of repeated dose of artemether and NC-ATM administered in vivo on heart morphology and function

Because cardiomyocyte contraction was decreased, we examined heart morphology and function by echocardiography at the end of the 4-day repeated-dose treatments (Figure S3). When compared to the control group (vehicle), free artemether had no effect on the diameter of the LV lumen (Figure S3A), measured as the LV internal diameter (LVID) in diastole, or the thickness of both the LV anterior wall (AW) and the LV posterior wall (PW) (Figure S3B,C). Functional parameters such heart rate (HR) and the ejection fraction (EF) reflecting systolic function were not modified (Figure S3D,E). The E/A ratio, reflecting the early passive (E-wave) and late atrial contraction (A-wave) components of LV filling, was also unchanged (Figure S3F). No parameters were affected by artemether administered in its encapsulated form (NC-ATM group; Figure S3). Altogether, these results showed that the 4-day repeated-dose treatment with artemether induced neither morphological nor functional changes in line with an absence of cardiac remodelling.

4 | DISCUSSION

Artemether is an effective drug for reducing parasitaemia in malaria and increasing the survival of mice infected with P. berghei (Souza et al., 2018). Remarkably, effectiveness of artemether and other antimalarial drugs can be improved by nanoencapsulation that prolongs their effect (Haas, Bettoni, de Oliveira, Gutierrez, & Dalla Costa, 2009; Leite et al., 2007; Mosqueira et al., 2004; Souza et al., 2018).
FIGURE 8  Nanoencapsulation prevents the effect of artemether on the action potential in single left ventricular myocytes following repeated-dose treatment of mice with ATM. (a) Representative AP of LV myocytes isolated from mice treated with vehicle (control; n = 15, N = 4), free-ATM (n = 9–12, N = 4), blank-NCs (n = 10, N = 4), or NC-ATM (120 mg·kg⁻¹; n = 16, N = 4); (b, c) time required to reach, respectively, 90% and 50% of AP repolarization (APR); (d) mean resting membrane potential of cardiomyocytes; (e) mean AP amplitude; and (f) maximum rate of AP depolarization. In panels (b)–(f), data shown are means ± SEM; n values as indicated above.

However, a legitimate question is whether higher efficacy induces artemether toxicity, as suggested by QT and QTc interval prolongation in mice, and whether nanoencapsulation can prevent adverse cellular effects. Our present results have shown that free artemether modifies cellular electromechanical coupling and induces proarrhythmogenic effects. Our data also provided evidence that the nanoencapsulation of artemether prevented its adverse cardiac effects.

The cardiotoxicity risk of antimalarial drugs has received much attention. These drugs can affect myocardial depolarization and repolarization. For example, primaquine, quinine and quinidine block inward Na⁺ current, which depresses the maximal upstroke velocity of the AP (Grace & Camm, 1998; Orta-Salazar, Bouchard, Morales-Salgado, & Salinas-Stefanon, 2002). Risks of VA have also been identified from QTc interval prolongation due to delayed repolarization (Yin et al., 2014). Chloroquine, halofantrine, lumefantrine, and mefloquine have reported molecular effects including the blockade of L-type Ca²⁺ currents and K⁺ currents, such as I₉ of I₉K, involved in the AP plateau and repolarization (White, 2007). Anti-cholinergic action of artesiminin on I₉Ca,M also may also occur via inhibition of the muscarinic K⁺ channel and/or associated GTP-binding proteins (Hara et al., 2007).

Here, we report critical cellular effects of repeated oral administration of artemether (120 mg·kg⁻¹; twice daily, 12/12 h) for 4 days in mice. The in vivo treatment reproduced the effects of acute myocyte exposure to free artemether on AP duration and abnormal Ca²⁺ events. Overall, these results are in line with a direct and rapid effect of the drug (de Vries & Dien, 1996). This also excluded the possibility that the effects seen after repeated administration in vivo result from cardiac remodelling, unlikely to occur within the 4-day treatment (Svoboda, Poprach, Dobes, Kiss, & Vyzula, 2012), which was confirmed by our investigation of heart morphology and function by echocardiography. Incidentally, the reduction of myocyte contraction after treatment with free artemether was not found on myocardial function, either because this effect is modest (or not significant upon acute exposure of single cells) or because the negative inotropy is compensated by one or more mechanisms external to the cell, such as neurohormonal system(s).

One major result of our study was that NCX overactivity accounts for the artemether-induced lengthening of AP duration (Figure 5). The delay in AP repolarization occurs at potentials less than −50 mV; that is, when the fast repolarizing K⁺ current I₉ is mostly inactivated. Free artemether consistently had no effect on I₉. Although attenuation of I₉ can prolong late AP repolarization and the QT interval (Xu et al., 1999), we discarded this possibility. Likewise, we also discounted any effect of artemether on I₉Ca,M (on both amplitude and decay kinetics), as well as a promotion of the I₉Current, as ranolazine failed to inhibit the effect of artemether. On the other hand, the NCX inhibitor SEA-0400 successfully abolished AP lengthening (Figure 5), supporting the idea that artemether increases the activity of the NCX operating in forward mode, that is, extruding one Ca²⁺ ion out, in exchange for the entry of three Na⁺ ions into the cell (Venetucci, Trafford, O'Neill, & Eiser, 2007). This mechanism is also
consistent with lowered diastolic intracellular Ca\(^{2+}\) (Figures 2 and 6). Our results are in line with a previous report showing that SEA-0400 shortens AP duration and increases diastolic [Ca\(^{2+}\)]\(_j\) (Bourgonje et al., 2013).

QTC prolongation, a surrogate marker of VA risks, provides a substrate for earlier afterdepolarizations (EADs). Our study demonstrated the involvement of NCX in the QTC prolongation induced by artemether (Souza et al., 2018). As women have longer QTc and a greater intrinsic sensitivity to QT prolonging drugs than men (Darpo et al., 2014), extrapolarization of the NCX-dependent AP and QTc (Souza et al., 2018) prolongations in mice may suggest a critical drug-induced delay in cardiac repolarization reserve in women. This aspect warrants further investigation in a more appropriate experimental model (Salama & Bett, 2014). Our results disclosed another type of risk due to occurrence of abnormal spontaneous intracellular Ca\(^{2+}\) events during diastole (Figures 5 and 8) that can trigger Ca\(^{2+}\)-dependent VA (Pasquié & Richard, 2009). Increased Na\(^+\) entry through NCX may generate sufficient depolarization for ectopic activation of voltage-gated Na\(^+\) channels and the triggering of abnormal APs and Ca\(^{2+}\) transients during diastole. In addition to NCX, artemether also boosted the activity of a set of Ca\(^{2+}\) handling proteins involved in excitation–contraction coupling, but also in arrhythmias in case of imbalance due to pathological situations. In normal conditions, Ca\(^{2+}\) removal from the cytosol by the SERCA2a pump, responsible for intracellular Ca\(^{2+}\) uptake and storage in the cardiac SR, prevails over NCX activity. PLB phosphorylation enhances the SR Ca\(^{2+}\) load through enhanced SERCA2a activity, whereas RyR2 phosphorylation produces the opposite effect by promoting SR Ca\(^{2+}\) leak (Dennis et al., 2018; Hagemann & Xiao, 2002; Niggl et al., 2013). Our results showed that artemether induces complex effects known to favour, on one hand, an increased activity of SERCA2a (via PLB phosphorylation) and NCX, and on the other hand, Ca\(^{2+}\) leak from the SR via RyR2. As the decline in Ca\(^{2+}\) transients was unchanged, a balance may have been established between the different factors. Finally, enhanced extrusion of Ca\(^{2+}\) through NCX may explain the decrease in the Ca\(^{2+}\) transient and cell contraction because less Ca\(^{2+}\) would be available for SR Ca\(^{2+}\) uptake (Schillinger, Fiolet, Schlotthauer, & Hasenfuss, 2003).

Artemether rapidly phosphorylated the CaMKII-specific Thr\(^{17}\). PLB site, but not the PKA-specific Ser\(^{16}\)-PLB, following acute exposure to the drug. Similarly, free artemether promoted phosphorylation of the cardiac ryanodine receptor, RyR2, at Ser\(^{2814}\) (but not that at Ser\(^{2808}\)). CaMKII-dependent phosphorylation has been associated with pathological RyR2 conformational change that induces abnormal SR Ca\(^{2+}\) leak (Uchinomi et al., 2016). Incidentally, CaMKII activity is increased in the heart under stress or pathological conditions where oxidation is elevated (Anderson, 2015). Remarkably, artemether has an endoperoxide bridge, the opening of which by iron leads to the production of free radicals, causing oxidative stress in the parasite (Cumming, Ploypradith, & Posner, 1997). Artemether has multiple cellular targets through the involvement of ROS (Kavishe, Koenderink, & Alifrangis, 2017) such as ion channels and transporters that are often implicated in VA (Niggl et al., 2013; Wagner, Rokita, Anderson, & Maier, 2013). RyR2 contains several redox-sensitive cysteine residues and is highly sensitive to ROS, which promotes aberrant SR Ca\(^{2+}\) release (Terentyev et al., 2008). Redox-mediated SR Ca\(^{2+}\) depletion was proposed to involve reciprocal regulation of SERCA and NCX via direct oxidative modification of both proteins (Kuster et al., 2010). The NCX also has reactive thiols and can be activated by ROS (Goldhaber, 1996; Reeves, Bailey, & Hale, 1986), which could account for the artemether-induced increase of NCX activity. Moreover, exposure to modest oxidative stress causes a contractile phenotype characterized by reduced contraction, reduced Ca\(^{2+}\) transient, and no effect on diastolic Ca\(^{2+}\) (Kuster et al., 2010), relatively similar to modifications induced by artemether. Therefore, artemether-induced ROS production may disturb, directly or indirectly, a set of Ca\(^{2+}\) handling proteins critically involved in VA. Taken together, the proarrhythmogenic effects of artemether are clearly multifactorial and resemble many effects of ROS. These aspects and the determination of the primary artemether targets will require further investigation.

Artemisinin-based combination therapies (ACTs) are recommended for uncomplicated malaria (Plewes, Leopold, Kingston, & Dondorp, 2019; WHO, 2018). Artemether is given as first-line oral treatment in combination with lumefantrine to treat malaria due to P. falciparum (Belew et al., 2019) and Plasmodium vivax (Abreha et al., 2018). As a medical emergency, severe malaria requires rapid parenteral administration. Unfortunately, cardiotoxicity is a major concern with reports of significant prolongation of the QTc interval of ECG in healthy volunteers treated with ACTs (Falade et al., 2008; Funck-Brentano et al., 2019). In an experimental model, artemether induced high mortality (50%) and electrocardiographic disorders in mice surviving the parasitaemia (Souza et al., 2018). To circumvent this problem, artemether nanocarriers have been developed, including lipid nanoparticles (Aditya, Patankar, Madhusudhan, Murthy, & Souto, 2010), liposomes (Chimanuka, Gabriëls, Detaevenier, & Plaizier-Vercammen, 2002), nanocrystals (Shah et al., 2016), and nanoemulsions (Laxmi, Bhardwaj, Mehta, & Mehta, 2015). Oral drug delivery is a convenient administration route for patients (Attilli-Quadri et al., 2013). NC-ATM administered orally not only improved the efficacy of artemether against the Plasmodium but also prevented QTc prolongation in mice (Souza et al., 2018). Here, we provide additional evidence for the protective effects of NCs against adverse effects of artemether shown at the cellular and molecular levels (AP prolongation and Ca\(^{2+}\) handling modifications). The lymphatic pathway is involved in the absorption of NCs, protecting docetaxel from degradation either in the intestine or systemic circulation, thereby leading to higher blood levels than those achieved with the free drug injected intravenously (Attilli-Quadri et al., 2013). A similar mechanism may account for the safer profile of NC-ATM. Another possibility may relate to modified drug distribution and bioavailability, with NCs acting as a reservoir enabling a slow, sustained, release of lower doses of free artemether in the circulation, rather than a large but short peak of concentration with free artemether, as for halofantrine and lynchopholide (Branquinho, Pound-Lana, et al., 2017; Branquinho, Roy, et al., 2017; Leite et al., 2007). Such a system would be expected to provide more continuous drug coverage for the duration of treatment and therefore increase the effectiveness of therapy.
This may also result in more limited exposure of the heart to the free drug. This advantage has a genuine pharmaceutical potential, as the underlying principle may be extended to other organs and to various parasitic diseases, including toxoplasmosis (Sordet, Aumajaud, Fessi, & Derouin, 1998), Chagas disease (De Mello et al., 2016), leishmaniasis (Chaurasia et al., 2015), and malaria (Mosqueira et al., 2004; Souza et al., 2018). It is worth noting that the therapeutic potential of artemether goes far beyond the framework of malaria, as this drug also shows promise for treating leishmaniasis (Mortazavi Dehkordi, Ghaffarifar, Mohammad Hassan, & Esavand Heydari, 2013), schistosomiasis (El-Beshbishi et al., 2013), toxoplasmosis (Mikaeloo, Ghaffarifar, Dalimi, Sharifi, & Hassan, 2016), and cancer, with fewer adverse effects than conventional chemotherapy (Alcântara et al., 2013).

In conclusion, our study showed that oral administration of free artemether had toxic effects on cardiomyocytes following a repeated-dose treatment regimen. Major effects involved NCX-dependent prolongation of the AP, explaining QTc prolongation and disrupted Ca²⁺ handling with a potential to trigger Ca²⁺-dependent arrhythmias. However, these effects were totally prevented by nanoencapsulation of the drug in polymeric NCs. This strategy has the potential to improve therapeutic approaches to treat malaria, as well as other parasitic diseases, and to expand anti-tumour therapy, with artemether.

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AUTHOR CONTRIBUTIONS

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR
This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, Immunoblotting and Immunohistochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

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