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Simultaneous Administration of 2-Aminoethyl Diphenylborinate and Chloroquine Reverses Chloroquine Resistance in Malaria Parasites

Ehab Mossaad,[a,b,c] Wakako Furuyama,[a] Masahiro Enomoto,[d] Satoru Kawai,[e] Katsuhiko Mikoshiba,[f] Shin-ichiro Kawazu[a]

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan[a]; The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Japan[b]; College of Veterinary Medicine, Sudan University of Science and Technology, Khartoum, Sudan[c]; Princess Margaret Cancer Centre, Toronto, Canada[d]; Laboratory of Tropical Medicine and Parasitology, Dokkyo Medical University, Tochigi, Japan[e]; Laboratory for Developmental Neurobiology, RIKEN Brain Science Institute, Saitama, Japan[f]

A nearly complete reversal of chloroquine (CQ) resistance in the CQ-resistant *Plasmodium falciparum* K-1 strain, with a significant decrease in the mean ± standard deviation (SD) 50% inhibitory concentration (IC_{50}) from 1,050 ± 95 nM to 14 ± 2 nM, was achieved *in vitro* by the simultaneous administration of 2-aminoethyl diphenylborinate (2-APB). The CQ resistance-reversing activity of 2-APB, which showed the same efficacy as verapamil, was also observed in an *in vivo* mouse infection model with the CQ-resistant *Plasmodium chabaudi* AS(30CQ) strain.

Malaria continues to be a worldwide public health problem, causing significant morbidity and mortality. In addition, the resistance of the causative parasite to existing antimalarial drugs has been a growing problem in countries where the disease is endemic (1). Thus, there is an urgent need to develop new antimalarial drugs (2–4) and find novel agents to reverse antimalarial resistance. Understanding the signaling pathways governing the blood-stage growth of the causative parasite may aid in the discovery of new therapeutic targets for antimalarial drugs. As such, the calcium ion (Ca^{2+}) homeostasis and signaling pathways in the malaria parasite *Plasmodium* might be promising targets. Ca^{2+} is a ubiquitous intracellular signal responsible for controlling a wide range of cellular activities in eukaryotic cells (5). In protozoan parasites, Ca^{2+}-mediated signaling controls various vital functions, such as protein secretion, motility, cell invasion, and differentiation (6–9). We recently, for the first time, demonstrated spontaneous Ca^{2+} oscillation in *Plasmodium falciparum*. Furthermore, we showed that the blockage of this oscillation in the trophozoite stage by 2-aminoethyl diphenylborinate (2-APB) inhibited 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release (10–12), resulting in the death of the parasite (13). Chloroquine (CQ) is thought to exert its toxic effect in the intraerythrocytic parasite at the digestive vacuole (14). The compound was also found to induce Ca^{2+} release and disrupt Ca^{2+} and H^+ homeostasis in the cytoplasm of *Plasmodium chabaudi* cells (15, 16). Therefore, with regard to the disruption of intracellular Ca^{2+} homeostasis, it was hypothesized that the potentiation of CQ activity could be achieved in the malaria parasite with the simultaneous administration of 2-APB.

To evaluate the potential of 2-APB for reversing CQ resistance, a CQ-resistant K-1 strain of *P. falciparum* was cultured with the modified method of Trager and Jensen (17) in RPMI 1640 medium (Life Technologies Japan Co., Tokyo, Japan) supplemented with 0.5% AlbuMAX (Life Technologies Japan), 25 mM HEPES, 24 mM sodium bicarbonate, 0.5 g/liter L-glutamine, 50 mg/liter hypoxanthine, 25 μg/liter gentamicin, and human erythrocytes (from healthy Japanese volunteers) at a hematocrit level of 2%. Growth synchronization was achieved with 5% D-sorbitol (18). The outcome of the *in vitro* drug susceptibility test was assessed using the SYBR green I method (19). In brief, *P. falciparum*-infected erythrocytes were cultured with the standard method using a multigas incubator (5% O_2 and 5% CO_2). After reaching 1.5% ring-form parasitemia, the parasites were synchronized with 5% D-sorbitol for 30 min at room temperature and washed with RPMI 1640 medium twice by centrifugation at 1,000 × *g* for 5 min. Next, the erythrocytes were resuspended in the culture medium at 2% hematocrit. One hundred microliters of the erythrocyte suspension was then replaced in each well of a tissue culture plate (96-well flat-bottom plate; Corning Japan Co., Tokyo, Japan) in triplicate. For the CQ sensitivity test, chloroquine diphosphate (Sigma-Aldrich Japan Co., Tokyo, Japan) was added to the parasite culture (100 μl in total) in each well to give a series of dilutions from 1.25 to 10,240 nM. The sensitivity tests for 2-APB were performed as described for CQ. 2-APB (Sigma-Aldrich Japan Co.) was mixed with dimethyl sulfoxide (DMSO) (Sigma-Aldrich Japan Co.) before being added to the culture to give a series of dilutions from 25 to 200 μM. The simultaneous addition of the two compounds was performed by adding 50 μM 2-APB to the serially diluted CQ. After 72 h of incubation, each test plate was removed from the incubator, and 100 μl of lysis buffer (130.1 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.016 % [wt/vol] saponin, and 1.6 % [vol/vol] Triton X-100) containing SYBR green I (Life Technologies Japan) (2× final concentration) was added directly to each well in the plates and gently mixed. The plates were then covered with aluminum foil and incubated for another 24 h at room temperature in the dark. The relative fluorescent units per well were determined using a Fluoroskan Ascent (Thermo Fisher

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Address correspondence to Shin-ichiro Kawazu, skawazu@obihiro.ac.jp.

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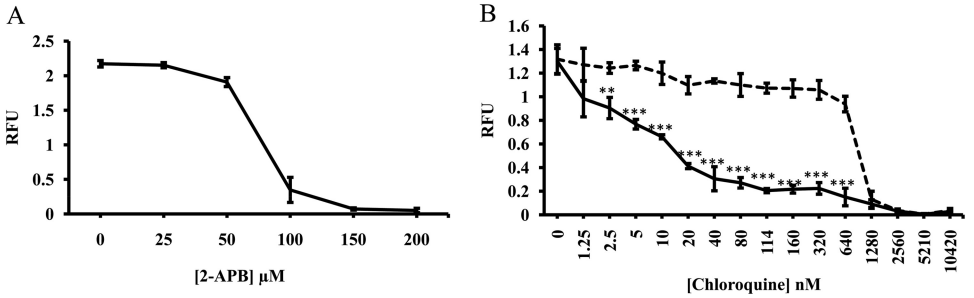

FIG 1 Dose-dependent activities of 2-APB and chloroquine (CQ) on *P. falciparum* synchronized cultures of CQ-resistant K-1 strain. Shown are various concentrations of 2-APB (A) and CQ plus DMSO as solvent control (dashed line) and CQ plus 50 μM 2-APB (solid line) (B). The statistical significance of the differences between the treatments was assessed with Student's *t* test. **, $P < 0.004$, and ***, $P < 0.0005$. The results are presented as the mean ± SD from three independent experiments. RFU denotes relative fluorescence units in SYBR green I assay (see Materials and Methods).

Scientific K.K., Yokohama, Japan), with the excitation and emission wavelength bands set at 485 and 530 nm, respectively. The concentration of antimalarial drug inhibiting parasite growth by 50% (IC_{50}) was calculated using the probit method, as described previously (20). The mean ± standard deviation (SD) IC_{50}s of 2-APB and CQ were determined to be 73.5 ± 3 μM and 1,050 ± 95 nM, respectively (Fig. 1A and B). The addition of the suboptimal dose of 2-APB (50 μM) to the series of CQ concentrations, which causes a minimum effect on the growth of the parasite *in vitro* (Fig. 1A), reversed CQ resistance and resulted in a significant decline in the IC_{50} (mean ± SD) from 1,050 ± 95 nM to 14 ± 2 nM (Fig. 1B). Similar experiments with the CQ-resistant *P. falciparum* Dd2 strain showed results comparable to those seen in *P. falciparum* K-1 (data not shown).

The strategy was then applied to the malaria mouse infection model, as previously described (21), with some modifications. Briefly, 6-week-old female ICR mice (CLEA Japan, Tokyo, Japan; 3 mice/group) were infected with the CQ-resistant *P. chabaudi* AS(30CQ) strain by an intraperitoneal injection of 5×10^6 parasitized erythrocytes. 2-APB (at 0.1 and 1 mg/kg of body weight) and CQ (3 mg/kg) were administered separately (via intraperitoneal injection) to different groups of mice to assess their antimalarial activities at days 0, 1, and 2. Thin blood films were prepared at days 1 to 4 and stained with Giemsa. The number of parasitized erythrocytes per 10,000 erythrocytes in each stained preparation was counted, with the mean values obtained from 3 preparations used as an index of parasitemia (%). Antimalarial activity was evaluated at day 4 as follows: antimalarial activity = (parasitemia in the compound-treated group)/(parasitemia in DMSO control group). The results showed that 1 mg/kg 2-APB exhibited an antimalarial effect but 0.1 mg/kg 2-APB did not (Fig. 2A). The potential of 2-APB to reverse CQ resistance was evaluated at day 4 as follows: antimalarial activity = (parasitemia in the two-compound-treated group)/(parasitemia in the CQ-treated group). The results showed that the simultaneous intraperitoneal injection of a lower concentration of 2-APB (0.1 mg/kg), which presented minimal antimalarial activity, produced a CQ resistance-reversing effect in the mice (Fig. 2B). It is noteworthy that the potency of 2-APB as a reverser of CQ resistance was equivalent to that of verapamil (Wako Chemical Co, Osaka, Japan) (at 20 mg/kg via intraperitoneal injection) (21). Verapamil is the first Ca^{2+} channel antagonist that has been reported to exhibit a resistance-reversing effect in CQ-resistant *P. falciparum* (22). These outcomes suggest the complete reversal of CQ resistance with 2-APB *in vitro* and *in vivo*; this is probably due to the disturbance of Ca^{2+} homeostasis in the parasite cell. To the best of our knowledge, this is the first observation of a CQ resistance-reversing effect induced by an IP_3 receptor inhibitor in the malaria parasite.

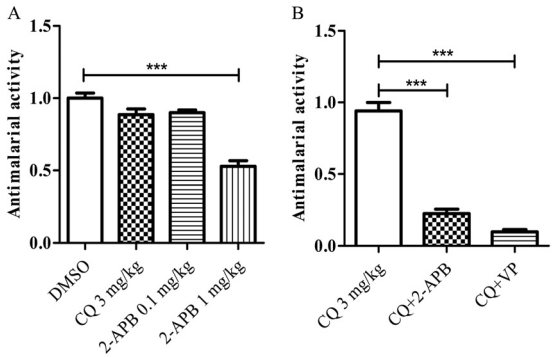

FIG 2 *In vivo* activities of chloroquine (CQ) and 2-APB on CQ-resistant *P. chabaudi* AS(30CQ) strain (A), and the potential of 2-APB in reversing CQ resistance (B). The antimalarial activity of each experimental group is shown as the ratio of parasitemia relative to the control (DMSO in panel A and CQ 3 mg/kg in panel B). (B) CQ plus 2-APB and CQ plus verapamil (VP) represent 3 mg/kg CQ plus 0.1 mg/kg 2-APB and 3 mg/kg CQ plus 20 mg/kg VP, respectively. The statistical significance of the differences between the groups ($n = 3$) was assessed with one-way analysis of variance (ANOVA), followed by Dunnett's test. ***, $P < 0.0001$ versus the control group.

The present study provides a novel strategy, which mainly targets Ca^{2+} homeostasis through the IP_3 pathway for Ca^{2+} release, which is critical for the blood-stage development of the parasite (13). 2-APB and other functionally related compounds that block the IP_3 pathway might be promising candidates as leads in the search for novel resistance-reversing agents. We anticipate that future studies will be undertaken to develop a 2-APB analogue that selectively affects Ca^{2+} homeostasis in the parasite cell.

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