Antimalarial drugs impact chemical messenger secretion by blood platelets

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ABSTRACT

Background: Advances in antimalarial drug development are important for combating malaria. Among the currently identified antimalarial drugs, it is suggested that some interact directly with the malarial parasites while others interact indirectly with the parasites. While this approach leads to parasite elimination, little is known about how these antimalarial drugs impact immune cells that are also critical in malarial response.

Methods: Herein, the effects of two common antimalarial drugs, chloroquine and quinine, on platelets were explored at both the bulk level, using high performance liquid chromatography, and the single cell level, using carbon-fiber microelectrode amperometry, to characterize any changes in chemical messenger secretion.

Results: The data reveal that both drugs cause platelet activation and reduce the number of platelet exocytosis events as well as delay fusion pore opening and closing.

Conclusions: This work demonstrates how chloroquine and quinine quantitatively and qualitatively impact in vitro platelet function.

General significance: Overall, the goal of this work is to promote understanding about how antimalarial drugs impact platelets as this may affect antimalarial drug development as well as therapeutic approaches to treat malarial infection.

1. Introduction

Malaria is an infectious disease caused by the Plasmodium parasites that are transmitted through the bites of infected female Anopheles mosquitoes [1]. Five known Plasmodium species cause malaria in humans, with the most prevalent and dangerous being Plasmodium falciparum (mostly found in sub-Saharan Africa) and Plasmodium vivax (mostly found in South America, Asia, and Oceania) [1]. Overall, there were an estimated 219 million cases of malaria in 90 countries and 435,000 deaths in 2017, with high Plasmodium falciparum infection incidence rate being in regions of Africa, South-East Asia, the Eastern Mediterranean, and the Western Pacific regions, and high Plasmodium vivax being in region of the Americas [1]. Though there has been recent progress toward a vaccine, current treatments for malaria rely on antimalarial drugs, with the primary aim being elimination of the malarial parasites [2].

Although much of the action regarding antimalarial drugs and malarial parasite elimination have been well-characterized, few studies have been done on the effects of antimalarial drugs on critical immune cells. Of interest here are platelets, anuclear cell bodies circulating in large numbers within the bloodstream, which are known for their roles in multiple defense mechanisms and wound healing [3]. The role of platelets in immune response involves the release of chemical messengers from their alpha, dense, and lysosomal granules to activate other immune cells, such as leukocytes, and amplify the immune response toward malarial parasite destruction and clearance [4]. Thus, it is hypothesized that antimalarial drugs may also have an off-target effect on platelets, a key component in the immune response, and will influence their critical delivery of chemical messengers.

The work herein utilized both bulk platelets and single platelet measurements to gain deep biophysical understanding about how platelets, from two model species, are affected by different antimalarial drugs: chloroquine (CQ) and quinine (QN). The mouse model is widely used in malaria studies and is, thus, a reasonable model to study the effect of antimalarial drugs on platelets. Previous work exploring the role of platelets in malaria have used the mouse model [4–8]. Furthermore, although platelets are known to share common features, functional responses are known to be different among different species [9]. As such, both mouse and rabbit platelets were studied herein to investigate the generality of any impacts the antimalarial drugs may have on platelets. The two analysis methods used are high performance liquid chromatography (HPLC) coupled to an electrochemical detector, which focuses at the bulk cell level to quantitatively understand general platelet population information, and carbon-fiber...
microelectrode amperometry (CFMA) at the single cell level to quantitatively and qualitatively understand biophysical heterogeneity within the platelet population. Both methods take advantage of the redox activity of the serotonin, or 5-hydroxytryptamine (5-HT), that is found in dense granules [10–12]. HPLC coupled to an electrochemical detector was used to probe for bulk platelet exocytosis following drug treatment of naïve platelets to give information regarding how the drugs influence cell-cell interactions that are critical during immune response. Results suggest that the tested drugs did not inhibit platelet activation as indicated in the literature [13,14], but rather enhanced it. In CFMA, the small size of the microelectrode and fast time resolution (sub-millisecond) [15,16] enables study of real-time chemical messenger release from individual granules from single platelets [11], neuroendocrine cells [17], or mast cells [18].

Herein, CFMA was used to explore how common antimalarial drugs influenced platelet secretory machinery. Results indicate that both drugs reduce the number of granule fusion events and delay fusion pore opening and closing based on measured 5-HT release. Quantitative information from the bulk platelet measurements was shown to agree well with averaged information from a population of single cell measurements [19,20], suggesting that the antimalarial drugs QN and CQ do not selectively act on different subpopulations of platelets or platelet granules.

2. Materials and methods

2.1. Platelet and drug preparation

Blood from three mice and two rabbits were collected according to IACUC protocol #1702-34571 A and protocol #1610-34243 A, respectively. The number of biological replicates for the rabbits was based on limitations in approved IACUC protocols, though control results from the rabbit platelets are in good agreement with previous work when the rabbits used were, in fact, distinct biological replicates. Mouse blood was collected via cardiac puncture into pre-filled syringes with acid citrate dextrose solution (11.6 mg/L sodium octyl sulfate, 170 μM dibutylamine, 55.8 mg/L Na₂EDTA, 10% methanol, 203 mg/L anhydrous sodium acetate, 0.1 M citric acid, and 120 mg/L sodium chloride). The chromatographic technique works using a C18 column to separate di-phosphate and quinine dihydrochloride were prepared to find final concentrations of 0, 0.1, 10, and 1000 μM in Tyrode’s buffer for HPLC bulk cell studies and to 1000 μM for CFMA single cell studies. 0 – 10 μM concentrations were chosen based on a biologically relevant range [21,22] with the 1000 μM concentration chosen to see what effects can be observed at high, non-physiological concentrations. The highest concentration was chosen for single cell studies based on the effects observed in bulk cell studies. All drug concentrations were compared to the 5 U/mL thrombin (positive control).

HPLC analysis of prepared standards and samples were run according to a previously established protocol [23]. A series of 5-HT standards were prepared in 0.5 M perchloric acid to final concentrations of 0.0625, 0.125, 0.250, 0.500, and 1.00 μM with 5.00 μM dopamine added as an internal standard. A total of 125 μL of prepared platelet samples were combined with 125 μL of drug (diluted in Tyrode’s buffer) or a negative control (Tyrode’s buffer) and incubated for 1.5 h at 37 °C. Following incubation, samples were centrifuged at 800 × g, and the supernatant was saved for further analysis. Pelleted platelets were re-suspended in Tyrode’s buffer to 125 μL and incubated with 5 U/mL of thrombin (Sigma-Aldrich) for 1 h at 37 °C. Then, all samples including the lysis products were filtered using a Millipore 96 well Multi Screen HTS filter plate with a 0.45 μm pore and centrifuged for 5 min. All filtered samples were run on an Agilent 1200 HPLC with an auto sampler containing a 5 μm, 4.6 × 150 mm C18 column (Eclipse XDB-C18) attached to a Waters 2465 electrochemical detector with a glassy carbon-based electrode. The working potential was set at +700 mV vs. an in situ Ag/AgCl reference electrode with a current range up to 50 nA. Samples were separated in the HPLC column at a flow rate of 2 mL/min in an aqueous mobile phase mixture (11.6 mg/L sodium octyl sulfate, 170 μL/L dibutylamine, 55.8 mg/L Na₂EDTA, 10% methanol, 203 mg/L anhydrous sodium acetate, 0.1 M citric acid, and 120 mg/L sodium chloride). The chromatographic technique works using a C18 column to separate different platelet exocytosis products based on their interaction strength with the stationary phase; then, the redox-active chemicals can be detected electrochemically (Fig. 1A). Based on calibration with a series of 5-HT standards, the integrated peak area at ~2 min can be used to quantify

Fig. 1. Schematic diagram of HPLC set up for bulk platelet chemical messenger detection. (A) The bulk platelet exocytosis products were injected and separated on the C18 HPLC column and then detected electrochemically at + 0.7 V vs. Ag/AgCl. (B) The relationship between peak area and 5-HT concentration was characterized in advance to find the concentration of released serotonin after drug stimulation of bulk platelets.
the released 5-HT from platelets under various conditions (Fig. 1B). Neither CQ nor QN interfere with detection of 5-HT at +700 mV vs Ag/AgCl.

2.3. Single cell CFMA experiments

Carbon-fiber microelectrodes were fabricated in the laboratory as described in previous literature [24]. Prior to usage, the electrodes were polished to 45° on a diamond polishing wheel (Sutter Instruments) and stored in isopropyl alcohol. The electrodes were backfilled with electrolyte solution (3.0 M potassium acetate and 30.0 mM potassium chloride) prior to being mounted on a platinum-coated silver wire (Squires Electronics) situated on a headstage mounted on an Axon Instruments Axopatch 200 B potentiostat (Molecular Devices Inc.) using low-pass Bessel filtering (5 kHz), a sampling rate of 20 kHz, and gain amplification of 20 mV/pA controlled by locally written LabVIEW software and National Instruments data acquisition boards. This potentiostat controlled the applied voltage at +700 mV vs. a silver/silver chloride (Ag/AgCl) reference electrode (BASi). A pulled glass capillary micropipette loaded with 5 U/mL of thrombin (Sigma-Aldrich) connected to a Picospritzer III (Parker Hannifin) was used to control delivery of the stimulating thrombin solution to the cells. Both the headstage of the mounted electrodes and micropipette were mounted on Burleigh PCS-5000 piezoelectric micromanipulators (Olympus America Inc.) to control electrode and stimulant placement relative to individual platelets. A drop of platelet suspension was placed onto a poly-l-lysine-coated glass coverslip containing Tyrode’s buffer. Individual platelets were visually monitored using an inverted microscope equipped with phase contrast optics (40× magnification) (Nikon Instruments, Melville, NY). Upon the start of a measurement, the stimulant was applied to a 3 s dose of the stimulant, inducing platelet degranulation. The current was recorded with time resolution of milliseconds so that it was possible to compare the real-time secretion behavior after different drug treatments. The carbon-fiber microelectrode, which is large enough to cover the entire diameter of the platelet, was placed close to the platelet surface so that, after stimulation, the released 5-HT molecules were oxidized and detected by the electrode (Fig. 2). Further analysis of current versus time traces for individual cells provides both quantitative and qualitative information to allow comparison of platelet exocytosis events.

Collected amperometric traces were processed using Tar Heel software (courtesy of Dr. Michael Heien), processed at 200 Hz with a low-pass Bessel filter, and analyzed using Minianalysis software (Synaptosoft, Inc.) with the signal-to-noise ratio set at 2. Individual current spikes within an amperometric trace represent individual granular release events. For each platelet trace, the area under each spike (Q), number of spikes per platelet (N), and time at full-width half-maximum of each spike (T$_1/2$) were measured, averaged, and statistically analyzed for outliers. Additional analyzed parameters consisted of 1) the time from 10% to 90% of the peak current on the rising phase (T$_{rise}$), 2) the time from 90% to 10% of peak current on the decay phase (T$_{decay}$), and 3) the total 5-HT molecules released from an individual platelet. Since the oxidation of 5-HT involves a two electron transfer, the total 5-HT molecules released per platelet measurement was calculated using Faraday’s Law after taking the product of Q and N. If a cell was a statistical outlier for any analyzed parameter, the trace was eliminated altogether. Statistical determination of outliers included taking the log of the individual average spike parameter, obtaining the average from the individual log parameter, determining the standard deviation from the log parameter, and discarding any amperometric traces whose log value fell outside two standard deviations of the average of the individual log parameter. Experimental condition averages were calculated for each spike parameter using one-way ANOVA with 95% confidence level as threshold for statistical significance.

3. Results and discussion

3.1. Effects of chloroquine and quinine on bulk platelets secretion of 5-HT

Literature on the effects of CQ suggests that the drug inhibits platelet aggregation through the interaction between CQ and membrane glycoproteins and receptors, as well as the binding between CQ and membrane phospholipids [25]. Likewise, QN has been shown to affect platelet structure by perturbing the membrane as well as function through aggregation [26,27]. To characterize the effects of CQ and QN on platelets beyond aggregation, HPLC was used to quantitatively determine secretion of 5-HT following varied treatment with CQ or QN; these data were used to choose the drug concentration to be used for the single cell studies. Furthermore, both drugs were explored for their abilities to either promote or prevent platelet activation via 5-HT secretion following thrombin stimulation. Although there is no known role for 5-HT secretion in the presence of CQ and QN, in this work, 5-HT is a measurable species that is co-released with other granule chemical messengers during platelet activation. Thus, 5-HT secretion is used as a proxy for other platelet chemical messengers such as platelet factor 4 that have known roles in malaria and are co-localized and co-released with 5-HT to understand how platelet function is affected [6,28].

In the first set of experiments, the ability of each drug, at various concentrations, to directly activate platelets was explored. The data...
show that both drugs (at 1 mM) are able to induce 60–80 times greater platelet release of 5-HT for both mouse and rabbit platelets when compared to the non-drug-treated negative control conditions; also, CQ induces nine times greater release of 5-HT from rabbit platelets at doses as low as 10 μM (Fig. 3A). Although previous morphology studies on mouse and rabbit platelet aggregates showed no differences among platelets from the two species [29], the species likely possess different receptors or functional responses, resulting in the observed sensitivity differences. In the second set of experiments, platelets incubated in drugs were monitored for their ability to promote or prevent further platelet activation via thrombin. Results confirmed that platelets from 1 mM CQ or QN conditions had indeed been activated previously, as indicated by significantly lower secreted 5-HT vs. control (pre-incubation only in Tyrode’s buffer) (Fig. 3B). In all cases but one, pre-exposure of platelets to CQ and QN appears to mitigate or not impact further activation by thrombin. The one outlier was in the case of rabbit platelets, where pre-exposure to 0.1 μM CQ led to a two-fold enhancement in released 5-HT vs. control (pre-exposure to buffer). (Fig. 3B). Overall, the results suggest that the secretion efficiency of 5-HT from platelets and activation upon thrombin stimulation are enhanced rather than inhibited upon low concentration of pre-CQ exposure, whereas no effects are observed from low concentration of pre-QN exposure, and the low 5-HT release observed with the highest CQ and QN concentration is likely due to direct drug-induced platelet activation.

3.2. Effects of drugs on single platelet secretion of serotonin

From bulk cell studies, it was clear that 1 mM CQ and QN were able to directly activate both mouse and rabbit platelets. Herein, the effect of the 1 mM drug stimulation was explored at the single cell level using CFMA to provide quantitative, high time-resolution information to complement the HPLC data and qualitative understanding about the action of the secretion machinery during platelet exocytosis. Additionally, the high concentration of drugs was chosen for CFMA considering the short exposure time of platelets with the drugs into a large volume during the 3-s stimulation and drug diffusion in the CFMA setup. These conditions were chosen to ensure that platelets undergo full exocytosis in the positive control. Because we hypothesized that the effects from the drug incubation on the platelets would occur ahead of thrombin stimulation, the intense single cell exocytosis measurements show the end result of those impacts. Here, no pre-drug incubation studies were completed as pre-treatment with drugs caused platelet activation before CFMA studies, and all drug-based stimulations were compared to stimulation by thrombin as the positive control. Concerns about potential apoptosis of platelets based on high drug concentrations [30] were allayed by the fact that the single cell platelet response rates to stimulation were not decreased compared to naïve controls.

Representative traces show the character of platelet secretion following drug-induced activation; in all cases, the drugs do induce secretion albeit showing a smaller response than induced by thrombin (Fig. 4) and a larger response than measured from unstimulated platelets or platelets stimulated only with buffer (Fig. S1). Detailed analysis of all individual spikes from each single cell measurement indicated that platelets from both mouse and rabbit species responded similarly to stimulation by QN vs. control, with slight differences observed for CQ-based stimulation vs. control (Table 1) (also see supplemental information, Fig. S2 and Fig. S3 for detailed individualized parameters). From CQ stimulation on mouse platelets vs. control, there were 58% fewer exocytosis events, 78% fewer total 5-HT molecules released, and 55% longer pore opening time; whereas, CQ stimulation on rabbit platelets vs. control showed 99% higher average charge from each exocytosis event, 107% longer time at full width half max, and 109% longer pore closing time. From QN stimulation on mouse platelets vs. control, there were 38% fewer exocytosis events, 53% fewer 5-HT molecules secreted, 49% longer time at full width half max, and 57% longer pore closing time; whereas from QN stimulation on rabbit platelets vs. control, there were, statistically significant, 61% fewer exocytosis events, 61% fewer 5-HT molecules secreted, 74% longer time at full width half max, and 76% longer pore closing time. Based on previous studies, mouse and rabbit platelets are known to present different concentrations of phospholipids, cholesterol, and SNARE proteins within their cell membranes to assist with the exocytosis process [9]; it is likely these differences that account for the differences in pore stability observed between the two species.

Overall, results from CQ and QN support that platelets are being activated by the drugs, although less intensely than that achieved with thrombin (5 U/mL) used as the positive control. The significant differences in $T_{1/2}$ and $T_{\text{decay}}$ include contributions from the expansion/ dissolution of the inorganic polyphosphate matrix [31] in the platelet dense granules as granule-bound 5-HT is secreted as well as diffusion of oxidized 5-HT away from the working electrode and platelet pore closing. Additional analysis on the mode of platelets exocytosis were explored by examining “foot events”, current shoulders that sometimes occur before or after full fusion current spikes. This analysis shows
slight variation in pre- and post-foot features among the two different species (supplemental information, Fig. S4 and Fig. S5); these data give insight into how long the platelet maintains a small diameter fusion pore before it dilates to allow maximal chemical messenger secretion. The observable differences seen with CQ and QN-induced platelet activation of both mouse and rabbit platelets emphasized that, although both model species do share common features, there are subtle functional differences among them that are model specific. Although both CQ and QN drugs can cause platelet activation as indicated by both bulk and single cell studies at high drug concentrations, mouse and rabbit platelets respond differently to CQ at lower concentrations (0.1 and 10 μM). Additionally, this difference was also apparent when considering the functional responses obtained from the single cell studies with 1 mM CQ. Clearly, regardless of model species or antimalarial drug, the drug shows potential off-target impacts on platelets. These in vitro results do not necessarily reflect in vivo off-target effects but do suggest that they should be considered during future in vivo studies with antimalarial drugs.

4. Conclusion

Overall results show that the tested antimalarial drugs, CQ and QN, impact naïve platelets and influence their critical delivery of chemical messengers, an important characteristic to keep in mind during malaria treatments. The nuance of the drug effects in these bulk studies shows the importance of using different species to study the effects of antimalarial drugs. Furthermore, this work shows the importance of considering platelet behaviors beyond aggregation to understand the effects of antimalarial drugs on platelets for drug development as the findings here suggest that the drugs induce platelet activation.

Author contributions

Kang Xiong-Hang: Conceptualization, Methodology, Formal Analysis, Investigation, Writing- Original draft preparation, Visualization Jiayi He: Methodology, Investigation, Writing- Original draft preparation, Visualization Kaila Kemnetz-Ness: Validation, Investigation. Christy L. Haynes: Conceptualization, Writing – Critical Review & Editing, Supervision, Funding Acquisition.

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Table 1

| Parameter         | Mouse | Rabbit |
|-------------------|-------|--------|
|                   | Thrombin | CQ | QN | Thrombin | CQ | QN |
|                   | (n = 23) | (n = 24) | (n = 26) | (n = 20) | (n = 24) | (n = 23) |
| Avg Qspike (fC)   | 170.50 ± 23.37 | 176.80 ± 36.08 | 141.80 ± 11.51 | 129.50 ± 15.82 | 258.00 ± 40.14* | 134.00 ± 28.45 |
| Total spikes      | 232 | 102 | 162 | 297 | 216 | 134 |
| Avg spikes        | 10.1 ± 1 | 4.3 ± 0.6*** | 6.2 ± 0.6** | 14.9 ± 2.8 | 9.0 ± 1.6 | 5.8 ± 0.6** |
| Total 5-HT (10^6 molecules) | 5.33 ± 0.86 | 1.20 ± 0.39*** | 2.53 ± 0.22** | 5.53 ± 1.09 | 5.30 ± 0.80 | 2.18 ± 0.34** |
| Avg Trise (ms)    | 14.03 ± 1.17 | 21.75 ± 2.57* | 20.89 ± 1.40* | 11.42 ± 0.84 | 23.59 ± 2.36**** | 19.91 ± 1.92** |
| Avg Tdecay (ms)   | 6.63 ± 0.64 | 9.12 ± 1.37 | 9.43 ± 1.14 | 6.06 ± 0.52 | 8.94 ± 1.13 | 6.95 ± 0.75 |
| Avg T1/2 (ms)     | 13.37 ± 1.17 | 18.46 ± 2.22 | 20.41 ± 1.22** | 10.17 ± 0.81 | 21.24 ± 1.84**** | 17.64 ± 1.45** |
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100758.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100758

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