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Quinine localizes to a non-acidic compartment within the food vacuole of the malaria parasite *Plasmodium falciparum*

Elaine B Bohórquez¹, Michael Chua² and Steven R Meshnick^{3*}

Abstract

Background: The naturally fluorescent compound quinine has long been used to treat malaria infections. Although some evidence suggests that quinine acts in the parasite food vacuole, the mechanism of action of quinine has not yet been resolved. The *Plasmodium falciparum multidrug resistance (pfmdr1)* gene encodes a food vacuolar membrane transporter and has been linked with parasite resistance to quinine. The effect of multiple *pfmdr1* copies on the subcellular localization of quinine was explored.

Methods: Fluorescence microscopy was used to evaluate the subcellular localization of quinine in parasites containing different *pfmdr1* copy numbers to determine if copy number of the gene affects drug localization. The acidotropic dye LysoTracker Red was used to label the parasite food vacuole. Time-lapse images were taken to determine quinine localization over time following quinine exposure.

Results: Regardless of *pfmdr1* copy number, quinine overlapped with haemozoin but did not colocalize with LysoTracker Red, which labeled the acidic parasite food vacuole.

Conclusions: Quinine localizes to a non-acidic compartment within the food vacuole possibly haemozoin. *Pfmdr1* copy number does not affect quinine subcellular localization.

Keywords: Quinine, *Plasmodium falciparum*, *Pfmdr1*, Copy number

Background

Quinine (QN) was isolated from the bark of the Peruvian Cinchona tree in the mid-1800s [1] and quickly became the treatment of choice for intermittent fever worldwide [2]. Although efficacious, QN use as an anti-malarial requires a relatively long treatment regimen and has substantial side effects. As a result, artemisinin-based combination therapy (ACT) has now been implemented as first-line treatment regimens due to drug efficacy and better patient tolerance. However, QN remains an important treatment option for severe malaria infections [3-5]. Despite the longevity of its use as an anti-malarial, the mechanism of action of QN has not yet been fully resolved.

Early investigations into the anti-malarial activity of QN demonstrated the ability to inhibit chloroquine-induced clumping of haemozoin in the parasite food vacuole [6]. Subsequent evidence found that, like chloroquine, QN interferes with haem crystallization [7,8], indicating that QN acts in the food vacuole. In recent years, parasite resistance to QN has been reported in Africa and Southeast Asia, prompting investigations into resistance mechanisms with a focus on the food vacuole [4,9-13]. Decreased QN sensitivity has been strongly linked to a protein on the food vacuolar membrane, encoded by the *Plasmodium falciparum multidrug resistance (pfmdr1)* gene [10-12,14-16]. Whereas some studies have found that *pfmdr1* mutations exert a significant effect on *in vitro* QN sensitivity [10,13,14], other studies have found no association between *pfmdr1* mutations and QN sensitivity [17-19]. Elevated *pfmdr1* gene copy number, however, has been linked to reduced parasite susceptibility to QN in both *in vitro* and clinical

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studies [12,16,18-20]. Thus, *pfmdr1* has an effect on quinine activity.

Quinine is an aromatic alkaloid that naturally fluoresces under ultraviolet (UV) light. Because of its relatively constant fluorescence quantum yield, QN is commonly used in photochemistry as a fluorescence standard [21]. Surprisingly, the inherent fluorescence of QN has not previously been exploited to investigate its mechanism of action. In this study, the fluorescent properties of QN were exploited to evaluate subcellular localization of the drug by fluorescence microscopy in parasites containing different *pfmdr1* copy numbers in order to determine if copy number of the gene affects drug localization.

Methods

Parasite cultivation

Plasmodium falciparum cultures of two clonal parasite strains (3D7 and Dd2) with different *pfmdr1* copy numbers and quinine (QN) sensitivity were used in this study. The 3D7 strain has one *pfmdr1* copy and is sensitive to QN. The Dd2 strain has four *pfmdr1* copies [22] and is resistant to QN. Cultures were maintained in candle jars at 37°C under the Trager and Jensen method for malaria parasite culturing [23]. Media was changed every 24 hours. Type O+ serum at 10% and red blood cells (Research Blood Components, LLC: Boston, MA, USA) at 2% haematocrit in malaria culture media (MCM) [24] were used in culturing and experimental conditions.

Imaging of parasites using fluorescence microscopy

Live asynchronous cultures at 5-8% parasitaemia with 1µM of QN were imaged over an 11-hour period using fluorescence microscopy. Parasitized RBC preparations were plated onto 35mm glass bottom dishes (MatTek, Corp.: Ashland, MA, USA) coated with Poly-L-lysine. A coverslip was carefully applied, and the excess fluid gently squeezed out. The coverslip was sealed with warmed Vaseline to prevent the sample from drying out. Time-lapse imaging of multiple parasites was carried out with a Prior Scientific (Prior Scientific: Rockland, MA, USA) motorized x-y stage. An incubator box maintained the microscope work area including the objective at 37°C. Because *P. falciparum* grows well at 3% oxygen but will tolerate levels as low as 0.5% [25], air was in the incubator box, and the samples were sealed during imaging as stated above. LysoTracker Red DND-99 (Invitrogen: Carlsbad, CA, USA) (50nM) was used to stain the food vacuole and DRAQ5 (Biostatus Limited: Leicestershire, UK) (1µM) was used to stain DNA [26]. Fluorescence images were acquired using an Olympus FV1000 inverted IX81 microscope with a 63x 1.42 NA oil immersion objective at the UNC-Chapel Hill Michael Hooker Microscopy Core Facility. Serial sections with

step sizes of 0.20 or 0.25µm were gathered using sequential scanning at 405nm (QN), 559nm (LysoTracker Red), and 635nm (DRAQ5) excitation, and 435±25nm, 595±25nm and 705±50nm emission filters for QN, LysoTracker Red, and DRAQ5 respectively. Confocal pin-hole was set to maximum (800µm, ~8 Airy units). Laser transmitted light differential interference contrast (DIC) images were collected at an optimum z-level separately. Image stacks were deconvolved using the iterative blind deconvolution method with Volocity Image Analysis software, version 5.52 (Perkin Elmer: Waltham, MA, USA). Occasionally, Tetraspec 0.1µm beads (Molecular Probes, OR) were added to the sample and used for deconvolution with measured point spread functions. Three-dimensional (3-D) opacity volume rendering and 3-D co-localization analysis was carried out with Volocity.

Results

Quinine localizes to the parasite food vacuole

Two-dimensional (2-D) fluorescence and DIC images of both 3D7 (one *pfmdr1* copy) and Dd2 (four *pfmdr1* copies) parasites showed that quinine (QN) co-localizes with haemozoin, which suggests localization in the parasite food vacuole (Figure 1). Similar results were found using a Leica SP2 microscope with a 351nm excitation laser (data not shown). The co-localization was highly repeatable and seen in four fields in at least 10 experiments. Parasites were also imaged at earlier time points following the addition of QN (beginning at one hour of exposure through 11 hours). Quinine fluorescence was found in the food vacuole as early as one hour of exposure and remained there for the duration of the experiment (Figure 2). This effect appears to be independent of *pfmdr1* copy number as experiments in both strains rendered similar results. The same pattern was seen regardless of whether the infected red cells were maintained on the heated microscope stage or in a candle jar, indicating that photobleaching was not a factor. The parasites did not significantly progress through the intraerythrocytic cell cycle during this 11-hour period consistent with a previously observed maturation delay in the intraerythrocytic cell cycle following QN exposure at IC50 and IC99 concentrations for 12 hours [27]. Thus, the apparent halt in cell cycle progression observed in the current study is likely due to the previously reported QN-induced maturation delay phenomenon.

To further characterize subcellular localization of QN, co-localization of QN with either LysoTracker Red, which localizes to acidic compartments, or DRAQ5, which localizes to the nucleus, was investigated using 3-D reconstruction of z-stack fluorescence images. As expected, no co-localization with DRAQ5 was seen. However, QN was found to localize in a separate but adjacent compartment from that in which LysoTracker Red was

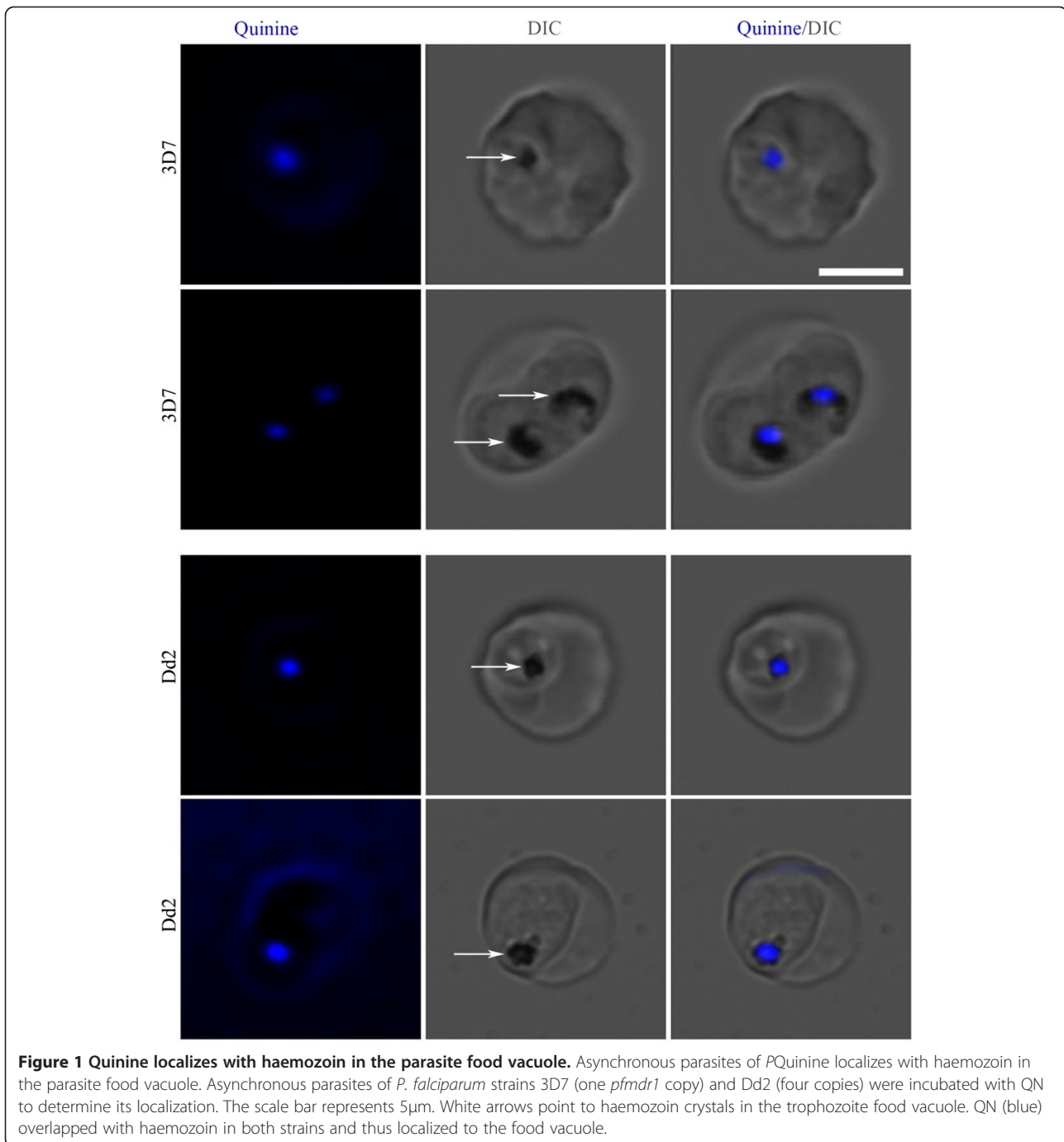


Figure 1 Quinine localizes with haemozoin in the parasite food vacuole. Asynchronous parasites of *P. falciparum* Quinine localizes with haemozoin in the parasite food vacuole. Asynchronous parasites of *P. falciparum* strains 3D7 (one *pfmdr1* copy) and Dd2 (four copies) were incubated with QN to determine its localization. The scale bar represents 5 μ m. White arrows point to haemozoin crystals in the trophozoite food vacuole. QN (blue) overlapped with haemozoin in both strains and thus localized to the food vacuole.

localized (Figure 3). This 3-D imaging method does not allow for haemozoin to be visualized. The pattern of colocalization was the same for 3D7 and Dd2 parasites, despite their differences in *pfmdr1* copy number. These observations suggest that QN localizes to a distinct, non-acidic compartment within the parasite food vacuole, possibly overlapping with haemozoin in parasites with both low and high *pfmdr1* copy number.

Discussion

Although QN was the first therapeutic compound used to treat malaria infection [1], its mechanism of action has never been fully resolved [6-8]. Some evidence suggests that parasite resistance to QN is associated with mutations and/or elevated copy number of the *pfmdr1* gene, which encodes for a transporter protein found in the membrane of the parasite food vacuole

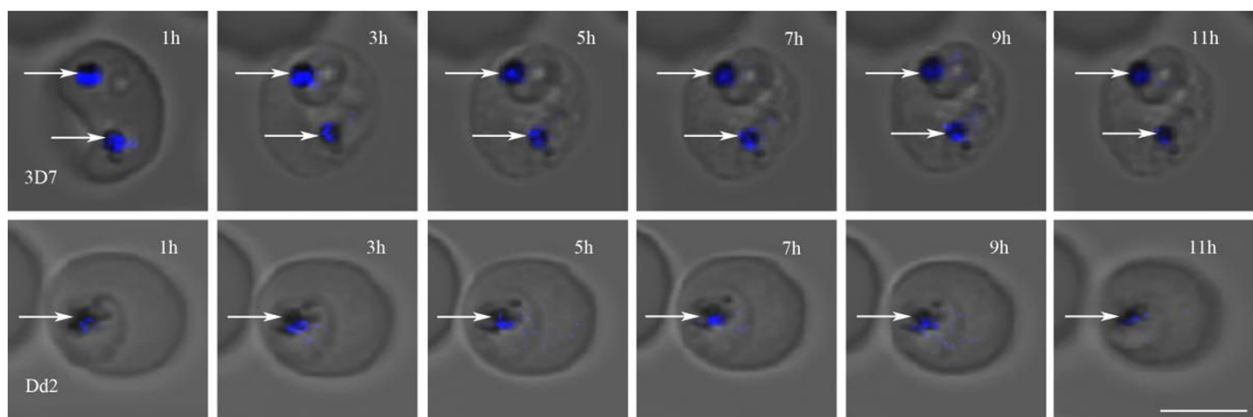


Figure 2 Quinine localization over time in *P. falciparum*. Asynchronous parasites were incubated with quinine (blue) to determine subcellular localization of the drug over an 11-hour period. White arrows point to haemozoin crystals in the food vacuole of trophozoites. Quinine overlapped with haemozoin crystals at all time points assessed. The scale bar represents 5µm.

[9,10,12-14,16,18,20]. Here, the natural fluorescent properties of QN were exploited to obtain insight into the mechanism of action of the drug. Although knowledge of QN's fluorescent properties has been around since the late-1800s [28], this is the first study to employ the QN's fluorescence for imaging in the malaria parasite.

Fluorescence microscopy was employed to image QN subcellular localization in two *P. falciparum* strains that contained different *pfmdr1* copy numbers. Quinine consistently overlapped with the haemozoin crystals in both strains when evaluated by 2-D microscopy (Figure 1). However, upon 3-D reconstruction of serial z-stack

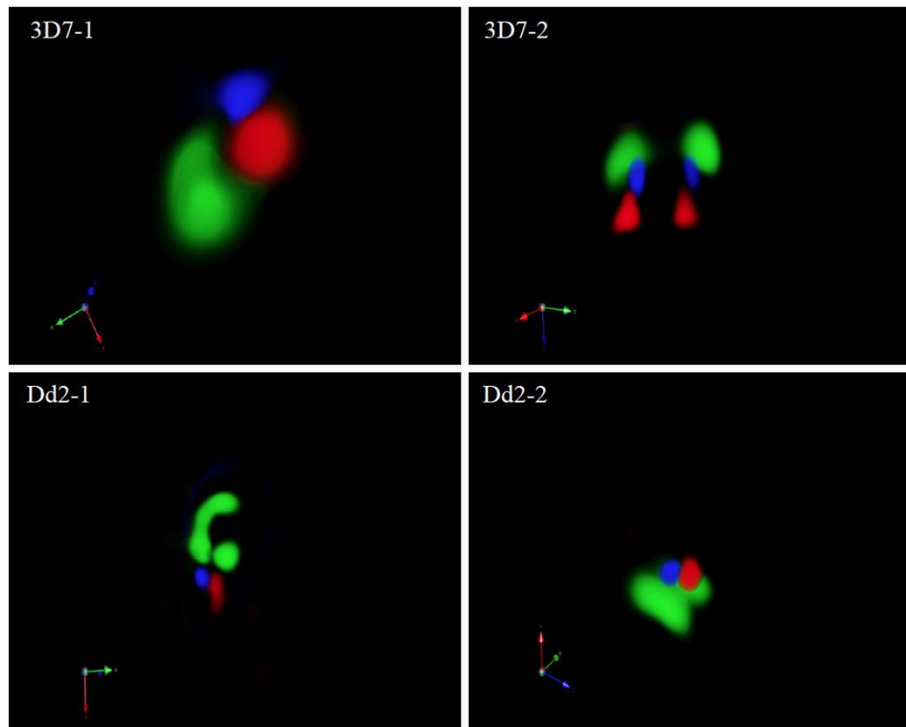


Figure 3 3-D reconstruction of *P. falciparum* parasites. Z-stack images of each parasite shown in Figure 1 were used for 3-D volume rendering and colocalization analysis. No QN colocalization with LysoTracker Red was observed in either the 3D7 (left) or the Dd2 (right) strain. Because LysoTracker Red is highly specific for acidic organelles, QN appears to be in a separate and non-acidic compartment of the food vacuole. QN is shown in blue, LysoTracker Red in red, and the DNA stain DRAQ5 in green.

images, QN was found to reside in a distinct compartment, which is contiguous to, but separate from, the compartment stained by LysoTracker Red. The lack of co-localization with the acidotropic dye suggests that QN resides in a non-acidic compartment within the food vacuole, possibly the same one occupied by haemozoin. This would be consistent with previous reports that quinolone compounds including QN interact with haemozoin crystals directly or with enzymes involved in the haemozoin crystallization process, as previously reported [7,8,29-33]. In summary, these findings suggest that QN is localized in a non-acidic compartment in the food vacuole, possibly that which contains haemozoin.

This study underscores the importance of utilizing the 3-D reconstruction software in imaging studies, since the localization of QN into this novel compartment would not have been detected otherwise. A recent study revealed that QN-haem adducts exhibit fluorescence at least seven-fold greater than QN alone [34]. Thus, it is possible that QN exists in other areas of the food vacuole but cannot be visualized due to the fluorescence intensity of the QN-haem adducts present in these parasites.

Although there was no apparent difference in localization in strains containing different *pfmdr1* copy numbers, the possibility that the *pfmdr1* gene has a role cannot be ruled out. Single nucleotide polymorphisms in *pfmdr1* have also been associated with decreased sensitivity to QN [10,13,14]. Because the protein encoded by *pfmdr1* is a membrane transporter that pumps solutes into the food vacuole, it is possible that mutations within the *pfmdr1* gene could affect the transporter function of the protein by altering the conformation or function of the transporter protein.

In summary, this study is novel because it is the first to exploit quinine fluorescence to study the intracellular distribution of the drug. Here, QN was shown to enter a distinct, non-acidic compartment inside the parasite food vacuole. These results are important because they provide visual support for the hypothesis that QN interferes with haemozoin production, which could guide future studies to investigate a possible interaction between QN and enzymes involved in the haemozoin formation process.

Conclusions

Quinine localizes to a non-acidic compartment within the food vacuole and *pfmdr1* copy number does not affect QN subcellular localization. These results support previous findings that QN acts in the parasite food vacuole, perhaps interfering with haemozoin formation. This is the first study to provide evidence for QN localization in a sub-compartment of the food vacuole.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EBB participated in the design of the study, carried out all of the fluorescence microscopy experiments and data analysis, and drafted the manuscript. MC participated in the acquisition and analysis of microscopy data. SRM conceived of the study and participated in its design. All authors read and approved the final manuscript.

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