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Mode of action of quinoline antimalarial drugs in red blood cells infected by *Plasmodium falciparum* revealed in vivo

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The most widely used antimalarial drugs belong to the quinoline family. Their mode of action has not been characterized at the molecular level in vivo. We report the in vivo mode of action of a bromo analog of the drug chloroquine in rapidly frozen *Plasmodium falciparum*-infected red blood cells. The *Plasmodium* parasite digests hemoglobin, liberating the heme as a byproduct, toxic to the parasite. It is detoxified by crystallization into inert hemozoin within the parasitic digestive vacuole. By mapping such infected red blood cells with nondestructive X-ray microscopy, we reveal that bromoquine caps hemozoin crystals. The measured crystal surface coverage is sufficient to inhibit further hemozoin crystal growth, thereby sabotaging heme detoxification. Moreover, we find that bromoquine accumulates in the digestive vacuole, reaching submillimolar concentration, 1,000-fold more than that of the drug in the culture medium. Such a dramatic increase in bromoquine concentration enhances the drug’s efficiency in depriving heme from docking onto the hemozoin crystal surface. Based on direct observation of bromoquine distribution in the digestive vacuole and at its membrane surface, we deduce that the excess bromoquine forms a complex with the remaining heme deprived from crystallization. This complex is driven toward the digestive vacuole membrane, increasing the chances of membrane puncture and spillage of heme into the interior of the parasite.

**Significance**

The most widely used antimalarial drugs belong to the quinoline family. The question of their mode of action has been open for centuries. It has been recently narrowed down to whether these drugs interfere with the process of crystallization of heme in the malaria parasite. To date, all studies of the drug action on heme crystals have been done either on model systems or on dried parasites, which yielded limited data and ambiguity. This study was done in actual parasites in their near-native environment, revealing the mode of action of these drugs in vivo. The approach adopted in this study can be extended to other families of antimalarial drugs, such as artemisinins, provided appropriate derivatives can be synthesized.

**Author contributions:** S.K., L.L., and J.A.-N. designed research; S.K. performed research; T.-S., Yang Yang2, J.L., A.J.P.-B., E.P., Yang Yang1, S.W., and P.G. contributed new reagents/analytic tools; S.K., L.L., and J.A.-N. analyzed data; S.K., L.L., and J.A.-N. wrote the paper; T.S. prepared malaria cultures and did ICP analysis; Yang Yang2 and J.L. synthesized bromoquine; A.J.P.-B., E.P., S.W., and P.G. facilitated and assisted in soft X-ray tomography collection; and Yang Yang1 facilitated and assisted in X-ray fluorescence measurements.

The authors declare no competing interest.

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between a quinoline molecule and 2 accessible heme propionic acid groups spanning 2 unit cells on the well-expressed \{100\} face. However, to date, all growth inhibition studies have been done on synthetic hemozoin.

Various approaches were employed to investigate whether and how such drugs inhibit hemozoin crystallization (9, 18, 19). In vitro studies monitored crystallization of synthetic hemozoin in the presence of drug molecules, whose affinity to the crystal surface and inhibition of crystallization was tested, but in media different from that of the parasite digestive vacuole (9, 20, 21).

A more direct mode of investigation involved localization of the drug ferroquine within the malaria parasite itself (22), albeit in a dried red blood cell.

In order to validate the proposed mechanisms of action of antimalarial quinoline drugs we raised the following questions. What is the concentration of the drug in the digestive vacuole of the parasite? Does the drug indeed bind to the hemozoin crystal faces and persist at these crystal surfaces in vivo? If so, would the crystal surface coverage by the drug be sufficient to inhibit regular heme dimer adsorption onto the crystal faces? Finally, can we detect or envisage binding of the drug to free heme?

With these considerations in mind, we undertook a correlated X-ray microscopy study to establish the mode of action of established drugs like chloroquine via its localization within hydrated malaria parasites in their native, albeit rapidly frozen, environment. We provide evidence that both mechanisms take place and argue that the interaction is sufficient to disrupt heme detoxification. We used bromoquine (BrQ), an analog of chloroquine (cf. Fig. 1A), to take advantage of the identifiable X-ray fluorescence signal of the bromine (Br) signal of BrQ. In order to assess the efficiency of hemozoin growth inhibition by capping with BrQ we measure the BrQ coverage on the surface of hemozoin crystals.

To provide evidence of strong attachment of BrQ to the hemozoin crystal surface we compare BrQ coverage of crystals within the digestive vacuole with those isolated from the parasites. We find a similar coverage in both cases, suggesting strong interaction between BrQ and the hemozoin crystal surface.

To validate that our calculations do not include background Br signal from culture medium, we analyze the Br distribution in BrQ-treated and BrQ-free samples. We find that background Br originating from bovine serum used to culture the parasites does not significantly enter the digestive vacuole.

We identify an increased concentration of BrQ at the membrane of the digestive vacuole. This can be explained by BrQ exceeding that in the culture medium. Within the digestive vacuole, BrQ caps the hemozoin crystal surface. Such a coverage, defined as the fraction of surface docking sites capped by BrQ and found to range between 4% and 15%, is sufficient to hinder the deposition of oncoming heme molecules. We also observed that BrQ covers hemozoin crystals isolated from the parasites to the same extent as in the digestive vacuole. This suggests a persistent binding of BrQ to the crystal surface. We deduce that BrQ forms a complex with isolated heme, which accumulates at the digestive vacuole membrane, possibly spreading to other parasitic membranes, leading to their disruption.

Based on these in vivo observations, we present in the closing words of this paper a model of the antimalarial mode of action by BrQ and, by extension, that of related quinolines.

Results

To verify the first hypothesis that quinoline drugs cap hemozoin crystals, we colocalize the iron (Fe) signal of hemozoin crystals with the bromine (Br) signal of BrQ. To assess the efficiency of hemozoin growth inhibition by capping with BrQ we measure the BrQ coverage on the surface of hemozoin crystals.

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complexion with free heme (see Discussion), thereby validating the second hypothesis that quinoline drugs interact with free heme.

Finally, to verify immediate availability of BrQ at the site of hemozoin nucleation and growth we measure concentration of BrQ within the digestive vacuole but not attached to hemozoin crystals. We find it accumulates in the digestive vacuole at a thousandfold higher concentration than in the culture medium, confirming previously reported estimates (25–27).

**BrQ Caps Hemozoin Crystals in Infected Red Blood Cells.** A red blood cell, which happened to be invaded by 2 parasites (Fig. 1), was treated with BrQ drug 30 h after infection. The cell was non-destructively imaged by soft X-ray cryotomography with the photon energy within the so-called water window range in which oxygen atoms are practically transparent and carbon atoms are heavily absorbing. This generates strong contrast between lipid, cytoplasm, and hemozoin crystals (Fig. 1B), enabling direct tomographic mapping of cellular structure within entire parasites without the need for staining.

A sample with infected red blood cells, frozen in 20-μm-thick ice, was mapped by soft X-ray cryotomography. From the tomographic data it was possible to render a complete 3-dimensional (3D) picture of an infected red blood cell, shown in Fig. 1C in artificial colors.

The cell was subsequently mapped by X-ray fluorescence cryomicroscopy. This involves raster scanning with a 30-nm-diameter hard X-ray beam, which causes fluorescent X-rays to be emitted uniformly in all directions, with an energy spectrum reflecting the element distribution in the irradiated volume. The scan provides a distribution map for each atomic element, but here we consider only the contributions of Fe and Br as shown in Fig. 1D and E. The similarity between the Fe map in Fig. 1D and the calculated soft X-ray projection in Fig. 1B enables an unambiguous association between Fe and Br distribution and structure of parasite 1, including hemozoin crystals and its digestive vacuole. Clearly, large Br concentrations are located precisely in the same regions as the large Fe concentrations of the hemozoin crystals. Similar overlap has been observed in every BrQ-treated parasite, while no such overlap has been observed in any of the BrQ-free parasites. In total, 7 BrQ-treated and 9 BrQ-free parasites were mapped by X-ray fluorescence cryomicroscopy.

**BrQ Caps One-Tenth of Hemozoin Surface Docking Sites within Parasites.** First, we assume there is no occlusion of the BrQ in- to hemozoin crystals since no direct evidence was found for the existence of quinine or chloroquine within synthetic hemozoin grown in their presence (21). Hence, BrQ is always located at the existence of quinine or chloroquine within synthetic hemozoin to hemozoin crystals since no direct evidence was found for the existence of quinine or chloroquine within synthetic hemozoin grown in their presence (21). Hence, BrQ is always located at the

An estimate of the fluorescence signal ratio, \( \frac{I_{Br}}{I_{Fe}} = 40/(8 \times 10^{3}) = 1:200 \), can be obtained by inspection of the vertical color scales of the 2 maps (Fig. 1D and E). A more precise measure is obtained by summing up the intensities in the Fe map (Fig. 1D) within the pixel area of hemozoin crystals and in the same pixel area in the Br map (Fig. 1E) with a constant background subtracted. The result is a ratio of 1:272.

What remains to be assessed is the ratio \( \frac{N_{bulk}}{N_{surf}} \) for all of the hemozoin crystals in the digestive vacuole. This was done by measuring the volume and surface area of the cluster of hemozoin crystals using soft X-ray tomography datasets, as detailed in SI Appendix, section 4.1, resulting in the value \( \frac{N_{bulk}}{N_{surf}} = 10^{6}/3.9 \times 10^{4} \) for parasite 1.

We thus estimate the resulting fractional coverage \( C_{cvrg} \) to be

\[
C_{cvrg} = \frac{4}{5.4} \frac{I_{Br}}{I_{Fe}} \frac{N_{bulk}}{N_{surf}}
\]

At this point we take into account that the measured X-ray fluorescence intensity \( I \) per atom of Br is 5.4 times higher than that for Fe, as detailed in SI Appendix, section 2. Therefore,

\[
\frac{N_{BrQ}}{N_{Fe}} = \frac{1}{5.4} \frac{I_{Br}}{I_{Fe}} C_{cvrg} \frac{N_{surf}}{N_{bulk}} \frac{1}{4}
\]

From this equation we derive the surface coverage:

\[
C_{cvrg} = \frac{4}{5.4} \frac{I_{Br}}{I_{Fe}} \frac{N_{bulk}}{N_{surf}}
\]

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For this doubly invaded red blood cell (iRBC labeled C, Fig. 1) the BrQ drug was introduced into the parasite culture at a concentration of 150 nM, which is in the range of the therapeutic dose of chloroquine (28).

In another infected red blood cell (iRBC labeled A in Fig. 4), the concentration at which BrQ was introduced into the culture was 40 nM, in keeping with previous measurements reported in the literature (22). Based on the same analytical procedure, the coverage onto hemozoin by BrQ was measured to be \( \sim 10 \pm 4\% \). This extent is nearly the same as that for iRBC C, even though the drug was introduced at an almost 4 times lower concentration.

**Isolated Hemozoin Crystals outside Red Blood Cells Are Capped with Bromoquine.** The Fe X-ray fluorescence map shown in Fig. 2 reveals several isolated hemozoin crystals outside infected red blood cells. Regarding the origin of these ex vivo crystals, we present 2 scenarios. The crystals may have originated from rupture of previous-generation parasites in the process of release of their daughter parasites at the end of the parasitic asexual red blood cell cycle (29). It is also possible that these crystals were released from those few parasites that were destroyed by BrQ. These ex vivo crystals are marked Hz1, Hz2, and so on in Fig. 2A. A close-up view of Hz1 is shown both as Fe and Br maps in Fig. 2B, providing prima facie evidence that the BrQ molecule has a distinct affinity to be absorbed onto the surface of hemozoin crystals.

Below we present a determination of BrQ coverage on crystal Hz1, making once again use of the equation

\[
C_{cvrg} = \frac{4}{5.4} \frac{I_{Br}}{I_{Fe}} \frac{N_{bulk}}{N_{surf}}
\]

We have also analyzed 7 other samples of isolated hemozoin crystals (shown in SI Appendix, Fig. S3) and incorporate all 8 measurements (SI Appendix, Table S1 in SI Appendix, section 5) for estimating the average coverage. This estimate is along the same lines as for the analysis within an infected red blood cell (discussed above). However, all isolated crystals were outside the focal plane for soft X-ray tomography measurements, so the determination of
surface area $S_{H}$ of each crystal was carried out solely by an analysis of its X-ray fluorescence maps, as detailed in SI Appendix, section 4.2. Briefly, the number of unit cells in the bulk of the crystal, $N_{\text{bulk}}$, was derived from the number of Fe atoms comprising the crystal measured by X-ray fluorescence with the knowledge that the unit cell of hemozoin consists of 2 Fe atoms. The number of surface unit cells $N_{\text{surf}}$ was derived from the crystal surface divided by the weighted average area of surface unit cells. For crystal Hz1 the ratio $N_{\text{bulk}}/N_{\text{surf}}$ was determined to be equal $1.2 \cdot 10^6/1.5 \cdot 10^5$. See SI Appendix, section 4.2 for the detailed calculation.

The average X-ray fluorescence intensities of Br and Fe across Hz1 are, $I_{\text{Br}} = 15$ and $I_{\text{Fe}} = 700$, respectively (Fig. 2B). The coverage of BrQ on crystal Hz1 is then

$$C_{\text{covg}} = \frac{4}{5.4} \frac{I_{\text{Br}}}{I_{\text{Fe}}} \frac{N_{\text{bulk}}}{N_{\text{surf}}} = \frac{4}{5.4} \frac{15}{700} \frac{1.2 \cdot 10^6}{1.5 \cdot 10^5} \approx 0.12.$$ 

The same analysis for all 8 isolated crystals yielded an average BrQ coverage of around $0.070 \pm 0.035$, that is, $7 \pm 3.5\%$. Further experiments with a higher spatial resolution are required to determine the different affinities of BrQ to the {100} and the {010} faces of hemozoin (8).

### Bromine from Bovine Serum Does Not Cap Hemozoin Crystals

We have identified 2 sources of Br atoms in the examined samples. One is the BrQ drug introduced at the ~100 nM level. The second source is background Br originating from bovine serum used for cultivating the malaria parasites, reportedly present in the form of 2-octyl $\gamma$-bromooctaoacetate (30, 31). Measured concentrations of the background Br range up to 4 $\mu$M. As shown below, we have been able to effectively separate the total Br signal arising from BrQ and the serum and establish unambiguously that only BrQ decorates hemozoin crystals and the digestive vacuole membrane.

Inspection of the Fe map and the Br map in Fig. 1 shows clearly that Br tends to accumulate on hemozoin crystals, both for parasites 1 and 2. There is also some lower Br signal in the parasites but outside their hemozoin clusters. This signal may arise from the drug BrQ as well as from Br in the serum. In order to distinguish between these 2 sources of Br, we analyze Br distribution in BrQ-treated and the BrQ-free parasites.

We analyzed 7 BrQ-treated and 9 BrQ-free parasites. In BrQ-treated parasites, we identify distinct areas of Br overlaying hemozoin (Figs. 1 and 3A). In BrQ-free parasites there is no such overlap (Fig. 3B). However, the difference in overlap is not always as clear to the naked eye as shown in other examples given in SI Appendix, Fig. S6. We have taken up this challenge by quantifying the extent of overlap of Br with hemozoin in BrQ-treated and BrQ-free parasites. This extent is given in form of an overlap parameter $O_{\text{Br,Hz}}$. When there is no overlap $O_{\text{Br,Hz}} = 0$. In case there is an overlap $O_{\text{Br,Hz}} > 0$. The overlap parameter for several BrQ-treated and BrQ-free parasites is plotted in Fig. 3C. The exact definition and calculation of the overlap parameter are presented in SI Appendix, section 9.

For 9 BrQ-free cells $O_{\text{Br,Hz}}$ varied between $-0.2$ and 0.2, the olive-colored dots in Fig. 3C. In the cell shown in Fig. 1, where the overlap is obvious to the eye, the overlap parameter came out to be $O_{\text{Br,Hz}} > 1.0$. This is recorded in Fig. 3C by 2 red dots marked “C” belonging to the 2 parasites residing in this doubly invaded cell. Therefore, the figure shows quantitatively that, although not always as visible as in Fig. 1, there is indeed overlap between Br signal and Hz crystals in BrQ-treated cells, while no such overlap occurs in the BrQ-free samples.

The average overlap parameter in the BrQ-treated cells is $0.57 \pm 0.40$. In the BrQ-free cells it is 0.09 ± 0.12.

### BrQ Accumulates at the Membrane of the Digestive Vacuole

In most of the parasites examined, an elevated Br X-ray fluorescence signal appears to decorate the parasitic membranes, including the parasitic nucleus. Br also appears to decorate the digestive vacuole but only in BrQ-treated parasites. In order to examine whether it is the membranes that are decorated by Br, a Br distribution image was simulated by virtually placing Br atoms along the 3D positions of the membranes of the digestive vacuole, the nucleus, and the parasite as shown in Fig. 4. In order to accomplish this task, we have made a crude estimate of Br membrane coverage by summing the Br atoms along the periphery of the digestive vacuole membrane.

![Image](image_url)
A closer inspection of parasite membranes seen in Br maps in Fig. 3 and SI Appendix, Fig. S6 reveals that the digestive vacuole membrane is decorated by Br only in BrQ-treated parasites with the digestive vacuole membrane well-delineated in 4 parasites occupying iRBCs A, B, D, and E. We therefore conclude that the Br signal at hemozoin crystals and the digestive vacuole membrane indeed originates from BrQ and not from Br found in bovine serum.

Similar observations were made by Dubar et al. (22), who reported the presence of the quinoline drug, ferroquine, close to the digestive vacuole membrane of a Plasmodium-infected red blood cell imaged by transmission electron microscopy, and by Woodland et al. (32), who used visible light fluorescence microscopy to locate covalently labeled chloroquine at the digestive vacuole membrane and other parasitic membranes.

BrQ Reaches High Concentrations in the Digestive Vacuole. We measured concentration of Br in the lumen of the parasitic digestive vacuole in BrQ-treated parasites by counting Br atoms in Fig. 3.

### Fig. 3.
Overlap between Fe and Br distribution in BrQ-treated samples and BrQ-free samples. (A) Fe and Br X-ray fluorescence maps of an infected red blood cell (iRBC D) treated by BrQ. DV denotes the digestive vacuole and Hz the hemozoin crystals. (B) Fe and Br signals in a BrQ-free infected red blood cell (iRBC K). (C) Overlap parameter, \( O_{BrQ,Hz} \), between hemozoin position and Br fluorescence signal in 7 BrQ-treated and 9 BrQ-free samples. Each dot represents a measurement within an individual parasite carrying the name of its host iRBC. iRBCs named A through K are shown in SI Appendix, Fig. S6; others are labeled with a hash mark. iRBCs C and A are shown in Figs. 1 and 2, respectively. The overlap parameter = 0 if there is no overlap between Br and hemozoin and >0 if there is such an overlap.

### Fig. 4.
Surface rendering, measured and simulated X-ray fluorescence maps of a BrQ-treated infected red blood cell (iRBC) labeled A. (A) Surface rendering of a soft X-ray tomography segmentation. (B) Measured Fe X-ray fluorescence map. (C) Measured Br X-ray fluorescence map. (D) Simulated Br X-ray fluorescence map. Br atoms were evenly distributed over the surface of the digestive vacuole (DV) membrane, the parasite nucleus, and the parasite membrane with the density of \( 5 \times 10^7 \) atoms per square micrometer, and on the surface of hemozoin (Hz) crystals with a density corresponding to 10% BrQ surface coverage. (E) Same simulation as D but without Br at the surface of Hz crystals.
We now address the question of the ability of BrQ to smother the hemozoin crystalline surface in the early stages of parasitic development, say a couple of hours after hemozoin crystals begin to form. In a 2-μL digestive vacuole of a young trophozoite (SI Appendix, Table S1; electron microscopy data in ref. 34), assuming that half the digestive vacuole volume would be freely occupied by BrQ at 150 μM concentration, the amount of BrQ would be ~90,000 molecules. This amount would be sufficient to block 11% of hemozoin crystal surface developed at this stage (SI Appendix, section 11), implying, all in all, that BrQ would be efficient all the way from the onset of parasitic production of hemozoin crystals.

We now address the observation of an elevated BrQ presence at the membrane of the digestive vacuole. This membrane is clearly visible in soft X-ray tomographic images. In the BrQ-treated parasite the membrane is highlighted in X-ray fluorescence maps of Br, indicating the presence of BrQ molecules. The presence of background Br in parasites precludes analysis of BrQ molecule distribution in other parasitic membranes. Nonetheless, in a recent study by Woodland et al. (32), chloroquine molecules covalently labeled for light fluorescence microscopy were detected also in other parasitic membranes, but not on red blood cell membranes, which is in accordance with our observations. We note that in their work little signal from the labeled molecule over hemozoin-rich area was detected, which is possibly due to signal absorption by the crystals. It is debatable whether the water-soluble drug would, by itself, tend to accumulate at the lipid membranes. However, the drug molecule has high affinity to complex with free heme in the aqueous medium of the digestive vacuole with the acidic pH. Indeed, the possibility of quinoline drug complexation with free heme has been proposed in the literature (7–16). The affinity to water of this complex is presumably higher than that of free heme but lower than that of BrQ alone. We therefore rationalize that this complex would be driven toward and accumulate at the lipid membrane of the digestive vacuole. Supporting this hypothesis is a report of chloroquine–hemin complex association with lipid membranes observed in vitro (35–37). A prolonged exposure of the digestive vacuole membrane to an increased presence of BrQ–heme complex might lead to intercalation of the heme or the complex with the membrane, leading to its local puncture and spilling of the toxic heme into the interior of the cell (36, 37).

We found that BrQ reaches the same concentrations in the digestive vacuole of chloroquine-resistant strain FCR3 as in the BrQ-sensitive 3D7 strain and displays similar distribution in both strains (SI Appendix, Fig. S6). We interpret this observation as either lack of resistance to BrQ by the FCR3 strain or a resistance mechanism other than the previously suggested reduction of BrQ concentration in the digestive vacuole (1). Further investigation needs to be conducted to clarify this point.

Conclusion

Using a correlative X-ray microscopy approach, we have identified that in vivo BrQ—the bromo analog of chloroquine—covers a substantial part of available docking sites at the surface of hemozoin crystals formed in the digestive vacuole of the Plasmodium parasites. Quantification of quinoline-type molecule in vivo coverage of hemozoin crystals in fully hydrated cells was achieved. This coverage was as high as 10 ± 4%, enough to prevent oncoming heme docking onto the crystals. The drug was found in abundance in the digestive vacuole. We have provided evidence, albeit indirect, that the drug complexes with free heme, given that the drug binds to the hemozoin surface. This complex accumulates at the membrane of the digestive vacuole, as observed by bromine X-ray fluorescence signal, and possibly spreads to other membranes. In other words, both hypotheses mentioned in the Introduction take place in vivo. This model can be generalized to quinoline drugs, such as quinine, which can...
stereospecifically bind to the \{100\}, \{011\}, and \{001\} faces of hemozoin.

Last, but not least, the approach described here would be applicable to test whether other antimalarial drugs, such as the widely used artemisinin as an in vivo adduct with heme (38), will bind to hemozoin crystals (21, 39), provided an appropriate atom detectable by X-ray fluorescence can be attached to the drug.

BrQ Synthesis and Methods

Synthesis of BrQ and the NMR spectrum of the synthesized product are given in SI Appendix, section 12.

Malaria parasite culture and strain verification are described in SI Appendix, section 13. Tight synchronization resulting in 30- to 32-h-postinvasion parasites is detailed in SI Appendix, section 14. IC\(_50\) (concentration that inhibits parasite growth by 50\%) values of chloroquine and BrQ for chloroquine-sensitive (3D7) and chloroquine-resistant (FCR3) strains and the measurement procedure are given in SI Appendix, section 15.

Preparation of vitrified samples for cryo X-ray microscopy is described in SI Appendix, section 16. Cryo X-ray fluorescence and soft X-ray cryotomography instruments are described in SI Appendix, section 17. Software used in data analysis and presentation is described in SI Appendix, section 18.

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