Characterization of the choline carrier of *Plasmodium falciparum*: a route for the selective delivery of novel antimalarial drugs

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New drugs are urgently needed to combat the growing problem of drug resistance in *Plasmodium falciparum* malaria. The infected erythrocyte is a multicompartmental system, and its transporters are of interest as drug targets in their own right and as potential routes for the delivery of antimalarial drugs. Choline is an important nutrient that penetrates infected erythrocyte membranes through the endogenous carrier and through parasite-induced permeability pathways, but nothing is known about its transport into the intracellular parasite. Here we present the first characterization of choline transport across the parasite membrane. Transport exhibits Michaelis-Menten kinetics with an apparent K_m of 25.0 ± 3.5 μM for choline. The carrier is inhibitor-sensitive, temperature-dependent, and Na$^+$-independent, and it is driven by the proton-motive force. Highly active bis-amidine and bis-quaternary ammonium compounds are also known to penetrate the host erythrocyte membrane through parasite-induced permeability pathways. Here, we demonstrate that the parasite choline transporter mediates the delivery of these compounds to the intracellular parasite. Thus, the induced permeability pathways in the host erythrocyte membrane and the parasite choline transporter described here form a cooperative transport system that shows great promise for the selective targeting of new agents for the chemotherapy of malaria. (Blood. 2004;104: 3372-3377)

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Introduction

Plasmodium falciparum malaria causes immense public health and economic problems in most developing countries. For many years, these problems have been intensified by the emergence and spread of resistance to the currently available antimalarial drugs. As a result, discovering and developing novel antimalarial agents is one of the greatest challenges facing malaria control today.

In the search for new antimalarial agents, parasite transporters are of great interest as drug targets[1-4] and as potential selective drug delivery routes in blood-stage parasites.[5,6] In this study, we have focused on the parasite choline carrier because the parasite is known to take up choline from the external medium for the de novo synthesis of phosphatidylcholine (PC). Cholinephosphate cytidylyltransferase is a regulatory step in this pathway, and choline transport (which regulates the supply of precursor) is a rate-limiting step.[7]

Phospholipid metabolism is an ideal target for new chemotherapy because of its vital importance to the parasite. Phospholipid metabolism is absent from normal mature human erythrocytes,[8] but after malarial infection, the erythrocyte phospholipid content increases by as much as 500%.[9,10] PC and phosphatidylethanolamine (PE) are the major phospholipids of the infected erythrocyte, representing approximately 85% of the total phospholipid pool. De novo pathways for PC and PE biosynthesis from choline and ethanolamine, respectively, have been thoroughly described in *Plasmodium*-infected erythrocytes.[11]

In the past few years, a large number of compounds have been synthesized that were designed to mimic the structure of choline. Lead compounds of bis-quaternary ammonium or diamidine structure have been shown to exhibit potent in vitro activity against *P falciparum* and *Plasmodium vivax*[5,12] and good in vivo activity against *P falciparum* and *P cynomolgi* in Aotus and rhesus monkeys, respectively.[13]

In spite of the efficacy of these new drugs and the possible dependence of the parasite on exogenous choline, the choline transport process has not been fully characterized in the *P falciparum*–infected erythrocyte. It is known that in infected erythrocytes, choline penetrates the host erythrocyte membrane through the endogenous choline carrier[14] or through the parasite-induced new permeation pathway (NPP) (for an in-depth review, see Kirk[15]). However, the mechanism by which choline penetrates the intracellular parasite is unknown. Similarly, it has been reported that antimalarial choline analogs penetrate the host erythrocyte membrane through the NPP and that they eventually accumulate in the intracellular parasite,[5,16] but the means by which they cross the parasite plasma membrane is unknown.

Here we present the first report of the biochemical properties of choline transport into isolated parasites of *P falciparum* and its inhibition by antimalarial choline analogs. These choline analogs (which rely on high levels of accumulation to exert their antimalarial activity) are not transported by the host erythrocyte choline

carrier, and penetration of the host cell compartment is solely through the NPP. Remarkably, however, these compounds are transported by the parasite choline carrier, providing an additional level of selectivity over and above interactions with the intracellular target(s).

Materials and methods

Parasite, culture, and drug sensitivity assays

Intraerythrocytic stages of *P falciparum* (TM6, K1, and HB3 strain) were maintained in continuous culture using standard methods. Briefly, cultures contained a 2% suspension of O^+ erythrocytes in RPMI 1640 (R8758) medium supplemented with 10% pooled human AB^+ serum, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), and 20 μM gentamicin sulfate. [^{3}H]-Labeled and unlabeled T16; 1,12-dodecanemethylene bis[4-methyl-5-ethylthiazolium] were synthesized at the Laboratoire des Aminoacides, Peptides et Proteines (CNRS, UMR 5810 Université Montpellier II) (E. Richier, H.J.V., M.Calas, unpublished data). All transport experiments were performed on trophozoite-stage cultures, 24 hours after predominantly ring-stage cultures were synchronized with 5% sorbitol. The sensitivity of *P falciparum*–infected erythrocytes to choline analogs was determined over 48 hours using the [^{3}H]-hypoxanthine incorporation method. These experiments used an inoculum size of 0.5% parasitemia (ring-stage), and 1% hematocrit. IC_{50} values were calculated using the 4-parameter logistic method (Grafit program; Erithacus Software, Surrey, United Kingdom).

Transport of radiolabeled choline and choline analogs into free parasites

Free parasites were prepared from aliquots of infected erythrocytes (approximately 8×10^9 cells/mL) by adding 5 vol 0.15% (wt/vol) saponin in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.76 mM K_2HPO_4, 8.0 mM Na_2HPO_4, 5.5 mM D-glucose, pH 7.4) for 1 minute, followed by 3 washes by centrifugation and resuspension in HEPES-buffered RPMI. Freed parasites were then resuspended into appropriate solutions (RPMI or Ringers buffer [122.5 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.5 mM D-glucose, 1.0 mM K_2HPO_4, 10 mM HEPES, pH 7.4], typically $3-5 \times 10^8$ cells/mL). Ringers buffer was used when an external choline concentration of 0 was required. The suspensions contained 50 nM [^{3}H]-pentamidine, 50 nM [^{3}H]-T16, or 20 nM [^{3}H]-choline. All the radiolabeled compounds were added at time zero. Where not shown, a preliminary time course was performed to ensure that the uptake of radiolabeled compounds was measured under initial rate conditions throughout. Transport experiments were performed at room temperature (in this case 22°C) instead of 37°C to avoid the temperature fluctuations that are inevitable when moving samples in and out of a heated water bath. For the concentration-dependence experiments (Figures 1B-C, 3, 4), the radiolabel was diluted with unlabeled compound to give the final concentrations indicated. After incubation, aliquots of the suspension were overlaid onto oil (a 5:4 mixture of dibutyl phthalate/dioctyl phthalate) and were centrifuged (10 000*g*, 20 seconds), sedimenting the cells below the oil. Preliminary experiments using [^{3}H]-inulin established that there was no significant carryover of medium to the cell pellet after centrifugation

through the phthalate oil mixture (data not shown). Cell pellets were lysed by the addition of distilled water (100 μL) and then solubilized and decolorized by the addition (100 μL) of a cocktail containing 5 parts tissue solubilizer, 2 parts H_2O_2 (30%), and 2 parts glacial acetic acid. Samples were then counted by liquid scintillation counting.

Transport inhibitors were tested for their ability to inhibit [^{3}H]-choline uptake. Compounds were dissolved in dimethyl sulfoxide (DMSO) or methanol at a stock concentration of 100 mM. All compounds were preincubated with parasites for 5 minutes before the addition of [^{3}H]-choline. Control samples were treated with appropriate concentrations of DMSO or methanol alone.

Transport of radiolabeled choline and choline analogs into uninfected erythrocytes

This procedure was essentially the same as that used for free parasites. Erythrocytes were suspended in RPMI, typically at 10^8 cells/mL, and were incubated in the presence of 50 nM [^{3}H]-T16 or 50 nM [^{3}H]-pentamidine, with various concentrations of unlabeled choline, for 30 minutes at 22°C.

### Ability of drugs to inhibit the parasite choline kinase

Removing the substrate by phosphorylation is important for maintaining linearity of the time course of choline uptake.[17] When measuring the effect of potential transport inhibitors, it is important to determine whether inhibitors are affecting only the transport phase or whether they also inhibit the subsequent phosphorylation step. The ability of inhibitors to inhibit the phosphorylation of choline was assessed by using a parasite lysate assay. This procedure was performed as described previously for glucose analogs.[18] Drugs were added to cell lysate at concentrations up to 1 mM, and the ability of the drugs to inhibit the parasite choline kinase was assessed.

Results

Kinetic characterization of choline transport into isolated parasites of *P falciparum*

Figure 1A shows a time-course of choline uptake into isolated parasites of *P falciparum*. Choline uptake (10 μM) was measured at room temperature (22°C) and at 0°C. At room temperature, choline accumulation is linear as a function of time for at least 20 minutes, with a rate of 1.82 ± 0.08 pmol $(10^6$ cells$)^{-1}$/min (linear regression, $r^2 = 0.98$). By contrast, little uptake of [^{3}H]-choline was found at 0°C, even after 1 hour (data not shown). In further experiments, choline uptake was measured as a function of extracellular choline concentration (after 10 minutes of incubation at 22°C, well within the linear phase of transport). Choline uptake into isolated parasites is a high-affinity, saturable process that predominates at physiologic choline concentrations (Figure 1B). Saturable choline uptake exhibited Michaelis-Menten kinetics with an apparent concentration of substrate leading to half-maximal velocity (K_m) of 25.0 ± 3.5 μM and reaching a maximum/limiting velocity (V_{max}) of 4.6 ± 0.2 pmol $(10^6$ cells$)^{-1}$/min (Figure 1B).

Figure 1. Kinetics of choline uptake. (A) Time course of [^{3}H]-choline uptake into free parasites of *P falciparum*. Uptake is measured at 22°C (○) and 0°C (●). Data points are means of duplicate observations from 2 individual experiments. (B) Concentration dependence of choline uptake into free parasites of *P falciparum*. Data points are means of duplicate observations from 2 individual experiments. Data were fitted to a function describing simple ligand binding at a single site by nonlinear regression analysis (Marquart method) using an iterative procedure to generate the best fit (χ^2) of the curve to the data. Standard errors were calculated for each parameter using the matrix inversion method (Grafit user manual). (C) Hill plot of the data presented in panel B.
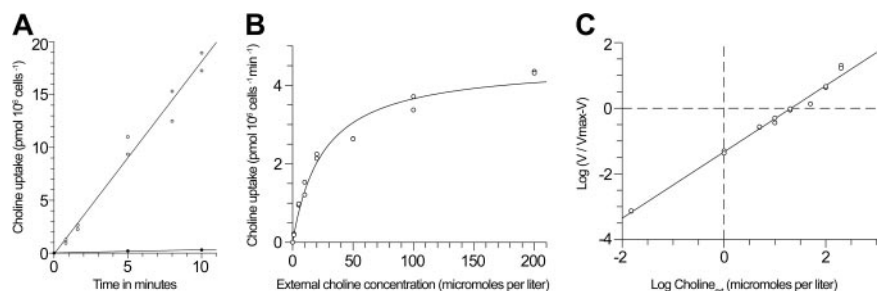
Kinetic parameters were generated by nonlinear curve fitting, assuming transport by a single carrier (Erithacus Software). The accurate fit of the curve to the data suggests that choline uptake is largely mediated by a single type of transporter (Hill coefficient of 1.01 ± 0.03; Figure 1C).

To assess any energy requirements for choline transport, uptake by isolated parasites was measured in the presence of various compounds known to affect sodium or proton gradients. Ouabain, an inhibitor of the eukaryotic plasma membrane Na^+/K^+-ATPase, had no effect on choline uptake at a concentration of 100 μM (Figure 2A). Neither was there any effect on choline uptake of substituting sodium for N-methyl-D-glucamine in the incubation medium (Figure 2A). However, bafilomycin A1 (a specific inhibitor of V-ATPase proton pumps) and the protonophore FCCP (at 100 nM and 20 μM, respectively) significantly reduced the transport of choline into free parasites (Figure 2A). These inhibitors are known to reduce the plasma membrane proton gradient in free parasites of *P falciparum*.[19] Reducing the plasma membrane proton gradient also partially collapses the parasite plasma membrane potential,[20] and we suggest that choline transport is at least partly energized by the proton-motive force across the parasite plasma membrane. This viewpoint is strengthened by the observation that choline uptake is stimulated slightly by adding 10 mM cesium chloride to the bathing medium (Figure 2A), a procedure that causes small hyperpolarization of the parasite plasma membrane.[20]

Inhibition of choline uptake into isolated parasites by antimalarial choline analogs and transport inhibitors

To investigate whether known choline transport inhibitors or antimalarial choline analogs could inhibit the *P* choline transporter, we examined the effect of these analogs on the transport of [³H]-choline in isolated parasites (Figure 2B).

Furosemide (200 μM), a potent inhibitor of choline transport through the NPP on the infected host cell membrane,[21] had no effect on the parasite plasma membrane choline transporter. By contrast, choline uptake in isolated parasites is significantly inhibited by the cation transport inhibitors amiodorone and bepridil (both at 200 μM), hemicholinium-3 (HC-3) (50 μM), and the choline analogs pentamidine and T16 (both at 1 μM). The possibility that these inhibitors also affect the phosphorylation step was investigated as well. With the exception of HC-3, which

caused a 50% inhibition at 50 μM, none of the compounds significantly inhibited the phosphorylation of choline in the cell lysate assay (data not shown). Therefore, with the exception of HC-3, it is likely that these drugs inhibit the uptake of choline primarily by blocking its transmembrane transport. On the other hand, HC-3 may exert most of its effects on the phosphorylation of choline.

The inhibition of choline uptake by the 2 most potent inhibitors, pentamidine and T16, was investigated in more detail. T16 shows a potent and concentration-dependent inhibition of choline uptake using a physiologically relevant buffer choline concentration of 15 μM (Figure 3A). The IC_{50} for inhibition of choline uptake by T16 under these conditions is approximately 140 nM. Pentamidine also showed potent and concentration-dependent inhibition of choline uptake. The effect of 10 μM pentamidine on choline uptake was determined and compared with control (no pentamidine) over a range of choline concentrations. The data are presented as a Lineweaver Burke plot, and the intersection of the lines at the ordinate clearly indicates (Figure 3B) that the inhibition of choline uptake by pentamidine is competitive, with a calculated inhibition constant (K_i) of 3.3 μM, raising the possibility that pentamidine itself is a transport substrate.

Antimalarial choline analogs are transported by the parasite choline carrier but not by the host cell choline carrier

Transport of [³H]-pentamidine and [³H]-T16 into free parasites was strongly inhibited by 5 mM choline (Figure 4A). Figure 4B shows the concentration dependence of inhibition of pentamidine uptake by choline, with a K_i of 19 ± 2.5, similar to the K_m for [³H]-choline transport. Similar results were found with T16 (data not shown), and it is very likely that the choline carrier directly transports both drugs. This argument is supported by the similar inhibitor profile of drug uptake to choline uptake (Figure 4C). [³H]-Choline transport and [³H]-pentamidine transport were equally sensitive to the inhibitors furosemide, amiodarone, and bepridil ($P > .05$). In the case of bafilomycin, there is a much greater effect on the transport of pentamidine than on the transport of choline. This can be rationalized on consideration that the proton-motive force is likely to drive the accumulation of bis-cations such as pentamidine to much higher levels than that of mono-cations such as choline.

It is likely that transport of pentamidine and T16 through the choline carrier is important to the antimalarial activity of these drugs. However, the inhibition of drug uptake by choline is incomplete: approximately 25% of the uptake of each drug is insensitive to choline (Figure 4A), suggesting that other transporters (or endocytosis with ferriprotoporphyrin IX; see "Discussion") may have a role to play in the accumulation of the drugs. Nonetheless, it is clear from the data presented in Figure 5 that drug uptake by the choline carrier does play an important role in determining the sensitivity of infected erythrocytes to these drugs. This graph compares the effect of different levels of choline in the medium on the uptake and the antimalarial activity of T16. Increasing the choline concentration causes a dose-dependent inhibition of T16 uptake that is directly proportional to the inhibition of antimalarial activity (linear regression, $r^2 = 0.91$).

We have further investigated whether pentamidine or T16 is transported by the host erythrocyte choline carrier. Previously, we have shown that the uptake of pentamidine and T16 by infected cells is effectively inhibited by furosemide, a specific NPP inhibitor.[5,16] Furosemide does not inhibit the endogenous choline carrier, suggesting that both drugs enter the infected cell exclusively through the NPP, not through the erythrocyte choline
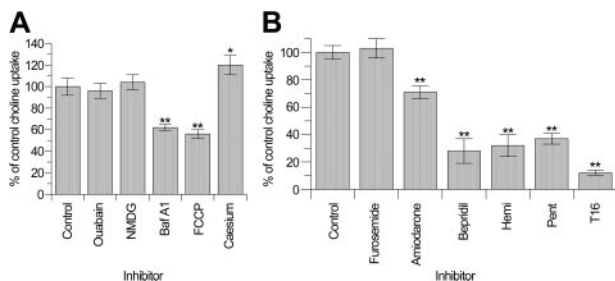
Figure 2. Effect of inhibitors on choline uptake. (A) Effect of ouabain (100 μM), substitution of sodium for N-methyl-D-glucamine, bafilomycin A1 (100 nM), FCCP (20 μM), and cesium chloride (10 mM) on the uptake of [³H]-choline (20 nM) into isolated *P falciparum* parasites over 10 minutes at 22°C. Data are mean ± SE for duplicate observations from 4 individual experiments. **$P < .001$ and *$P > .001 < .05$, Mann-Whitney U test for significance of difference between means. (B) Effect of known choline transport inhibitors furosemide (200 μM), amiodarone (200 μM), bepridil (200 μM), and HC-3 (50 μM) and antimalarial choline analogs T16 (1 μM) and pentamidine (1 μM) on the uptake of [³H]-choline (20 nM) into isolated *P falciparum* parasites over 10 minutes at 22°C. Data are mean ± SE for duplicate observations from 4 individual experiments. **$P < .001$ and *$P > .001 < .05$, Mann-Whitney U test for significance of difference between means.

Figure 3. Inhibition of choline uptake by novel antimalarials. (A) Concentration-dependent inhibition by T16 of [^{3}H]-choline (15 µM) uptake into isolated *P falciparum* parasites over 10 minutes at 22°C. Data are mean ± SD for duplicate observations from 3 individual experiments. (B) Lineweaver-Burke (double reciprocal) plot of choline uptake as a function of external choline concentration into isolated *P falciparum* parasites over 10 minutes at 22°C. Data are presented for choline alone (○) or choline in the presence of 10 µM pentamidine (●). Data are mean ± SD for duplicate observations from 3 individual experiments.
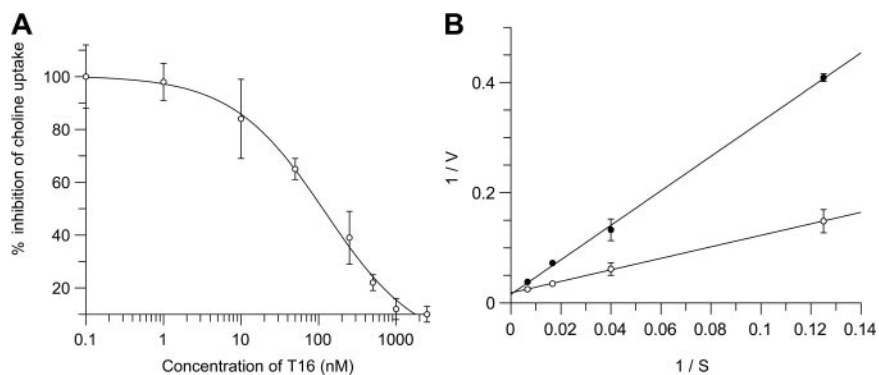

transporter. To test this hypothesis, we have investigated the effect of choline on the uptake of [^{3}H]-pentamidine or [^{3}H]-T16 by infected and uninfected erythrocytes. Infected erythrocytes exhibit cellular accumulation ratios (CARs) of several hundredfold after incubation with pentamidine or T16 for 1 hour.[5,16] A significant proportion (20%-30%) of the drug is found in the host cell compartment of the infected cell, probably because of weak binding of the drugs to hemoglobin.[5,16] By contrast, we found that uninfected erythrocytes take up both drugs very slowly, probably by passive diffusion. After 1 hour at room temperature, the CARs were approximately 3 for pentamidine and 1 for T16, and accumulation was again driven by binding to hemoglobin or other proteins Furthermore, this uptake was insensitive to the concentration of choline in the buffer (Figure 6). Thus, we can find no evidence that the erythrocyte choline carrier transports pentamidine or T16.

Discussion

We report the first characterization of choline transport into isolated parasites of the intra-erythrocytic stages of the human malaria parasite *P falciparum.* Choline transport has been studied before in other species of *Plasmodium,* but these studies used intact malaria-infected erythrocytes.[11] Without knowing which transport process is rate limiting, the earlier data are difficult to interpret because it is unknown whether transport kinetics measured in the multicompartment-infected erythrocyte reflect the activity of choline transporters on the host cell membrane or on the parasite plasma membrane. In this study, we used saponin to isolate *P falciparum* parasites

from their host cells. This technique has been effective for characterizing other *P falciparum* plasma membrane transporters.[22-24] Furthermore, we show that choline transport through the host cell membrane is rate limiting, revealing that unambiguous measurement of the properties of choline transport across the parasite plasma membrane can only be accomplished using isolated parasites.

Choline uptake by isolated parasites is a carrier-mediated process. The transporter has an apparent K_m for choline of 25.0 ± 3.5 µM and a V_{max} of 4.6 ± 0.2 pmol (10^6 cells)$^{-1}$/min (Figure 1B). This is equivalent to a flux of 2.8 mmol (10^{13} cells)$^{-1}$/hour at maximum capacity. Using intact erythrocytes infected with *P falciparum*, Kirk et al[21] report a maximum choline influx of 0.75 mmol (10^{13} cells)$^{-1}$/hour at an external choline concentration of 500 µM. Even allowing for differences in experimental conditions, this comparison suggests that the transport capacity of the parasite plasma membrane (PPM) carrier is much higher than the choline flux through the NPPs. At more physiologic external choline concentrations, this difference is even more pronounced. For example, at 10 µM external choline, the flux through the endogenous erythrocyte choline carrier will be approximately 0.017 mmol (10^{13} cells)$^{-1}$/hour This is similar to the flux through the NPPs of 0.015 mmol (10^{13} cells)$^{-1}$/hour at the same external choline concentration.[21] Thus, the combined choline flux through the endogenous and induced host cell transporters will be about 0.03 mmol (10^{13} cells)$^{-1}$/hour. This should be compared with the PPM carrier, which can transport choline at a rate of 1.8 mmol (10^{13} cells)$^{-1}$/hour under the same conditions. The enhanced transport rate of the PPM carrier relative to the host cell has 2 major
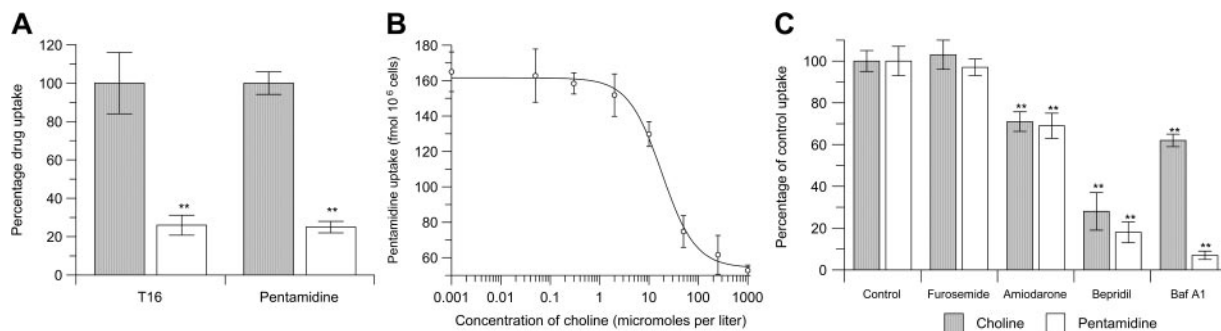
Figure 4. Antimalarial choline analogs are transported by the *P falciparum* choline carrier. (A) Uptake of [^{3}H]-T16 and [^{3}H]-pentamidine (both at 50 nM) into isolated parasites over 10 minutes at 22°C in the presence (□) or absence (▨) of 5 mM choline. Data are mean ± SE for duplicate observations from 4 individual experiments. (B) Concentration-dependent inhibition by choline of [^{3}H]-pentamidine (50 nM) uptake into isolated *P falciparum* parasites over 10 minutes at 22°C. Data are mean ± SD for duplicate observations from 3 individual experiments. (C) Effect of transport inhibitors furosemide (200 µM), amiodarone (200 µM), bepridil (200 µM), and bafilomycin A1 (100 nM) on [^{3}H]-choline (20 nM) and [^{3}H]-pentamidine (50 nM) uptake into isolated *P falciparum* parasites over 10 minutes at 22°C. Data are mean ± SD for duplicate observations from 3 individual experiments. **$P < .001$, Mann-Whitney U test for significance of difference between means.
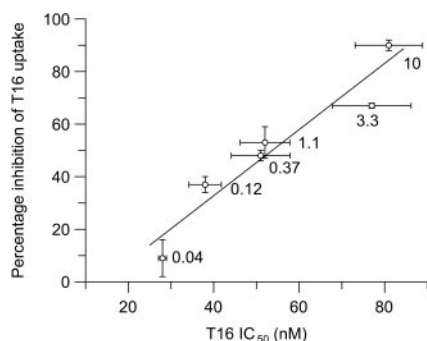
Figure 5. Activity of T16 depends on the external choline concentration. Effect of external choline concentration (0.04 mM, 0.123 mM, 0.37 mM, 1.1 mM, 3.3 mM, and 10 mM) on the uptake of [^{3}H]-T16 (50 nM) and the antimalarial IC_{50} of T16. Uptake of T16 was measured over 10 minutes at 22°C. Under these conditions, T16 uptake is in its linear initial phase (data not shown). Uptake experiments and sensitivity assays used intact infected erythrocytes. Uptake data are mean ± SE for duplicate observations from 3 individual experiments. Sensitivity assay data are mean ± SE for triplicate observations from 3 individual experiments.

implications. First, it means that choline transport kinetics measured using intact infected erythrocytes will relate to the rate-limiting processes on the host cell membrane rather than the PPM transporter. Second, at steady state, there is a reduced choline concentration in the host cell relative to the medium, and the PPM carrier can be likened to a choline vacuum cleaner for the host cell.

Choline uptake is sodium independent and appears to be energized by the plasma membrane proton-motive force (Figure 2A). We show that bafilomycin A1 at a concentration of 100 nM reduces the uptake of choline by approximately 40% to 50% (Figure 2A). This concentration of bafilomycin has been shown to almost completely inhibit the parasite plasma membrane proton pump,[19] yet only approximately half the choline uptake is blocked. Similarly, a 50% inhibition of choline uptake was seen with the proton uncoupler FCCP at 20 μM, a concentration sufficient to obliterate the plasma membrane proton gradient. Recently, it has been shown that the collapse of the plasma membrane proton gradient causes only a partial depolarization of the plasma membrane[20] and, thus, of the proton-motive force. We speculate that the choline uptake that remains after proton pump inhibition is attributed to a significant residual membrane potential that apparently is energized by outward K^+ diffusion.[20] This argument is supported by the small stimulation of choline uptake seen when potassium efflux is blocked with cesium (Figure 2A). Under these conditions, the plasma membrane is slightly hyperpolarized.[20] Similar phenomena have been well documented in other protozoa. In *Trypanosoma brucei* procyclics, for example, only approximately 60% of adenosine transport could be inhibited by CCCP, which was shown to induce partial depolarization of the plasma membrane potential and approximately 70% reduction of the proton-motive force.[25] Taken together, our data suggest that choline transport is an electrogenic process in *P falciparum*.

The *P falciparum* choline transporter was inhibited by the antiarrythmic drugs amiodarone and bepridil and possibly by HC-3, compounds previously shown to inhibit choline uptake in mammalian cells.[26,27] The low sensitivity of the *P falciparum* transporter to HC-3 distinguishes it from the high-affinity choline transporter in neurons, which is exquisitely sensitive to this inhibitor.[28] In many respects, including the dependence on proton-motive force, the *P falciparum* transporter is similar to the recently characterized choline transporter of *Leishmania major*,[29] though V_{max} and K_m of the malarial transporter are approximately 1 order of magnitude higher. Of particular interest is the high sensitivity of

choline transport of each parasite to quaternary ammonium compounds with antiprotozoal activity.[17,29,30] In the case of malaria parasites, recent studies indicate that bis-cations are more effective than mono-cations as inhibitors of phospholipid metabolism.[12] Accordingly, we have concentrated on choline analogs that are bis-cationic. Some of these compounds, such as T16, G25 (another bis-quaternary ammonium), and propamidine (a bis-amidine), exhibit potent (low nM) antimalarial activity,[5,13,16] and some have been shown to inhibit choline uptake into intact infected erythrocytes.[12] Malaria parasites are dependent on an external choline supply, and it has been speculated that the inhibition of choline uptake could form part of the antimalarial action of these compounds.[17,30] However, the existing data were difficult to interpret because of the multicompartmental nature of the infected red blood cell (RBC), and it remained unclear whether the drugs inhibit choline transport through the host erythrocyte choline carrier, the NPP, or the parasite choline transporter.

Some of these issues are resolved here. Both prototype antimalarial bis-cations, pentamidine and T16, are shown to be potent inhibitors of the *P falciparum* choline transporter. However, it is unlikely that this property forms the basis of the antimalarial action of these compounds. Pentamidine has an IC_{50} of 77.0 ± 4.5 nM against the TM6 strain used in these studies. At this concentration, pentamidine would inhibit only a small percentage of the total choline flux because its K_i for the choline transporter is 3.3 μM. The K_i for the inhibition of choline transport by T16 also appears to be more than 1 order of magnitude higher than its antimalarial EC_{50}. Our data suggest that the demonstrated inhibition of parasite phospholipid metabolism by these compounds[12,31] may not be related to the inhibition of choline transport, indicating that the later steps of the CDP-choline pathway are targeted. However, this question can only be fully resolved when the concentration of inhibitors in the host cell cytoplasm is accurately measured.

Although the mode of action remains to be fully elucidated, it is probable that the antimalarial activity of these compounds relies primarily on their accumulation to high levels in the intracellular parasite. We have reported that binding to ferriprotoporphyrin IX (FP) in the parasite makes a significant contribution to the accumulation and the antimalarial activity of these compounds.[5,16] There can be no doubt that binding to FP is important, but again it is unlikely to be the sole mode of action of these compounds, and it is likely that other intracellular targets exist. For example, it has recently been shown that mono-quaternary and bis-quaternary ammonium compounds exert their effects on PC synthesis *before* they inhibit nucleic acid synthesis, suggesting that other enzymes
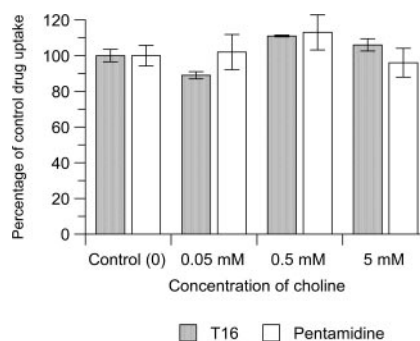
Figure 6. Choline analogs are not transported into uninfected erythrocytes. Effect of external choline concentration (0.0 mM, 0.05 mM, 0.5 mM, and 5 mM) on the uptake of [^{3}H]-T16 (50 nM) or [^{3}H]-pentamidine (50 nM) into uninfected erythrocytes over 30 minutes at 22°C. Data are mean ± SD for duplicate observations from 3 individual experiments.

in the Kennedy pathway could be targeted.[31] It should be noted that the mode of action of pentamidine in *T brucei brucei*, against which it has been used extensively for decades, has never been resolved. In addition, it is likely to be multifactorial[32] because it (pentamidine) accumulates to millimolar concentrations inside the parasite.[32] A similar situation exists for *P falciparum*. At minimum inhibitory external drug concentrations in the high nanomolar range, pentamidine and T16 are accumulated to millimolar concentrations by the intracellular parasite.[5,16] Given that the critical targets are inside the parasite and that the passive permeability of these drugs is low because of their positive charge, their effectiveness is directly dependent on efficient access through the membrane transporters of the infected cell.

We have previously demonstrated that pentamidine and T16 are substrates for NPP,[5,16] and now we show that the major route of entry of these drugs into the intracellular parasite is through the plasma membrane choline carrier. Although other routes of entry appear to exist, including a component of endocytosis alongside hemoglobin, transport of these drugs through the choline carrier is shown to be critical for their antimalarial activity. T16 itself is not phosphorylated (data not shown); hence, the inhibition of the antimalarial activity of T16 by high concentrations of choline is most likely to be related to the inhibition of drug transport (Figure 5). Neither T16 nor pentamidine is transported by the endogenous choline carrier of erythrocytes (Figure 6). We also have evidence that T16 is not transported by the dedicated choline transporter of yeast.[34] Thus, the *P falciparum* choline carrier is functionally distinct from the known dedicated eukaryotic choline carriers and is more akin to a polyspecific organic cation transporter.

In summary, the NPP and the parasite choline carrier form a transport network in the infected erythrocyte that offers 2 levels of selectivity for antimalarial choline analogs. We show that it is now possible to screen for compounds highly specific for the infected erythrocyte. In view of the number and effectiveness of the antimalarial choline analogs under development,[13,35,36] the parasite choline carrier, acting in concert with the NPP, should be regarded as a highly specific antimalarial drug delivery route of considerable promise.

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