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

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Abstract: There is an urgent requirement for new drugs to combat the growing problem of drug resistance in *Plasmodium falciparum* malaria. The infected erythrocyte is a multi-compartmental system and its transporters are of interest both 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 both via the endogenous carrier and via 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 $K_m$ value of 25.0 ± 3.5 $\mu$M for choline. The carrier is inhibitor-sensitive, temperature-dependent, Na$^+$-independent and 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 via 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.
Introduction

*Plasmodium falciparum* malaria causes immense public health and economic problems in most developing countries. For many years the situation has been intensified by the emergence and spread of resistance to the currently available antimalarial drugs. As a result, the discovery and development of 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 both as drug targets\(^1\)\(^-\)\(^4\) and as potential selective drug delivery routes in blood-stage parasites\(^5\)\(^-\)\(^6\). In this study we have focussed 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 due to its vital importance to the parasite: phospholipid metabolism is absent from normal mature human erythrocytes\(^8\) but after malaria 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 about 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\(^11\).

Over the last few years, a large number of compounds have been synthesized that were designed to mimic the structure of choline. Lead compounds of bis-quartenary ammonium or diaminidine structure have been shown to exhibit potent *in vitro* activity against *P.falciparum* and *P.vivax*\(^5\)\(^,\)\(^12\) as well as 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 *Plasmodium falciparum* infected erythrocyte. It is known that in infected erythrocytes, choline penetrates the host erythrocyte membrane via the endogenous choline carrier\(^14\) and/or via the parasite induced “new permeation pathway” (NPP) (see\(^15\) for an in-depth review). However, the mechanism by which choline penetrates the intracellular parasite is unknown. Similarly, it has been reported that the antimalarial choline analogues go through the host erythrocyte membrane via 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 *Plasmodium falciparum* and its inhibition by antimalarial choline analogues. 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 via 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. Intra-erythrocytic stages of *Plasmodium 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 (pH 7.4) and 20 µM gentamicin sulphate. [3H] Labelled and unlabelled T16; 1, 12-dodecanemethylene bis[4-methyl-5-ethylthiazolium] were synthesized in Laboratoire des Aminoacides, Peptides et Proteines, CNRS, UMR 5810, Richier et al. 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 analogues was determined over 48 hours using the [3H]-hypoxanthine incorporation method. These experiments employed an inoculum size of 0.5% parasitaemia (ring-stage) and 1% hematocrit. IC50 values were calculated using the four-parameter logistic method (Grafit program ; Erithacus Software, UK).

Transport of radiolabelled choline and choline analogues into free parasites. Free parasites were prepared from aliquots of infected-erythrocytes (~8 x 10⁹ cells/mL) by addition of 5 vol 0.15% (w/v) saponin in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.76 mM K₂HPO₄, 8.0 mM Na₂HPO₄, 5.5 mM D-glucose, pH 7.4) for 1 min, followed by 3 washes by centrifugation and re-suspension in HEPES-buffered RPMI. Freed parasites were then re-suspended into appropriate solutions (RPMI or Ringers buffer (122.5 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgCl₂, 5.5 mM D-glucose, 1.0 mM K₂HPO₄, 10 mM Hepes, pH 7.4), typically 3-5 x 10⁸ cells/mL). Ringers buffer was employed when an external choline concentration of zero was required. The suspensions contained either 50 nM [3H]-pentamidine, 50 nM [3H]-T16, or 20 nM [3H]-choline. All the radiolabelled compounds were added at time zero. Where not shown, a preliminary time course was performed to ensure that uptake of radiolabelled compounds was measured under initial rate conditions throughout. Transport experiments were performed at room temperature (in this case 22 ºC) instead of 37 ºC in order 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, 1C, 3 and 4) the radiolabel was diluted with unlabelled compound to give the final concentrations indicated. Following incubation, aliquots of the suspension were overlaid onto oil (a 5:4 mixture of dibutyl phthalate:diocyl phthalate) and centrifuged (10,000 g, 20 s) sedimenting the cells below the oil. Preliminary experiments using 3H 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 decolourized by the addition (100 µL) of a cocktail containing five parts tissue solubilizer, two parts H₂O₂ (30%) and two parts glacial acetic acid. Samples were then counted by liquid scintillation counting.

A number of transport inhibitors were tested for their ability to inhibit [3H] choline uptake. Compounds were dissolved in DMSO or methanol at a stock concentration of 100 mM. All compounds were pre-incubated with parasites for 5 min prior to addition of [3H]
choline. Control samples were treated with appropriate concentrations of DMSO or methanol alone.

**Transport of radiolabelled choline and choline analogues into uninfected erythrocytes.** This procedure was essentially the same as that employed for free parasites as described above. Erythrocytes were suspended in RPMI, typically at $10^8$ cells/mL and incubated in the presence of 50 nM [³H] T16 or 50 nM [³H] pentamidine, with various concentrations of unlabelled choline for 30 minutes at 22 ºC.

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

The removal of substrate by phosphorylation is important for maintaining linearity of the time course of choline uptake. When measuring the effect of potential transport inhibitors it is important to see if inhibitors are only affecting the transport phase or if 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 analogues. 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 Plasmodium 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 min, with a rate of 1.82 ± 0.08 pmol (10^6 cells)^{-1} min^{-1} (linear regression; r^2 = 0.98). By contrast, very little uptake of [3H] 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 min 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 physiological choline concentrations (Figure 1B). Saturable choline uptake exhibited Michaelis-Menten kinetics with an apparent \( K_m \) value of 25.0 ± 3.5 µM and reaching a \( V_{max} \) of 4.6 ± 0.2 pmol (10^6 cells)^{-1} min^{-1} (Figure 1B). The kinetic parameters were generated by non-linear curve fitting, assuming transport by a single carrier (Erithacus Software Ltd). 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 that are 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 *Plasmodium falciparum*\(^1^8\). Reducing the plasma membrane proton gradient also partially collapses the parasite plasma membrane potential\(^1^9\) 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 caesium chloride to the bathing medium (Figure 2A), a procedure that causes a small hyper-polarization of the parasite plasma membrane\(^1^9\).

**Inhibition of choline uptake into isolated parasites by antimalarial choline analogues and transport inhibitors.** To investigate whether known choline transport inhibitors or antimalarial choline analogues could inhibit the *Plasmodium* choline transporter, we have examined the effect of these analogues on 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\(^2^0\) 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 200 µM), hemicholinium-3
(50 µM) and the choline analogues 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 hemicholinium 3 (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 trans-membrane 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 two 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 IC₅₀ for inhibition of choline uptake by T16 under these conditions is around 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 to 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 Kᵢ value of 3.3 µM, raising the possibility that pentamidine itself is a transport substrate.

Antimalarial choline analogues 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 Kᵢ value of 19 ± 2.5, very similar to the Kₘ for [³H]choline transport. Similar results were found with T16 (data not shown) and it is very likely that both drugs are directly transported by the choline carrier. This argument is supported by the very similar inhibitor profile of drug uptake to choline uptake (Figure 4C). [³H]choline and [³H]pentamidine transport were equally sensitive to the inhibitors furosemide, amiodarone and bepridil (P>0.05). 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 upon consideration that the proton motive force is likely to drive the accumulation of bis-cations like pentamidine to much higher levels than that of mono-cations like choline.

It is likely that transport of pentamidine and T16 via the choline carrier is important to the antimalarial activity of these drugs. However, inhibition of drug uptake by choline is incomplete: approximately 25% of the uptake of both drugs 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 via 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; r² = 0.91).
We have further investigated whether either pentamidine or T16 is transported by the host erythrocyte choline carrier. Previously we have shown that uptake of pentamidine and T16 by infected cells is effectively inhibited by furosemide, a specific NPP inhibitor. As furosemide does not inhibit the endogenous choline carrier, this suggests that both drugs enter the infected cell exclusively via the NPP, and not via the erythrocyte choline transporter. To test this hypothesis, we have investigated the effect of choline on the uptake of [3H]pentamidine or [3H]T16 by infected and uninfected erythrocytes. Infected erythrocytes exhibit cellular accumulation ratios (CAR values) of several-hundredfold after incubation with pentamidine or T16 for 1 hour. A significant proportion (20% - 30%) of the drug is found in the host cell compartment of the infected cell, probably due to weak binding of the drugs to hemoglobin. By contrast, we found that uninfected erythrocytes take up both drugs very slowly, probably by passive diffusion. After 1 hour at room temperature, the CAR values were approximately 3 for pentamidine and 1 for T16, the accumulation again driven by binding to hemoglobin and/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 either pentamidine or T16 is transported by the erythrocyte choline carrier.
Discussion

We report the first characterization of choline transport into isolated parasites of the intra-erythrocytic stages of the human malaria parasite *Plasmodium falciparum*. Choline transport has been studied before in other species of *Plasmodium* but these studies employed intact malaria infected erythrocytes. Without knowing which transport process is rate limiting, the earlier data are difficult to interpret since it is not known whether transport kinetics measured in the multi-compartment infected erythrocyte reflect the activity of choline transporters on the host cell membrane or on the parasite plasma membrane. In this study we have employed *P. falciparum* parasites isolated from their host cells using saponin. This technique has been used to great effect to characterize other *Plasmodium falciparum* plasma membrane transporters. 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 $25.0 \pm 3.5 \, \mu M$ and a $V_{max}$ of $4.6 \pm 0.2 \, \text{pmol (10}^6 \text{ cells})^{-1} \text{min}^{-1}$ (Figure 1B). This is equivalent to a flux of $2.8 \, \text{mmol (10}^{13} \text{ cells})^{-1} \text{hour}^{-1}$ at maximum capacity. Using intact erythrocytes infected with *Plasmodium falciparum*, Kirk et al report a maximum choline influx of $0.75 \, \text{mmol (10}^{13} \text{ cells})^{-1} \text{hour}^{-1}$ at an external choline concentration of $500 \, \mu M$. Even allowing for differences in experimental conditions, this comparison suggests that the transport capacity of the PPM carrier is much higher than the choline flux through the NPP’s. At more physiological external choline concentrations this difference is even more pronounced. For example, at $10 \, \mu M$ external choline, the flux through the endogenous erythrocyte choline carrier will be about $0.017 \, \text{mmol (10}^{13} \text{ cells})^{-1} \text{hour}^{-1}$. This is similar to the flux through the NPP’s of $0.015 \, \text{mmol (10}^{13} \text{ cells})^{-1} \text{hour}^{-1}$ at the same external choline concentration. Thus the combined choline flux through the endogenous and induced host cell transporters will be about $0.03 \, \text{mmol (10}^{13} \text{ cells})^{-1} \text{hour}^{-1}$. This should be compared with the PPM carrier, which can transport choline at a rate of $1.8 \, \text{mmol (10}^{13} \text{ cells})^{-1} \text{hour}^{-1}$ under the same conditions. The enhanced transport rate of the PPM carrier relative to the host cell has two major implications: Firstly, 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. Secondly, at steady-state, there will be a reduced choline concentration in the host cell relative to the medium and the PPM carrier will act like 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 $100nM$ reduces the uptake of choline by about 40-50% (Figure 2A). This concentration of bafilomycin has been shown to almost completely inhibit the parasite plasma membrane proton pump and yet only approximately half of the choline uptake is blocked. Similarly, a 50% inhibition of choline uptake was seen with the proton uncoupler FCCP at $20\mu M$, 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\textsuperscript{19}, and thus of the proton-motive force. We speculate that the choline uptake that remains following inhibition of the proton pump is due to a significant residual membrane potential that apparently is energized by outward K\textsuperscript{+} diffusion\textsuperscript{19}. This argument is supported by the small stimulation of choline uptake that is seen when potassium efflux is blocked with caesium (Figure 2A). Under these conditions, the plasma membrane is hyperpolarized slightly\textsuperscript{19}. Similar phenomena have been well-documented in other protozoa. In \textit{T. brucei} procyclics, for example, only \~{}60\% of adenosine transport could be inhibited by CCCP, which was shown to induce a partial depolarization of the plasma membrane potential and a \~{}70\% reduction of the proton-motive force\textsuperscript{26}. Taken together, our data suggest that choline transport is an electrogenic process in \textit{P. falciparum}.

The \textit{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\textsuperscript{27,28}. The low sensitivity of the \textit{P. falciparum} transporter to HC-3 distinguishes it from the high affinity choline transporter in neurons, which is exquisitely sensitive to this inhibitor\textsuperscript{29}. In many respects, including the dependence on proton-motive force, the \textit{P. falciparum} transporter is very similar to the recently-characterized choline transporter of \textit{Leishmania major}\textsuperscript{30} although \textit{V}_{max} and \textit{K}_{m} of the malarial transporter are about an order of magnitude higher. Of particular interest is the high sensitivity of choline transport of both these parasites to quaternary ammonium compounds with anti-protozoal activity\textsuperscript{30-32}. In the case of malaria parasites, recent studies indicate that bis-cations are more effective than mono-cations as inhibitors of phospholipid metabolism\textsuperscript{12}. Accordingly, we have concentrated on choline analogues 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\textsuperscript{5,13,16} and some have been shown to inhibit choline uptake into intact infected erythrocytes\textsuperscript{12}. Malaria parasites are dependent on an external choline supply and it has been speculated that inhibition of choline uptake could form part of the antimalarial action of these compounds\textsuperscript{13,31,32}. However, the existing data were difficult to interpret because of the multi-compartmental nature of the infected red blood cell and it remained unclear whether the drugs inhibit choline transport via the host erythrocyte choline carrier, the NPP or the parasite choline transporter.

Some of these issues are resolved here: both of the prototype antimalarial bis-cations, pentamidine and T16, are shown to be potent inhibitors of the \textit{P. falciparum} choline transporter. However, it is unlikely that this property forms the basis of the antimalarial action of these compounds: Pentamidine has an \textit{IC}_{50} of 77.0 \pm 4.5 nM against the TM6 strain used in these studies. At this concentration it would inhibit only a small percentage of the total choline flux as its \textit{Ki} value for the choline transporter is 3.3 \mu M. The \textit{Ki} for inhibition of choline transport by T16 also appears to be well over an order of magnitude higher than its antimalarial \textit{EC}_{50}. Our data suggest that the demonstrated inhibition of parasite phospholipid metabolism by these compounds\textsuperscript{12,57} 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 both the accumulation and the antimalarial activity of these compounds. 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 very likely that other intracellular targets exist: For example, it has recently been shown that mono and bis-quarternary ammonium compounds exert their effects on PC synthesis before they inhibit nucleic acid synthesis, suggesting that other enzymes in the Kennedy pathway could be targeted. It should be noted that the mode of action of pentamidine in T. b. brucei, against which it has been used extensively for decades, has never been resolved and is also likely to be multi-factorial because it accumulates to millimolar concentrations inside the parasite. A very similar situation exists in P. falciparum: at minimum inhibitory external drug concentrations in the high nanomolar range, both pentamidine and T16 are accumulated to millimolar concentrations by the intracellular parasite. Given a) that the critical targets are inside the parasite and b) that the passive permeability of these drugs is very low due to 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 the NPP and now show that the major route of entry of these drugs into the intracellular parasite is via 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) and so the inhibition of the antimalarial activity of T16 by high concentrations of choline is most likely to be related to inhibition of drug transport (Figure 5). Neither T16 nor pentamidine are 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 (Roggero R, Zufferey R, Nastase M, Richier E, Calas M, Vial H, Mamoun CB. Antimicrob. Agents Chemother. 2004, in press) Thus the P. falciparum choline carrier is functionally distinct from the known dedicated eukaryotic choline carriers, being more akin to a poly-specific organic cation transporter.

In summary, the NPP and parasite choline carrier form a transport network in the infected erythrocyte that offers two levels of selectivity for antimalarial choline analogues. We show that it is now possible to screen for compounds that are highly specific for the infected erythrocyte. In view of the number and effectiveness of the antimalarial choline analogues that are currently under development, 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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Figure legends

Figure 1
A: Time course of [3H] choline uptake into free parasites of *P. falciparum*. Uptake is measured at 22 °C (open circles) and 0 °C (filled circles). Data points are means of duplicate observations from two individual experiments.
B: Concentration-dependence of Choline uptake into free parasites of *P. falciparum*. Data points are means of duplicate observations from two individual experiments. The data were fitted to a function describing simple ligand binding at a single site by non-linear regression analysis (Marquart method) using an iterative procedure to generate the best fit (Chi squared) of the curve to the data. Standard errors were calculated for each parameter using the matrix inversion method (Grafit user manual, Erithacus Software, Erithacus Software Ltd. P.O. Box 274, Horley, Surrey, RH6 9YJ UK).
C: Hill plot of the data presented in Figure 1B.

Figure 2
A: Effect of ouabain (100 µM), substitution of sodium for N-methyl-D-glucamine, bafilomycin A1 (100 nM), FCCP (20 µM) and caesium chloride (10 mM) on the uptake of [3H] choline (20 nM) into isolated *P. falciparum* parasites over 10 minutes at 22 °C. Data are means plus or minus standard error for duplicate observations from four individual experiments. Results of Mann-Whitney U test for significance of difference between means are given as follows; P < 0.001 = **, P > 0.001 < 0.05 = *
B: Effect of known choline transport inhibitors Furosemide (200 µM), amiodarone (200 µM), bepridil (200 µM) and hemicholinium-3 (50 µM) and antimalarial choline analogues 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 means plus or minus standard error for duplicate observations from four individual experiments. Results of Mann-Whitney U test for significance of difference between means are given as follows; P < 0.001 = **.

Figure 3
A: Concentration-dependent inhibition by T16 of [3H] choline (15 µM) uptake into isolated *P. falciparum* parasites over 10 minutes at 22 °C. Data are means plus or minus standard deviation for duplicate observations from three 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 (open symbols) or choline in the presence of 10 µM pentamidine (filled circles). Data are means plus or minus standard deviation for duplicate observations from three individual experiments.

Figure 4
Antimalarial choline analogues are transported by the *P. falciparum* choline carrier. A: Effect of 5 mM choline (unshaded bars) on the uptake of [3H] T16 and [3H] pentamidine (both at 50 nM) into isolated *P. falciparum* parasites over 10 minutes at 22 °C. Data are means plus or minus standard error for duplicate observations from four individual experiments. B: Concentration-dependent inhibition by choline of [3H] pentamidine (50
nM) uptake into isolated *P. falciparum* parasites over 10 minutes at 22 °C. Data are means plus or minus standard deviation for duplicate observations from three individual experiments. C: Effect of transport inhibitors furosemide (200 µM), amiodarone (200 µM), bepridil (200 µM) and bafilomycin A1 (100 nM) on [3H] choline (20 nM) and [3H] pentamidine (50 nM) uptake into isolated *P. falciparum* parasites over 10 minutes at 22 °C. Data are means plus or minus standard deviation for duplicate observations from three individual experiments. Results of Mann-Whitney U test for significance of difference between means are given as follows; P < 0.001 = **.

**Figure 5**
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 [3H] T16 (50 nM) and the antimalarial IC₅₀ 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 means plus or minus standard error for duplicate observations from three individual experiments. Sensitivity assay data are means plus or minus standard error for triplicate observations from three individual experiments.

**Figure 6**
Effect of external choline concentration (0, 0.05 mM, 0.5 mM and 5 mM) on the uptake of [3H] T16 (50 nM) or [3H] pentamidine (50 nM) into uninfected erythrocytes over 30 minutes at 22 °C. Data are means plus or minus standard deviation for duplicate observations from three individual experiments.
References


Biagini et al Figure 1A
Biagini et al Figure 1B
Log Choline (micromoles per liter)

Log \( \frac{V}{V_{\text{max}} - V} \)

Biagini et al Figure 1C
Biagini et al Figure 2A
Biagini et al Figure 2B
Biagini et al Figure 3A
Biagini et al Figure 3B
Concentration of choline (micromoles per liter)

Pentamidine uptake (fmol/10^6 cells)

Biagini et al Figure 4B
Biagini et al Figure 4C
Control (0) 0.05 mM 0.5 mM 5 mM

Percentage of control drug uptake

Concentration of choline

T16  Pentamidine

Biagini et al Figure 6
Characterization of the choline carrier of *Plasmodium falciparum*: a route for the selective delivery of novel antimalarial drugs