Identification of antimalarial compounds that require CLAG3 for their uptake by *P. falciparum*-infected erythrocytes

Running title: CLAGs and drug antimalarial resistance

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Abstract

During the intraerythrocytic asexual cycle malaria parasites acquire nutrients and other solutes through a broad selectivity channel localized at the membrane of the infected erythrocyte termed Plasmodial Surface Anion Channel (PSAC). The protein product of the *Plasmodium falciparum* clonally variant *clag3.1* and *clag3.2* genes determines PSAC activity. Switches in the expression of *clag3* genes, which are regulated by epigenetic mechanisms, are associated with changes in PSAC-dependent permeability that can result in resistance to compounds toxic for the parasite such as blasticidin S. Here we investigated whether other antimalarial drugs require CLAG3 to reach their intracellular target and consequently are prone to parasite resistance by epigenetic mechanisms. We found that the bis-thiazolium salts T3 (also known as albitiazolium) and T16 require the product of *clag3* genes to enter infected erythrocytes. *P. falciparum* populations can develop resistance to these compounds via selection of parasites with dramatically reduced expression of both genes. However, other compounds previously demonstrated or predicted to enter infected erythrocytes through transport pathways absent from non-infected erythrocytes, such as fosmidomycin, doxycycline, azithromycin, lumefantrine or pentamidine, do not require expression of *clag3* genes for their anti-malarial activity. This suggests that they use alternative CLAG3-independent routes to access parasites. Our results demonstrate that *P. falciparum* can develop resistance to diverse antimalarial compounds by epigenetic changes in the expression of *clag3* genes. This is of concern for drug development efforts because drug resistance by epigenetic mechanisms can arise quickly, even during the course of a single infection.

Background

Malaria is a major public health problem that affects half of the world's population. *P. falciparum* is the predominant species in Africa and the most deadly form of the parasite. It is responsible for half a million deaths every year, mostly in children and pregnant women (1). While chemotherapy is the main tool used for malaria control, *P. falciparum* has developed resistance to all antimalarial drugs, including artemisinin combination therapies (ACTs), which are the current frontline treatment (2, 3). Therefore, the appearance and spread of drug resistant parasites is a major obstacle to malaria control and elimination efforts and urges the discovery of new effective compounds to treat infections.

Most of the known mechanisms by which *P. falciparum* parasites develop resistance to antimalarial drugs are related to changes in the genome such as single nucleotide polymorphisms (SNPs) or gene amplifications (4). SNPs can occur in parasite genes encoding for the enzymes targeted by the drug, reducing the drug affinity as in the case of mutations in the *P. falciparum* dihydrofolate reductase (*pfdhfr)* and dihydropteroate synthase (*pfdhps)* genes (5, 6). Mutations associated with resistance can also occur in parasite-encoded transporters causing the active extrusion of the drug out of its site of action, as in the case of mutations in the chloroquine resistance transporter (*pfcrt)* gene associated with the efflux of chloroquine out of the digestive vacuole (7). Likewise, genetic amplifications can increase the expression of the target gene, or the expression of genes encoding transporters, as in the case of amplification of the multidrug resistance protein 1 (*pfmdr1*) gene that leads to accumulation of mefloquine in the digestive vacuole away from its predicted target (8).

Transport activities present in infected erythrocytes that mediate the uptake of solutes unable to enter uninfected erythrocytes are collectively referred to as new permeation pathways (NPPs) (9-

11). The Plasmodial Surface Anion Channel (PSAC) has been proposed to be the single channel responsible for NPPs. This broad selectivity channel, localized at the membrane of the infected erythrocyte, is essential for the uptake of nutrients and several other solutes (12-16). The protein product of *P. falciparum clag3.1* and *clag3.2* genes, part of the five-member *clag* family that encodes the CLAG/RhopH1 component of the RhopH complex (17), plays a key role for the activity of the PSAC (16, 18-20). Other members of the RhopH complex, RhopH2 and RhopH3, are also necessary for PSAC activity (21-23). The structure of the PSAC has not been determined yet, but protease sensitivity assays and experiments with various transgenic parasite lines suggest that CLAG3s (and possibly also RhopH2 and RhopH3) may participate directly in the formation of the channel rather than only activating a channel formed by other proteins (20, 22, 24). The RhopH complex is initially expressed at the schizont stage and localized in the rhoptries (25). About 20 h after reinvasion it is transported to the red cell membrane, where it determines PSAC activity (22, 23, 26). The sequence of *clag3.1* and *clag3.2* genes is 95% identical. These genes display clonally variant and mutually exclusive expression, such that commonly only one of the two *clag3* genes is expressed at a time (27). The latter property was observed in culture-adapted parasite lines of different genetic backgrounds (18, 19, 28, 29) and has been later confirmed in uncomplicated human malaria infections (30), although mutual exclusion is not strict (31).

Recently, an epigenetic mechanism of drug resistance involving changes in the expression of *clag3* genes was described in *P. falciparum* (32, 33). Previous studies demonstrated that blasticidin S and leupeptin require PSAC for their transport across the membrane of infected erythrocytes, and that *P. falciparum* resistance to these compounds is associated with changes in PSAC function (13, 34, 35). Later, we and others showed that changes in PSAC-mediated transport of blasticidin S were associated with switches in *clag3* genes expression regulated at the epigenetic level (32, 33). Resistance to low blasticidin S concentrations involved selection of

parasites that switched from *clag3.2* to *clag3.1* expression, whereas resistance to high concentrations of the drug was acquired by selection of parasites with both *clag3* genes simultaneously silenced (32). In all cases, *clag3* silencing is mediated by heterochromatin (31). The pattern of *clag3* expression in the selected parasites is transmitted to the next generations by epigenetic mechanisms even when the drug is no longer present. However, simultaneous silencing of the two genes poses a fitness cost for the parasite and in the absence of selection it is progressively reverted.

Whether other antimalarial drugs require the product of *clag3* genes to reach their intracellular targets and consequently are prone to parasite resistance by this epigenetic mechanism is not known. Most antimalarials are small hydrophobic compounds that can diffuse through lipid membranes and do not require specific channels to enter infected erythrocytes (36). However, large hydrophilic compounds such as blasticidin S and leupeptin require facilitated uptake through PSAC. Drug physicochemical parameters such as molecular size and hydrophobicity indexes, e.g. the *logP* value, can be used to predict which antimalarial drugs require PSAC-facilitated transport (36, 37). However, while such *in silico* predictions are informative, only experimental validation can determine which drugs are actually prone to parasite resistance by epigenetic silencing of *clag3* genes.

To address this question, here we compared the IC_{50} of selected antimalarial compounds between the blasticidin S-selected 10G-0.6-2 line, which has both *clag3* genes silenced and thus shows deficient PSAC transport, and the parental 10G line, which predominantly expresses *clag3.2* (32). In addition, we selected parasites with some of the drugs and monitored switches in the expression of *clag3* genes during selection. We also investigated PSAC transport in CLAG3-defficient parasites using the reporter compound 5-aminolevulinic acid (5-ALA).

Results

The 10G-0.6-2 line is a valid tool to investigate transport via CLAG3-containing PSAC.

We previously showed that the 10G-0.6-2 parasite line, derived from the 10G line selected with a high concentration of blasticidin S, shows dramatically reduced expression of the two *clag3* genes. The clonally variant *clag2* gene is silenced in both the 10G-0.6-2 and the parental 10G lines, whereas the non-clonally variant *clag8* and *clag9* genes are expressed in both lines (32). Silencing of *clag3* genes in 10G-0.6-2 results in reduced permeability to structurally diverse compounds such as blasticidin S, sorbitol and the canonical amino acid L-Alanine (32). To further demonstrate that the 10G-0.6-2 line is a valid tool to identify drugs that require CLAG3 to cross the membrane of the infected erythrocyte, we conducted immunofluorescence assays (IFAs) on 10G-derived lines selected with different concentrations of blasticidin S (32) using an anti-CLAG3 antibody that recognizes both CLAG3.1 and CLAG3.2 (19). By restricting the analysis only to schizonts positive for the mature schizont marker AMA1, we could unambiguously identify parasites in which absence of CLAG3 signal was attributable to epigenetic silencing rather than to parasite stage. We found that essentially all mature schizonts in the parental 10G line express CLAG3 (either CLAG3.1 or CLAG3.2), whereas the proportion of CLAG3-negative mature schizonts was 12% in 10G-0.4, 74% in 10G-0.6, and 98% in 10G-0.6-2 cultures (selected with 0.4, 0.6 and 2 μg/ml of blasticidin S, respectively) (Fig. 1A-B). These experiments at the single cell level show that the vast majority of parasites in 10G-0.6-2 cultures do not express CLAG3 proteins at detectable levels, validating the 10G-06-2 line as an appropriate tool to identify compounds that require CLAG3 to enter the cell.

Parasites with deficient PSAC transport due to silencing of *clag3* genes are resistant to bis-thiazolium salts.

To identify antimalarial compounds that require the product of *clag3* genes for efficient transport across the membrane of infected erythrocytes, we selected drugs to be tested based on two criteria. First, we included drugs of clinical relevance for which physicochemical properties suggest that they may not enter infected erythrocytes by passive membrane diffusion, i.e. doxycycline, azithromycin and lumefantrine (36). Second, we included drugs for which there is previous evidence for uptake through NPPs, i.e. fosmidomycin (38), pentamidine (39), and the bis-thiazolium salts T3 (also known as albitiazolium) and T16 (40, 41). As positive controls we included drugs for which there is already clear evidence of CLAG3-dependent uptake through PSAC, i.e. blasticidin S and leupeptin (32, 33). For the selected compounds, we compared the IC_{50} between the 10G-0.6-2 line and the parental 10G line.

Our criteria to consider that the uptake of a drug is impaired by the absence of CLAG3 was a 1.5 fold increase in the IC_{50} in 10G-0.6-2 vs 10G, plus a statistically significant difference ($p<0.05$). The former criterion was used because we consider that differences of lower magnitude are unlikely to have a major biological significance. Using these criteria, dose-response curves revealed that the 10G-0.6-2 line shows lower sensitivity to blasticidin S, T3, T16, and leupeptin than the 10G line (Fig. 2 and Table 1). These results support the idea that T3 and T16 require expression of *clag3* genes for their uptake, in addition to leupeptin and blasticidin S for which this was previously demonstrated (34) (13, 32, 33). The IC_{50} fold increase in 10G-0.6-2 compared to 10G was 3.3 for T3 and 1.8 for T16, which is lower than the 4.6 and 9.5-fold increase observed for leupeptin and blasticidin S, respectively (Table 1). On the other hand, we did not obtain evidence for *clag3* genes playing a role in the uptake of the other compounds

155 tested, as the difference in their IC_{50} between 10G and 10G-0.6-2 cultures was not statistically
156 significant and/or was of very low magnitude (Fig. 2 and Table 1).

157

158 **_clag3_ expression patterns after selection with different drugs.**

159 We and others have previously shown that adaptation to grow in the presence of blasticidin S is
160 associated with selection of parasites with altered *clag3* expression patterns, validating the
161 involvement of these genes in the transport of the drug (30, 32, 33). Here we investigated
162 whether sublethal concentrations of other drugs can also select parasites with specific *clag3*
163 expression patterns. In these experiments we tested drugs to which 10G-0.6-2 is less sensitive
164 than 10G (Fig. 2). Additionally, doxycycline and fosmidomycin were included in spite of not
165 showing differences between 10G and 10G-0.6-2 because previous studies suggested that they
166 may require NPPs for their transport into infected erythrocytes (36, 38). We considered that
167 selection experiments may be more sensitive than IC_{50} comparisons to detect the involvement of
168 CLAG3 in their transport.

169 We selected 10G cultures with the drugs at concentrations ranging from approximately the IC_{50}
170 to the IC_{80}. We used these relatively low concentrations because we predicted that this may
171 facilitate adaptation by selection of parasites expressing *clag3.1* or *clag3.2*, if expression of one
172 or the other paralog was associated with differential transport of the drug as in the case of
173 blasticidin S (32). However, for T3 and T16 we observed that toxicity is much higher in the
174 second and subsequent cycles than in the first cycle, such that selection with this range of drug
175 concentrations killed the cultures after only a few cycles. We determined the approximate IC_{50} of
176 T3 and T16 over two cycles and found that it was ~10 times lower than in a one-cycle assay; we

used T3 at 2.86 nM and T16 at 0.6 nM for selection. For doxycycline, which shows a delayed death effect, we also selected cultures with a concentration of the drug based on the IC_{50} in a two-cycles assay. Cultures were selected until we obtained evidence for adaptation (see Methods and Fig. S1) or for a maximum of 14 weeks. 10G cultures selected with blasticidin S (650 nM, corresponding to 0.3 µg/ml, a concentration that inhibits growth by ~60%) were used as a positive control. These cultures switched from predominant *clag3.2* to *clag3.1* expression after only 3 weeks of selection (80-fold increase in the *clag3.1/clag3.2* ratio), which is consistent with previous results (32) (Fig. 3A).

Cultures selected with T3 or T16 showed a prominent decrease in the expression levels of both *clag3* genes after only 2 weeks (Fig. 3A and Fig. S2). This was observed in selection experiments performed with the parasite lines 10G and also 3D7-A, a previously described stock of the *P.falciparum* clonal line 3D7, from which the 10G subclone was derived (27, 42). As expected from this result, in cultures selected with T3 the vast majority of parasites were resistant to sorbitol lysis (Fig. 3B) and negative for CLAG3 expression by IFA (Fig. 3C), similar to the blasticidin S-selected 10G-0.6-2 line. Together with the increased IC_{50} values observed for these drugs in the 10G-0.6-2 line, these results demonstrate that the uptake of T3 and T16 by infected erythrocytes requires CLAG3. As in the case of blasticidin S-selected cultures (32, 33), *clag3* silencing was reversible and removal of the drug resulted in a progressive selection of parasites that express *clag3* (Fig. S3). This is probably attributable to the fitness cost associated with simultaneous silencing of the two *clag3* genes (32). We also selected cultures with lower concentrations of T3, but we did not obtain evidence for any major alteration in the *clag3.1/clag3.2* ratio that would reflect selection of parasites expressing one or the other *clag3* gene (Fig. S4).

On the other hand, 10G cultures selected with fosmidomycin, doxycycline and leupeptin did not show any clear alteration in the expression of *clag3* genes compared to control 10G cultures maintained in parallel without drug (Fig. 3A).

Parasites with dramatically reduced levels of CLAG3 expression still acquire some compounds through the NPPs.

Previous reports demonstrated that the uptake of fosmidomycin or pentamidine depends on NPPs (38, 39), and the uptake of other compounds such as doxycycline, lumefantrine and azithromycin is strongly predicted to also require NPPs (36). Therefore, the results of the selection experiments with these drugs and especially the similar IC_{50} between 10G and 10G-0.6-2 were somehow unexpected. To test the possibility that the transport of specific compounds via NPPs is active in the 10G-0.6-2 line in spite of its dramatically reduced levels of CLAG3, we compared the uptake of 5-ALA between the 10G-0.6-2 line and the parental 10G. Inside the infected erythrocyte, 5-ALA is processed to the fluorescent compound Protoporphyrin IX (PPIX) that can be visualized by microscopy. 5-ALA has been previously observed to enter infected erythrocytes through NPPs and its uptake requires RhopH3 (a component of the RhopH complex), which indicates that it uses the PSAC (23, 43). We observed that the uptake of 5-ALA was impaired in RhopH3-defficient parasites, as previously reported (23). In contrast, essentially all mature parasite-infected erythrocytes acquired the compound in the 10G, 10G-0.6-2 and T3-selected lines (Fig. 4). This result indicates that CLAG3 is not essential for the acquisition of some specific PSAC substrates such as 5-ALA. This is in contrast to PSAC-mediated transport of blasticidin S, leupeptin, sorbitol, L-Alanine, T3 or T16, which appears to depend more strongly on CLAG3 expression.

Discussion

We and others have previously shown that epigenetic changes in the expression of *clag3* genes modify the permeability of the infected erythrocyte membrane through alterations in PSAC function, and that these changes can confer resistance to compounds such as blasticidin S and leupeptin (13, 32-35, 44). Here, we investigated whether other compounds with antimalarial activity require the product of *clag3* genes to enter the infected erythrocyte and are thus prone to parasite resistance due to epigenetic changes in *clag3* genes expression. For this aim, we compared the IC_{50} for several compounds with antimalarial activity between the parental 10G line and the 10G-0.6-2 line, which expresses dramatically reduced levels of *clag3* genes. Of note, the clonally variant gene *clag2*, which has also been implicated in infected erythrocyte permeability, is silenced in both the 10G and the 10G-0.6-2 lines (27, 32). This excludes changes in the expression of this gene as a confounding factor for the results obtained. In addition, we selected cultures with some of the drugs for several weeks, and measured *clag3.1* and *clag3.2* transcript levels to investigate if the drugs select for parasites with altered *clag3* expression. Both approaches led to the identification of T3 and T16 as new antimalarial compounds that require CLAG3 for their uptake.

We characterized the 10G-0.6-2 line by IFA using anti-CLAG3 antibodies and found that ~98% of the parasites in the population do not express CLAG3 at detectable levels, which is consistent with the previously described high level of resistance to sorbitol and blasticidin S and dramatically reduced *clag3* transcript levels in this parasite line (32). However, we also characterized PSAC functionality in this parasite line using 5-ALA, a compound that specifically enters infected erythrocytes and is then converted to fluorescent PPIX. The uptake of 5-ALA is inhibited by furosemide (a PSAC/NPPs inhibitor) or by conditional depletion of RhopH3 (23,

43), which demonstrates that it is a PSAC substrate that can be used as a convenient reporter for PSAC activity. Surprisingly, we observed PPIX fluorescence indicative of transport through PSAC in the 10G-0.6-2 line, despite silencing of both *clag3* genes. Although we cannot exclude the possibility that undetected quantitative differences may occur in the transport of 5-ALA between control and CLAG3-defficient parasites, these results suggest that the transport of some compounds into infected erythrocytes may be mediated by CLAG3-independent PSAC, possibly involving other CLAGs such as CLAG8 or CLAG9. These genes appear to be expressed by all parasites, as they have not been found to show clonally variant expression or to carry epigenetic marks of silencing (45-48). We hypothesize that PSAC involving CLAG8 or CLAG9 may ensure transport of some solutes even when total PSAC transport is highly reduced due to silencing of both *clag3* genes. The PSAC formed in the absence of CLAG3 appears to be able to mediate the uptake of compounds such as 5-ALA, but its ability to transport other compounds such as blasticidin S, sorbitol, L-Alanine, T3 or T16 is severely impaired. Altogether, these results indicate that the 10G-0.6-2 line provides an appropriate tool to measure drug uptake in the absence of CLAG3 and CLAG2. However, NPPs likely mediated by PSAC formed in the absence of CLAG3 are active in this parasite line, resulting in the uptake of some solutes. Considering that variant expression and epigenetic silencing has only been reported for CLAG3s and CLAG2, only drugs that show lower activity on the 10G-0.6-2 line are prone to parasite resistance by epigenetic silencing of *clag* genes. Drugs for which uptake may require PSAC but independently of the presence of CLAG3 are unlikely to develop parasite resistance by this mechanism.

Our results demonstrate that in addition to blasticidin S, leupeptin, sorbitol and L- Alanine (19, 32, 33), T3 and T16 also require the product of *clag3* genes to enter infected erythrocytes. The bis-thiazolium salts T3 and T16 are choline analogs that inhibit the synthesis of

phosphatidylcholine. They are able to cure malaria infections *in vivo* in mice, primates and humans, but their clinical development was discontinued because of rapid drug clearance in children (49). T3 concentrates massively in infected erythrocytes in an energy-dependent and saturable process, which underlies its antiplasmodial activity at low nanomolar concentration (50). We observed a clear increase in the IC_{50} values for T3 and T16 in 10G-0.6-2 compared to 10G, which together with silencing of both *clag3* genes after only 2 weeks of selection with these drugs demonstrates that *P. falciparum* can develop resistance to them by changes in the expression of *clag3* genes (30, 32). The concentration of T3 and T16 that was used in the selection experiments was below the IC_{50} of the drugs, which explains the difference with previous reports concluding that choline analogs are likely not prone to drug resistance (50). Resistance to drugs at low concentration is physiologically relevant because low drug concentration can be encountered by parasites at some points during a treatment course. However, in spite of selecting parasites with T3 and T16 at low concentrations, we observed silencing of both *clag3* genes, a pattern that in the case of blasticidin S was only observed when using high concentrations ($>>IC_{50}$) of the drug. While resistance to blasticidin S at low concentration can be acquired by selection of parasites that switched from expression of one *clag3* gene to expression of the other, which does not pose a fitness cost, this was not observed for T3. Thus, our experiments did not identify additional compounds for which there are differences in sensitivity (likely reflecting differences in transport efficiency) between parasites that express *clag3.1* and parasites that express *clag3.2*.

The increased IC_{50} for T3 and T16 in the 10G-0.6-2 line relative to 10G is moderate when compared to the increase observed for blasticidin S (~2-3-fold versus ~10-fold). This is consistent with previous reports showing that the transport of T3/T16 into the infected erythrocytes is highly reduced, but not eliminated, by treatment with the NPP inhibitor

furosemide. The authors concluded that transport of these compounds occurs mainly through PSAC/NPPs, but residual transport (~15%) is non-saturable and continues to occur even in the presence of high concentrations of the inhibitor (40, 41). Residual transport of T3/T16 through membrane diffusion or endogenous transporters may explain the relatively modest differences in IC_{50} values between 10G and 10G-0.6-2 cultures.

A previous study analyzing the effect of T4, a bis-thiazolium compound structurally related with T3 and T16, did not detect significant transcriptional changes in 3D7 cultures after 30 min to 36 h of exposure to the drug (51). The apparent discrepancy with our results reflects the very different experimental approaches used: while the previous study analyzed changes in mRNA levels soon after drug exposure to explore the occurrence of directed protective transcriptional responses, we studied adaptations at the transcriptional level after several cycles of selection with the drug. Our approach revealed changes in the expression of *clag3* genes linked to adaptation, indicating that selection of parasites with specific expression patterns of clonally variant genes can occur in the development of resistance to compounds to which the parasite is unable to mount a directed transcriptional response. Of note, natural selection of parasites with specific transcriptional patterns is the basis of bet-hedging adaptive strategies in *P. falciparum* (47, 48).

We did not observe major differences in the IC_{50} for doxycycline, fosmidomycin, azithromycin, lumefantrine or pentamidine between the 10G and 10G-0.6-2 lines. However, there is robust previous data for fosmidomycin and pentamidine indicating that these drugs require NPPs for their entry into infected erythrocytes: their uptake is abrogated by NPPs inhibitors and they concentrate massively inside the infected erythrocytes (38, 39). Hence, the most plausible explanation for the similar sensitivity to these compounds between the 10G-0.6-2 and 10G lines is that their uptake occurs via CLAG3-independent NPPs, as in the case of the PSAC substrate 5-

ALA. We consider it likely that PSAC involving non-variantly expressed CLAGs, which we predict to mediate 5-ALA uptake in CLAG3-defficient lines, also mediates the uptake of drugs such as fosmidomycin and pentamidine in these lines. However, we cannot fully exclude the possibility that the residual expression of CLAG3 in 10G-0.6-2, which is undistinguishable from background signal in IFA experiments, is sufficient to mediate transport of these compounds. In any case, given that doxycycline, fosmidomycin, lumefantrine, azithromycin and pentamidine can access and kill parasites expressing dramatically reduced levels of *clag3* genes, it is unlikely that *P. falciparum* can develop resistance to these compounds by selection of parasite subpopulations with altered *clag3* expression as in the case of blasticidin S, T3 or T16.

Altogether, we identified T3 and T16 as antimalarials that require CLAG3-containing PSAC to enter the infected erythrocyte, and thus these drugs are prone to parasite resistance by epigenetic changes in the expression of *clag3* genes. This is an important concern because resistance acquired at the epigenetic level can arise quickly during the course of a single infection and is easily reversible, providing the parasite with a level of plasticity towards susceptible drugs that would promptly render them ineffective. On the other hand, our results suggest that other compounds known or predicted to require NPPs for their uptake by infected erythrocytes may use PSAC involving CLAG8 or CLAG9, or other channels different from PSAC. To determine which drugs require any form of PSAC to access infected erythrocytes, experiments similar to the ones presented here could be performed using conditional KO lines for *rhoph2* or *rhoph3* (21-23). However, even if these experiments identified additional drugs that require PSAC for their uptake, these drugs would not be prone to the resistance mechanism regulated at the epigenetic level studied here if they show normal transport in the 10G-0.6-2 line.

Materials and Methods

Parasite cultures.

The 3D7-A stock of the clonal *P. falciparum* line 3D7 and the 10G subclone of 3D7-A have been previously described and characterized (27, 42, 52). The 10G-0.4 and 10G-0.6 lines were generated by selection of the 10G line with blasticidin S at 0.4 µg/ml and 0.6 µg/ml, respectively, whereas the 10G-0.6-2 line was generated by sequential selection with 0.6 µg/ml and 2 µg/ml (32). The 5F5 inducible *rhoph3* disruption line has been previously described (23). Parasites were cultured in O- erythrocytes at a 3% haematocrit with inactivated human serum under standard culture conditions, except for selection experiments with T3 and their controls that were performed with B+ erythrocytes and media containing Albumax II instead of serum. The 10G-0.6-2 parasite line was regularly cultured under blasticidin S pressure (2 µg/ml) to maintain silencing of both *clag3* genes (32). For synchronization, we used treatment with 5% sorbitol, except for the 10G-0.6-2 line, the 10G line selected with T3 or T16, and the unselected 10G controls analysed in parallel that were synchronised with L-Proline as previously described (31). To prepare RNA for transcriptional analysis, cultures were harvested when the majority of parasites were at the schizont stage, i.e. when *clag3* genes are expressed, and a small proportion of schizonts had already burst.

IFAs.

IFAs were performed on Percoll-purified parasites at the mature schizont stage because at this stage CLAG3 yields a strong and unambiguous signal in rhoptries, whereas detection at other stages in which the protein is only present in the surface of infected erythrocytes is less clear.

Air-dried smears were fixed for 10 min with 1% paraformaldehyde and permeabilised for 10 min with 0.1% Triton X-100 in PBS. Smears were incubated with rabbit anti-3D7 AMA1 (1:2,000; a kind gift from Dr. Robin F. Anders, La Trobe University, Australia) (53) and mouse anti-CLAG3 (1:2,000; from mouse 167#2, a kind gift from Dr. Sanjay A. Desai, NIAID-NIH, USA) (19) polyclonal antibodies. We used secondary anti-rabbit antibodies conjugated with Alexa Fluor 488 (Life technologies A-11034) and anti-mouse antibodies conjugated with Alexa Fluor 546 (Life technologies A-10036). Nuclei were stained with DAPI. Preparations were observed under a confocal Leica TCS-SP5 microscope with LAS-AF image acquisition software and processed using ImageJ. AMA1, which is expressed later than CLAG3 during intraerythrocytic development and does not show variant expression, was used to identify parasites that were mature enough for CLAG3 expression. This enabled us to distinguish between parasites that did not express CLAG3 because they were at a too early stage of development from parasites in which both *clag3* genes were simultaneously silenced at the epigenetic level. The analysis of CLAG3 expression was restricted to AMA1-positive parasites: the proportion of schizonts that have both CLAG3s silenced was determined by counting >100 AMA1-postitive mature schizonts in each replicate experiment.

Drugs.

Blasticidin S, lumefantrine, doxycycline, fosmidomycin, leupeptin, and azithromycin were purchased from Sigma-Aldrich (reference numbers 15205, L5420, D9891, F8682, L8511and PZ0007, respectively). T3, T16 and pentamidine were a kind gift from Dr. Henri J. Vial (CNRS, Montpellier, France). Stock solutions for each drug were prepared as follows: azithromycin and pentamidine in DMSO at 10 mM, blasticidin S in H_2O at 10 mM, doxycycline in methanol at 20

385 mM, fosmidomycin in PBS at 1 mM, T3 and T16 in H_2O at 10 mM, and lumefantrine at 1 mM in
386 a 1:1:1 volume mix of Tween 80, EtOH and linoleic acid (prepared from a 5 mM stock in
387 DMSO).

388

389 **Growth inhibition assays.**

390 To determine the IC_{50} of the different compounds in 10G and 10G-0.6-2 cultures, we used a
391 previously described SYBR Green-based assay (54) with some adjustments. We incubated
392 parasites in triplicate wells for 96 h for experiments with blasticidin S, leupeptin, lumefantrine,
393 fosmidomycin, pentamidine, T3 and T16, which kill the parasites in the first cycle after drug
394 administration, or for 144 h for drugs that produce a delayed death effect (55), killing parasites
395 only at the second cycle after adding the drug (doxycycline and azithromycin). Initial
396 parasitaemias were adjusted for each incubation time and parasite strain in order to prevent the
397 culture from collapsing, while ensuring a sufficiently high final parasitaemia (in the absence of
398 drug) for accurate determination of the inhibition levels. The parasitaemia of synchronized ring
399 stage cultures was determined by light microscopy and adjusted to 0.1-0.2% or 0.05% for 96 h
400 and 144 h experiments with the 10G line, respectively, and to 0.2-0.5% or 0.1% for 96 h and 144
401 h assays with 10G-0.6-2, respectively. In experiments with 10G-0.6–2 cultures, which were
402 regularly maintained under blasticidin S pressure, blasticidin S was removed 5 h before starting
403 the assay. After 96 or 144 h of incubation, parasites were exposed to one freeze/thaw cycle and
404 100 µl of lysis buffer (54) with SYBR Green 5X were added per well. Plates were incubated at
405 37ºC in the dark for 3 h and then fluorescence was measured on a plate reader (VICTOR X3
406 Perkin Elmer) with excitation and emission wavelength bands centred at 485 and 535 nm,
407 respectively. After LOG-transforming drug concentrations, data was fit to sigmoidal dose–

response curves using GraphPad Prism (version 5) setting the maximum to 100 and the minimum to 0. IC_{50} values were compared between 10G and 10G-0.6-2 using a two-tailed t-test for unpaired data (Stata version 12). We applied the Benjamini-Hochberg multiple testing correction of *p* values (56), with a false discovery rate of 0.1.

Drug selection experiments.

To select cultures with doxycycline, fosmidomycin, T3, T16, leupeptin or blasticidin S, the drugs were initially applied to cultures at the ring stage. Cultures were maintained under sublethal drug concentrations (determined in preliminary experiments) for a maximum of 14 weeks, or until clear evidence of adaptation to the drug was observed, i.e. an increase in the growth rate in the presence of the drug compared to the initial growth rate when the drug was first added (Fig. S1). Unselected cultures were maintained in parallel. In all selection experiments parasites were harvested for RNA extraction every 3-5 weeks.

Transcriptional analysis.

For RNA purification, erythrocyte pellets were collected in Trizol (Invitrogen) and phase separation was conducted following manufacturer indications. RNA was purified from the aqueous phase using the RNeasy Mini Kit (Qiagen) as previously described (30) and reverse transcribed using the Reverse Transcription System (Promega). To exclude gDNA contamination, parallel reactions were performed in the absence of reverse transcriptase. cDNAs were analysed by quantitative PCR in triplicate wells using Power SYBR Green Master Mix

(Applied Biosystems) in a StepOnePlus or a 7900HT Fast Real Time PCR System (Applied Biosystems). *clag3.1* and *clag3.2* expression values, in arbitrary units, were calculated using the standard curve method as previously described (30). The primers used have been previously described (30).

Analysis of erythrocyte membrane permeability by 5-ALA uptake.

The 5F5 inducible *rhoph3* disruption line was treated with rapamycin at the cycle prior to testing 5-ALA uptake to induce deletion of exons 4-6(23), or treated in parallel with DMSO as a control. 5-ALA uptake was determined as previously described (23, 43), with minor modifications. In brief, cultures of synchronous ring-stage parasites were incubated overnight in normal RPMI-based parasite culture medium with Albumax II supplemented with 200 µM 5-ALA (Sigma-Aldrich, #A3785). After uptake by infected erythrocytes, 5-ALA is converted to PPIX. Just prior to analysis, parasite nuclei were stained with 2 µg/ml Hoechst for 10min at 37ºC and washed with PBS. Samples were placed in an 8-well chamber slide for live cell fluorescence microscopy analysis using the Fluorescence Imaging System Leica AF6000. Images were captured with the same acquisition settings for all samples, so that signal intensities are directly comparable. Images were analysed using *ImageJ* software.

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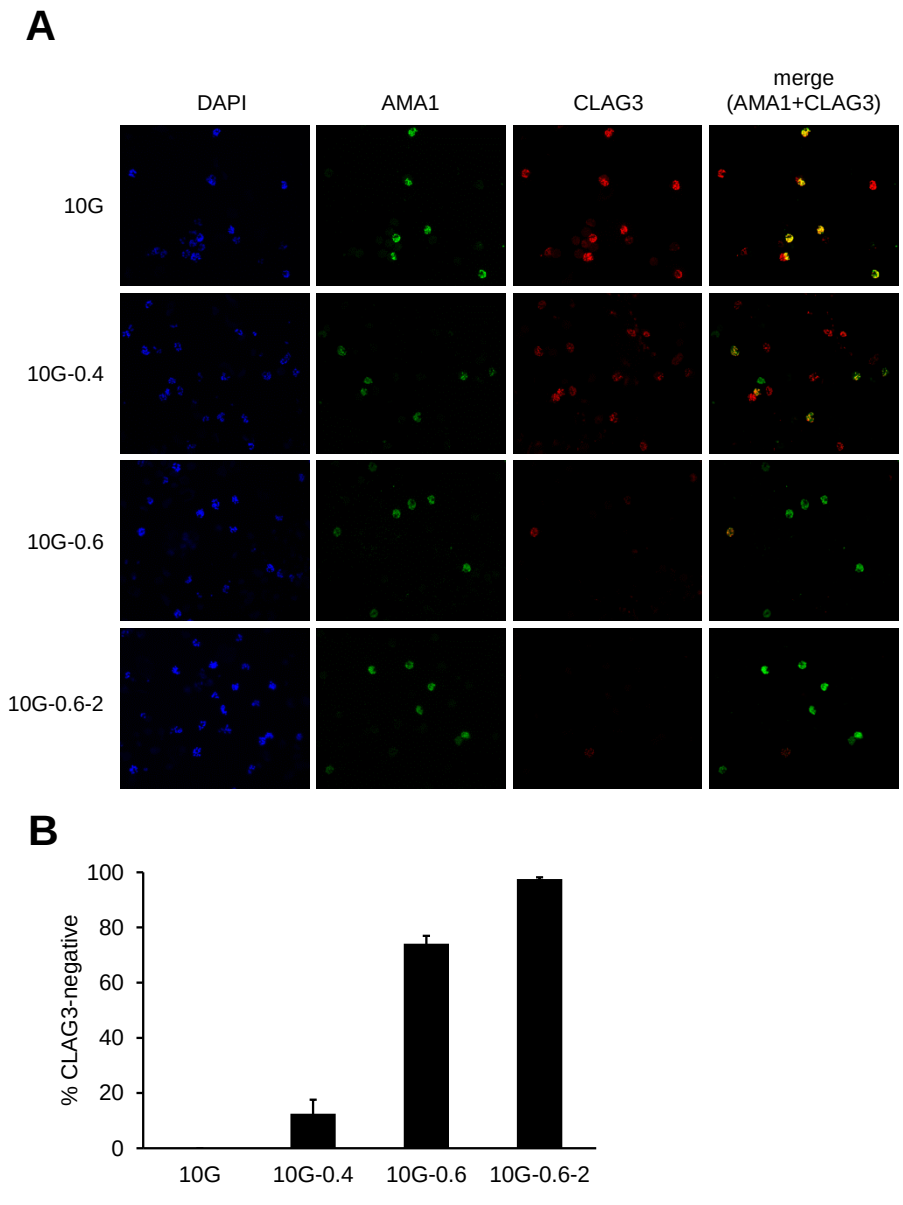
TABLE 1 IC_{50} of different drugs in the 10G and the 10G-0.6-2 lines.

DRUG	**IC_{50} (nM)**		**FOLD-change**	***p* value**
	10G	**10G-0.6-2**		
azithromycin	95 (22)	93 (19)	1.0	0.899
doxycycline	718 (11)	563(121)	0.8	0.091
fosmidomycin	841 (219)	985 (184)	1.2	0.434
leupeptin	1753 (1)	8010 (7)	4.6	0.039*
lumefantrine	36 (6)	40 (2)	1.1	0.394
pentamidine	106 (8)	132 (9)	1.2	0.019*
T3	26 (5)	85 (8)	3.3	0.003*
T16	10 (2)	18 (1)	1.8	0.002*
blasticidin S	530 (96)	5060 (1045)	9.5	0.002*

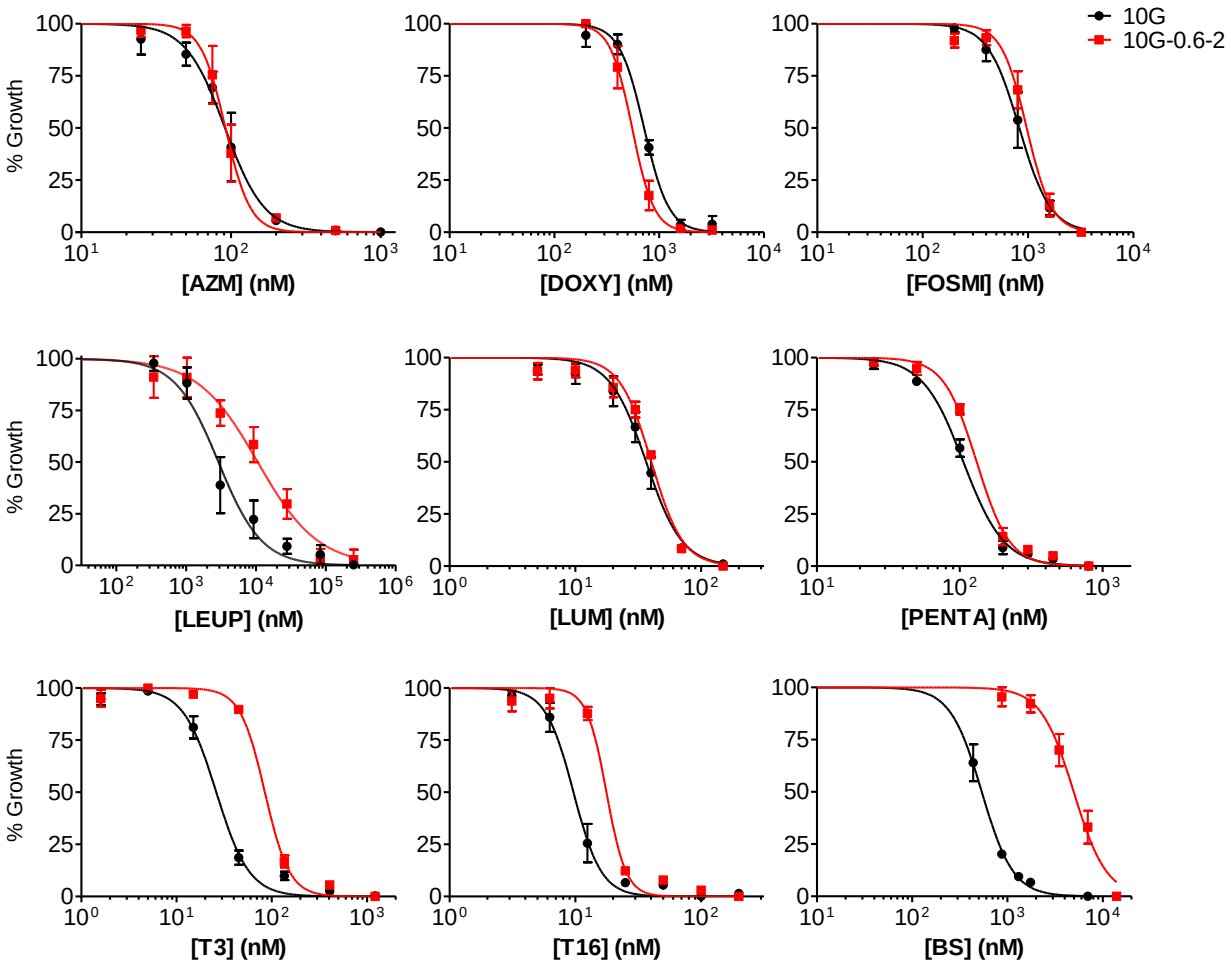
IC_{50} values are the average of three independent experiments (with SD in brackets) shown in Fig. 2. *p* values were calculated using a two-tailed unparied t-test. Significant differences between 10G and 10G-0.6-2 after applying the Benjamini-Hochberg correction for multiple testing with a false discovery rate of 0.1 are indicated with an asterisk.

Figures



680

681 **FIG 1** CLAG3 expression in parasite cultures selected with different concentrations of
682 blasticidin S. (A) Immunofluorescence Assay (IFA) analysis of mature schizont-infected
683 erythrocytes with anti-CLAG3 and anti-AMA1 antibodies in blasticidin S-selected lines and the
684 parental 10G line. DAPI marks parasite nuclei. Anti-AMA1 antibodies were used to identify
685 schizonts sufficiently mature for CLAG3-expression. (B) Proportion of AMA1-positive parasites
686 that were negative for CLAG3 fluorescence in each parasite line. Results are the average of two
687 independent biological replicates, with SD.



688

689 **FIG 2** Drug dose-response curves for parasite lines 10G and 10G-0.6-2. Comparison of the
690 susceptibility to azithromycin (AZM), doxycycline (DOXY), fosmidomycin (FOSMI), leupeptin
691 (LEUP), lumefantrine (LUM), pentamidine (PENTA), T3, T16 and blasticidin S (BS) between
692 the parasite lines 10G (predominantly expressing *clag3.2*) and 10G-0.6-2 (both *clag3* genes
693 silenced). Values are the average of three independent experiments, each performed in triplicate
694 wells, with SD.

695

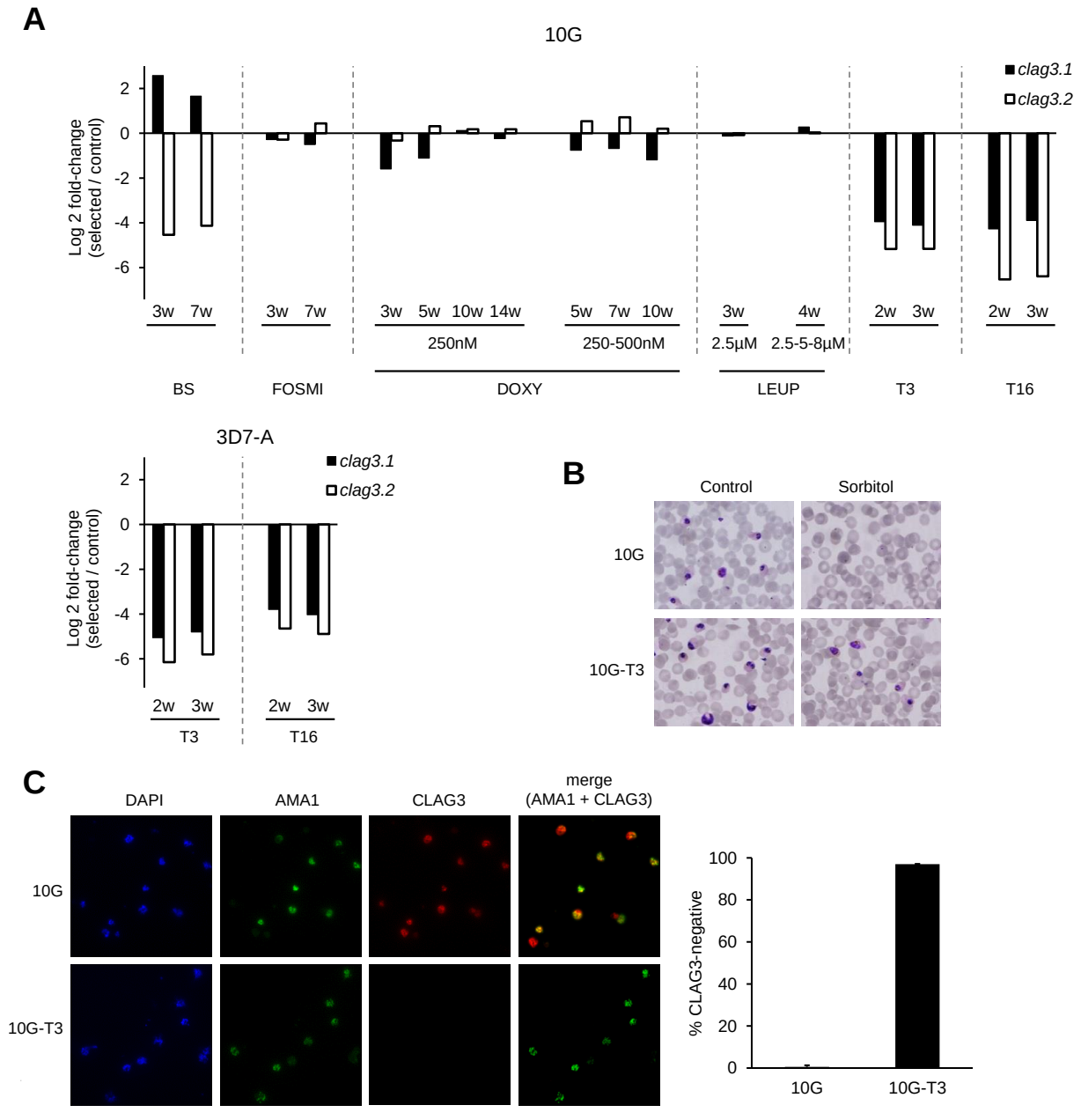
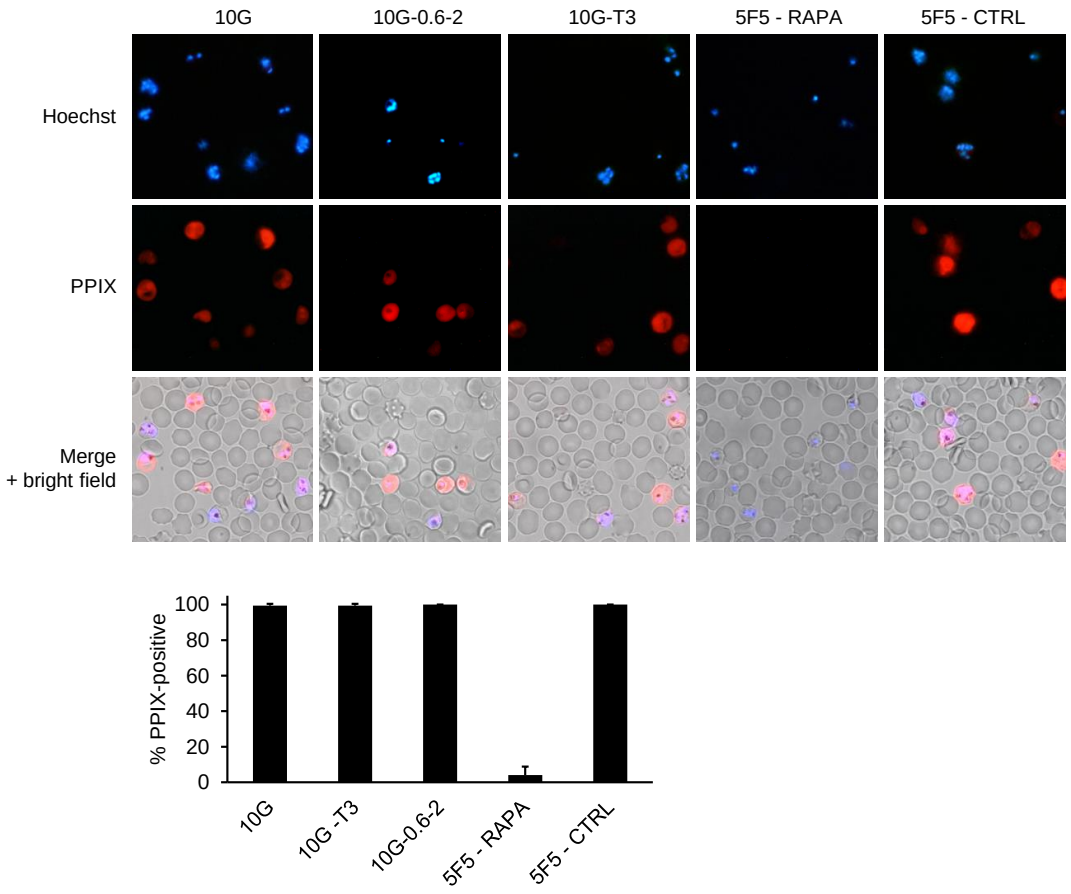


FIG 3 Changes in CLAG3 expression in cultures selected with different drugs. (A) Changes in the transcript levels of *clag3.1* and *clag3.2* in cultures selected with blasticidin S (BS), fosmidomycin (FOSMI), doxycycline (DOXY), leupeptin (LEUP), T3 and T16 compared to unselected control cultures maintained in parallel. The 10G or 3D7-A lines were used for these experiments as indicated. Cultures selected with DOXY were maintained with the drug at 250 nM for 14 weeks or at 250 nM for 3 weeks and then at 500 nM for 7 additional weeks. Cultures

selected with LEUP were first selected at 2.5 µM for 2 weeks, and then maintained for one additional week at the same concentration (total 3 weeks at 2.5 µM), or one additional week at 5 µM and one at 8 µM (2.5-5-8 µM bars). Transcript levels are normalized against *rhoph2*, which has a temporal expression dynamics similar to *clag3* genes along the asexual cycle. Values are the log2 of the normalized expression fold-change in drug-selected cultures versus cultures maintained in parallel in the absence of drug. Zero indicates the same expression in selected and control cultures, whereas positive values reflect an increase of expression in drug-selected cultures and negative values reflect reduced expression in drug-selected cultures. Individual values are the average of reactions performed in triplicate, but for each drug selection the result of independent biological samples collected at different times (indicated in weeks) is presented. (B) Resistance of late stage parasites (pigmented trophozoites and schizonts) to treatment with sorbitol in 10G cultures selected with T3 (10G-T3) or unselected 10G cultures maintained in parallel (10G). "Control" are the same cultures before sorbitol treatment. (C) Immunofluorescence Assay (IFA) analysis of mature schizont-infected erythrocytes with anti-CLAG3 and anti-AMA1 antibodies in T3-selected (10G-T3) or unselected 10G cultures. DAPI marks parasite nuclei. Anti-AMA1 antibodies were used to identify schizonts sufficiently mature for CLAG3-expression. The proportion of AMA1-positive infected erythrocytes that are negative for CLAG3 in 10G-T3 or unselected 10G cultures is shown. Values in the bar chart are the average of two independent biological replicates, with SD.



723

724 **FIG 4** Uptake of 5-ALA by erythrocytes infected with CLAG3-defficient parasites. Analysis
725 was performed with the CLAG3-defficient 10G-0.6-2 and T3-selected 10G lines, the parental
726 10G control line, and the previously described 5F5 transgenic line treated with rapamycin to
727 induce the deletion of *rhoph3* exons 4-6 (5F5 - RAPA) or treated in parallel with DMSO solvent
728 (5F5 - CTRL). Uptake of 5-ALA and its subsequent conversion to PPIX in infected erythrocytes
729 was visualized by fluorescence microscopy. Parasite nuclei were stained with Hoechst. The bar
730 chart shows the proportion of PPIX fluorescence-positive cells among pigmented parasite-
731 infected erythrocytes. Values are the average of three independent biological replicates, with SD.

732

Supplementary figures

Identification of antimalarial compounds that require CLAG3 for their uptake by *P. falciparum*-infected erythrocytes

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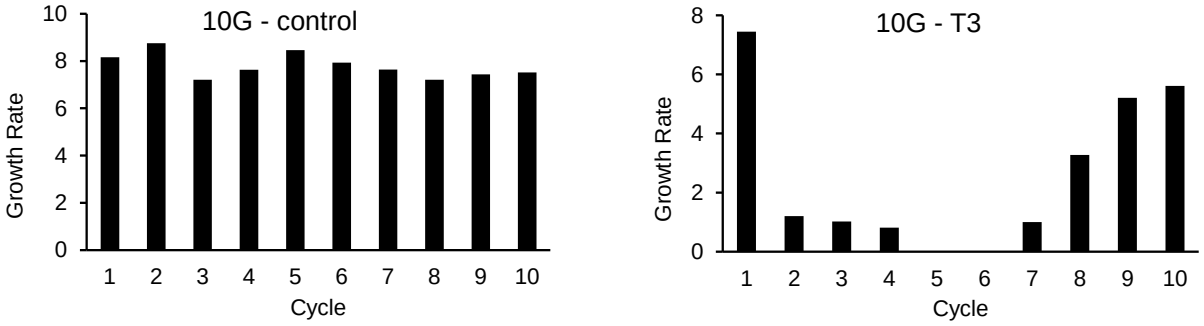

FIG S1 Parasite growth dynamics in a representative drug selection experiment (representative of two independent selection experiments with T3). Growth rate of 10G cultures during selection with the drug T3 (2.86 nM, 10G - T3) or maintained in parallel in the absence of drug (10G - control). There is strong growth inhibition in the initial cycles after drug addition. The clear increase in the growth rate observed from cycle 8 onwards reflects adaptation of the parasite population to the drug. Cultures were regularly synchronized with sorbitol or L-Proline. At each cycle, after determining parasitemia, cultures were diluted with fresh erythrocytes to a parasitemia of approximately 1%. Growth rate was determined at each cycle by dividing the parasitemia (measured at the ring stage) by the parasitemia at the previous cycle (after diluting the culture). Parasitemia was determined by flow cytometry after staining parasite nuclei with SYTO11.

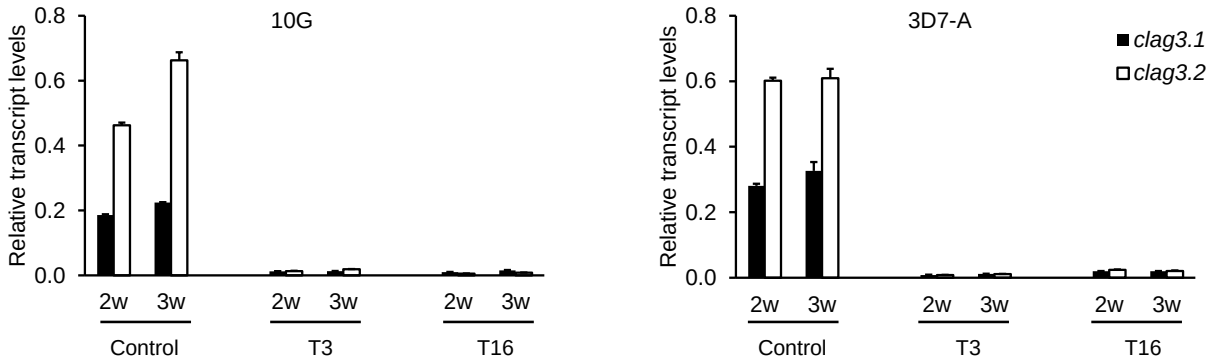

FIG S2 *clag3* expression in cultures selected with T3 or T16. Transcript levels of *clag3.1* and *clag3.2* (normalized against *rhoph2*) were measured in 10G and 3D7-A cultures selected with T3 (2.86 nM) or T16 (0.6 nM) and unselected cultures maintained in parallel (Control), at the times indicated in weeks (w). Values are the average of reactions performed in triplicate, with SD. These results show silencing of both *clag3* genes after only 2 weeks of T3 or T16 selection.

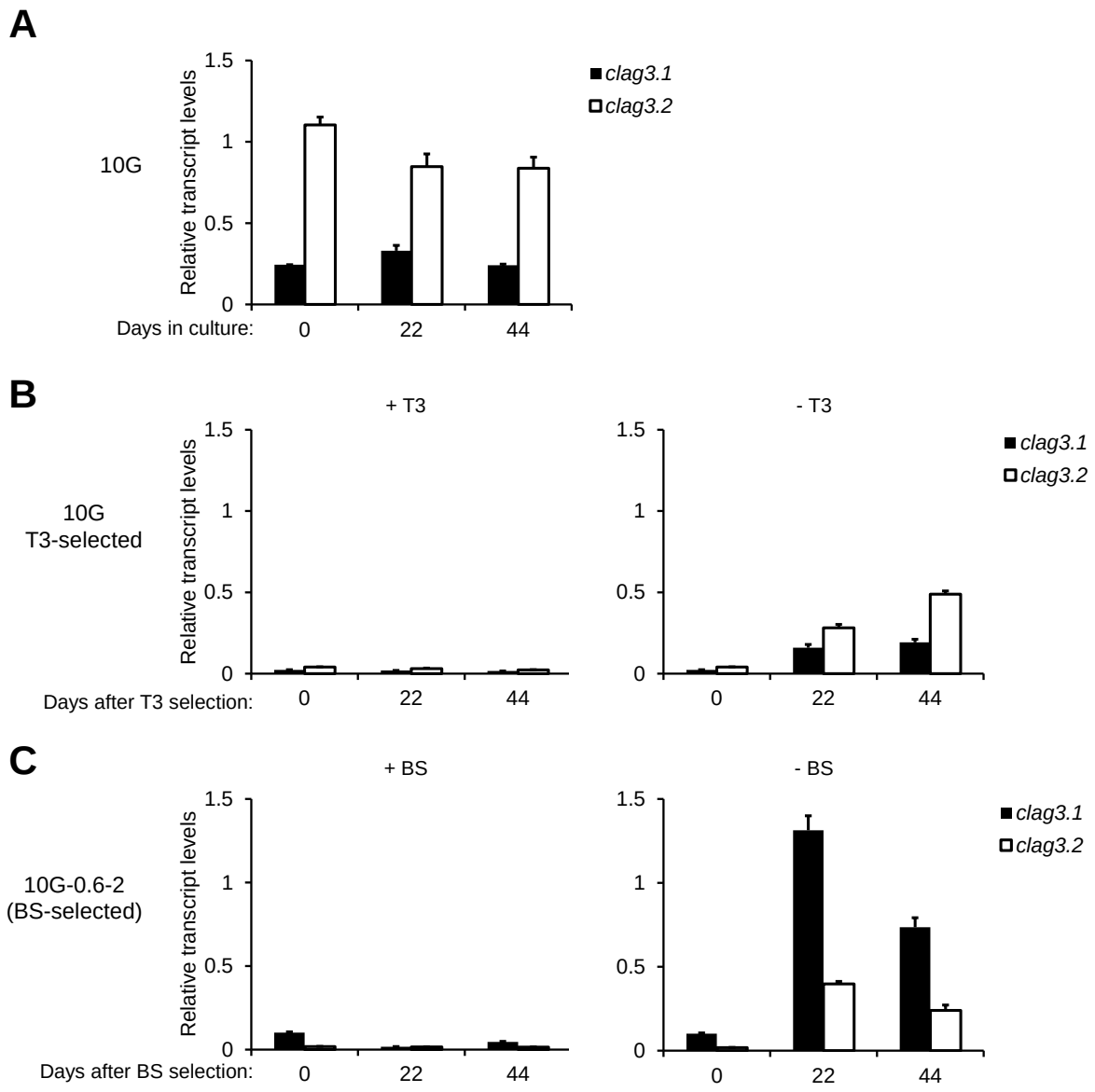

FIG S3 *clag3* expression in cultures selected with T3 or blasticidin S and then maintained either in the presence or absence of the drug. Relative transcript levels of *clag3.1* and *clag3.2* (normalized against *rhoph2*) in control 10G cultures (A), or in cultures previously selected with T3 (B) or blasticidin S (C). Cultures were maintained in parallel either in the presence of the selecting drug (+T3 and +BS bar charts) or after removing it (- T3 and - BS bar charts). Day 0 is the day at which previously selected cultures were split and maintained under either continued presence or absence of the drug. Values are the average of three technical quantitative PCR

replicates, with SD; independent biological samples for transcriptional analysis collected at different times are presented as separate data points (22 and 44 days after splitting the culture). These results show that silencing of *clag3* genes associated with resistance to the two drugs is reversible: in the absence of drug, parasites that re-activate *clag3* expression are progressively selected because simultaneous silencing of the two *clag3* genes poses a fitness cost. However, in blasticidin S-selected cultures, *clag3.1*-expressing parasites were predominant after drug removal, consistent with the previously reported partial protection against this drug associated with expression of this *clag3* paralog. This was not observed in T3-selected cultures, which maintained a *clag3.1*/*clag3.2* transcripts ratio similar to the parental 10G cultures.

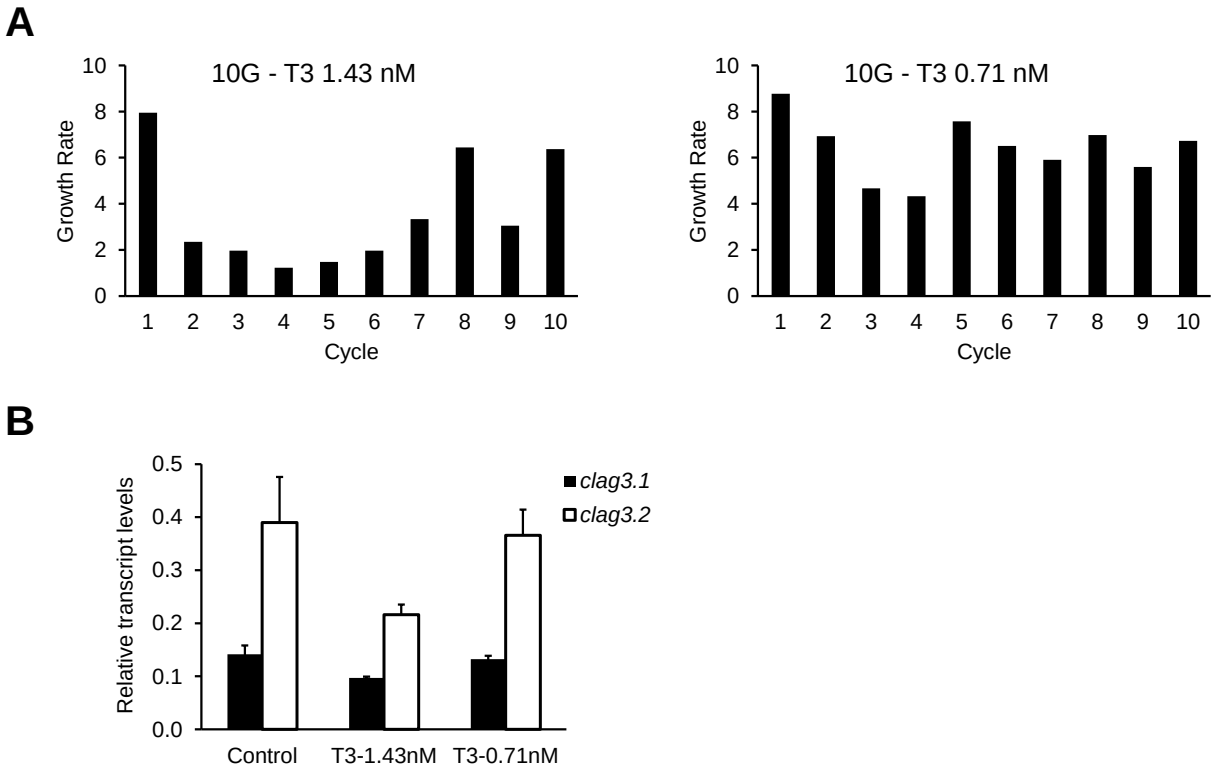

FIG S4 Selection of parasites with low concentrations of T3. Cultures were selected with 1.43 or 0.71 nM T3 (corresponding to 1/2 or 1/4 of the concentration used in the initial selection experiments shown in Fig. 3) to determine whether adaptation to low concentrations of the drug can occur by selection of parasites that express one or the other *clag3* gene. (A) Growth dynamics during selection with lower concentrations of T3, as in Fig. S1. (B) Relative transcript levels of *clag3.1* and *clag3.2* (normalized against *rhoph2*) in control cultures and in cultures selected for eight cycles (~17 days) with T3 at the concentrations indicated. No major alteration in the relative expression of the two genes compared to control cultures maintained in parallel was observed. Values are the average of two biological replicates, with SD.