Solute Restriction Reveals an Essential Role for clag3-Associated Channels in Malaria Parasite Nutrient Acquisition

Ajay D. Pillai,1 Wang Nguitragool,2 Brian Lyko,3 Keithlee Dolinta,4 Michelle M. Butler, Son T. Nguyen, Norton P. Peet, Terry L. Bowlin, and Sanjay A. Desai

Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland (A.D.P., W.N., B.L., K.D., S.A.D.); and Microbiotix, Inc., Worcester, Massachusetts (M.M.B., S.T.N., N.P.P., T.L.B.)

Received July 13, 2012; accepted September 4, 2012

ABSTRACT

The plasmodial surface anion channel (PSAC) increases erythrocyte permeability to many solutes in malaria but has uncertain physiological significance. We used a PSAC inhibitor with different efficacies against channels from two Plasmodium falciparum parasite lines and found concordant effects on transport and in vitro parasite growth when external nutrient concentrations were reduced. Linkage analysis using this growth inhibition phenotype in the Dd2 × HB3 genetic cross mapped the clag3 genomic locus, consistent with a role for two clag3 genes in PSAC-mediated transport. Altered inhibitor efficacy, achieved through allelic exchange or expression switching between the clag3 genes, indicated that the inhibitor kills parasite replication within host erythrocytes is responsible for most clinical sequelae of malaria. Plasmodium falciparum, the most virulent human pathogen, remodels its host erythrocyte by exporting proteins (Boddey et al., 2010; Russo et al., 2010), generating membrane-bound organelles within the host cytosol (Tamez et al., 2008), and increasing erythrocyte permeability to numerous solutes (Ginsburg et al., 1983; Kirk et al., 1994). The increased permeability is mediated by ion channels at the host erythrocyte membrane; it has been studied with tracer flux (Homewood and Neame, 1974; Saliba et al., 1998), osmotic fragility (Kutner et al., 1987), and patch-clamp (Alkhali et al., 2004, 2012; Staines et al., 2007) techniques. Although several different channels have been proposed, studies with highly specific inhibitors support a single, broad-selectivity ion channel known as the plasmoidal surface anion channel (PSAC) (Pillai et al., 2010). PSAC has an unusually small single-channel conductance for a broad-selectivity channel (20 pS in 1.1 M Cl–) and is functionally conserved in divergent Plasmodium species (Lisk and Desai, 2005). Two clag3 genes from the parasite have been impli-
cated in this channel activity through a molecular cloning strategy using an inhibitor that blocks channels from only the Dd2 parasite line (isolate-specific PSAC antagonist 28, ISPA-28) (Nguiragool et al., 2011).

Although direct evidence is lacking, circumstantial observations suggest that PSAC activity is essential for intra-erythrocytic parasite survival. First, both channel activity and the clag gene family are conserved in all Plasmodium species examined to date (Kaneko et al., 2001; Lisk and Desai, 2005). Single-channel patch-clamp studies determined that even biophysical properties such as ion channel gating, conductance, and functional copy number per cell are nearly identical in P. falciparum and Plasmodium knowlesi, two divergent malaria parasites (Lisk and Desai, 2005). Second, gene silencing and monoallelic expression of clag genes in P. falciparum suggest that channel function is important (Cornets et al., 2007); parasites invest in expression switching for key gene families to evade host immunity and to protect essential activities at the host cell surface (Schier et al., 2008). Third, quantitative permeability studies with some required nutrients suggested that their PSAC-mediated uptake is necessary for in vitro parasite cultivation (Gero and Wood, 1991; Saliba et al., 1998; Liu et al., 2006; Martin and Kirk, 2007). Finally, selections of parasite cultures with permuted channels has generated functional PSAC mutants (Hill et al., 2007; Lisk et al., 2008) but has not yielded complete loss of function. These mutant channels exhibit reduced toxin uptake but appear to satisfy the parasite’s transport demands. It remains possible, however, that channel activity is a nonessential byproduct of host cell invasion (Staines et al., 2007). The physiological roles served by PSAC are also debated, with proposals including nutrient uptake and metabolic waste removal (Desai et al., 2000), modification of host erythrocyte ionic composition (Brand et al., 2003), volume regulation of infected cells (Staines et al., 2001; Lew et al., 2004), and autocrine purinergic signaling (Akkaya et al., 2009).

We addressed these uncertainties with functional and molecular studies using ISPA-28. This and other PSAC inhibitors exhibit improved efficacy in parasite growth inhibition studies when the concentrations of key nutrients are reduced. Genetic mapping, DNA transfection, and in vitro selections implicate the clag3 genes in channel-mediated nutrient uptake required for parasite survival within erythrocytes.

Materials and Methods

Parasite Cultivation, Design of PGIM, and Growth Inhibition Studies. Asexual-stage P. falciparum laboratory lines were propagated with standard methods, in RPMI 1640 medium supplemented with 25 mM HEPES, 31 mM NaHCO3, 0.37 mM hypoxanthine, 10 µg/mL gentamicin, and 10% pooled human serum. Nutrient-deprivation experiments used this standard medium but with reduced concentrations of individual constituents; human serum was exhaustively dialyzed against distilled water before addition to those media. PGIM contained reduced concentrations of isoleucine (11.4 µM), glutamine (102 µM), and hypoxanthine (3.01 µM) and was supplemented with dialyzed serum.

The results of growth inhibition experiments were quantified by using a SYBR Green I-based fluorescence assay for parasite nucleic acid in 96-well microplates, as described previously (Pillai et al., 2010). Ring-stage synchronized cultures were seeded at 1% parasitemia and 2% hematocrit levels in standard medium or PGIM and were maintained for 72 h at 37°C in 5% O2/5% CO2 in nitrogen, without medium changes. Cultures were then lysed in 20 mM Tris, 10 mM EDTA, 0.016% saponin, 1.6% Triton X-100, pH 7.5, with SYBR Green I nucleic acid gel stain (Invitrogen, Carlsbad, CA) at 5000-fold dilution. After a 45-min incubation, parasite DNA contents were quantified through fluorescence measurements (excitation, 485 nm; emission, 528 nm). For each inhibitor concentration, the mean of triplicate measurements was calculated after subtraction of background fluorescence values determined with matched cultures that had been killed with 20 µM chloroquine. Growth inhibition studies with the HB3parasite were performed after transport-based selection with ISPA-28, to achieve expression of the chimeric clag3 gene generated through allelic exchange transfection. Limiting-dilution cultures to obtain parasite clones were performed in 96-well microplates; positive wells were detected by using the 5(and 6)-carboxy-seminaphthorhodafluor-1 method (Lyko et al., 2012).

Parasites were also cultivated in pooled human serum collected from nonfasting donors (Interstate Blood Bank, Memphis, TN). The serum was ultracentrifuged (300,000 g for 1 h) to remove buoyant lipoproteins and was used in 72-h cultivation experiments in microplates as described above. Growth was quantified as described above except that microplate wells were washed once with phosphate-buffered saline before the addition of buffer SYBR Green I gel stain, to eliminate serum-associated fluorescence artifacts.

Transport-Inhibition Assays. Inhibitor affinity for PSAC block was determined by using a quantitative transmittance assay based on the osmotic lysis of infected cells with sorbitol (Pillai et al., 2010). Parasite cultures were enriched at the trophozoite stage with the Percoll-sorbitol method, washed, and resuspended at 37°C and 0.15% hematocrit in 280 mM sorbitol, 20 mM Na-HEPES, 0.1 mg/mL bovine serum albumin, pH 7.4, with the indicated inhibitor concentrations. Osmotic lysis, which results from PSAC-mediated sorbitol uptake, was continuously tracked with transmittance measurements through the cell suspension (700-nm wavelength, DU640 spectrophotometer with Peltier temperature control; Beckman Coulter, Fullerton, CA). Inhibitor dose-response relationships were calculated from the times required to reach a fractional lysis threshold. ISPA-28 dose-response data were fitted to the sum of two Langmuir isotherms with the following equation: \( P = a/(1 + (x/b) + (1 - a)/(1 + (x/c))), \) where \( P \) represents the normalized sorbitol permeability in the presence of inhibitor at concentration \( x \), and \( a, b, \) and \( c \) are constants. Dose-response data for other inhibitors were fitted adequately with a single Langmuir isotherm.

To examine possible inhibitor metabolism in parasite cultures, we cultivated Dd2 parasites for 72 h at 37°C with 40 µM ISPA-28. After centrifugation, the culture supernatant was used as a source of ISPA-28 for comparison with freshly prepared compound in transport-inhibition studies.

QTL Analyses. We sought genetic loci associated with ISPA-28 growth-inhibitory efficacy in the Dd2 × HB3 genetic cross (Wellems et al., 1990) by using 448 previously selected polymorphic markers that distinguish the Dd2 and HB3 parental lines (Nguiragool et al., 2011). QTL analysis was performed with R/qtl software (freely available at http://www.rqtl.org/) as described (Broman et al., 2003), with conditions suitable for the haploid asexual parasite. A significance threshold of \( P = 0.5 \) was estimated through permutation analysis. Growth inhibition data at 0.3 and 10 µM ISPA-28 identified the same locus described with 3 µM ISPA-28. Additional QTLs were sought with secondary scans controlling for the clag3 locus.

Quantitative RT-PCR Assays. Two-step, real-time, PCR assays with allele-specific primers developed previously (Nguiragool et al., 2011) were used to quantify clag gene expression. RNA was harvested from schizont-stage cultures with TRIzol reagent (Invitrogen), treated with DNase, and used for reverse transcription with oligo(dT) primers and SuperScriptIII (Invitrogen). Negative control reactions without reverse transcriptase confirmed that there was no genomic DNA contamination. Real-time PCR assays were performed
with a QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA), an iCycler iQ multicolor, real-time, PCR system (Bio-Rad Laboratories, Hercules, CA), and clag gene-specific primers (Supplemental Table 1). Serial dilutions of parasite genomic DNA were used to construct the standard curve for each primer pair. PF7_0073 was used as a loading control, because it is expressed constitutively. Transcript abundance for each clag gene was determined from amplification kinetic data.

**PCR Studies of clag3 Recombination.** The clag3 locus of Dd2-PSIM28 was characterized with genomic DNA and the following allele-specific primers: 3.1f, 5′-GGCCAATATATAAATCTGATACTGACA-3′; 3.1r, 5′-AAGAAAAAATTGCAAACAAAGTGTTAGA-3′; 3.2f, 5′-GTTGAGTGAATCTAATGTCATTAATT-3′; 3.2r, 5′-AACCATATACTATTATGTTAATTACAC-3′. cDNA prepared from schizont-stage cultures was used with these primers to examine the expression of both native and chimeric clag3 genes.

**Southern Blotting.** A clag3-specific probe was prepared from Dd2 genomic DNA through PCR amplification with 5′-ATTATTACAAACAAAGAAAATGCCAAGAGGA-3′ and 5′-TTTCTCTATCTCTAATTCTTTAATTGTT-3′ in the presence of digoxigenin-dUTP (Roche Diagnostics, Basel, Switzerland). Probe specificity was confirmed through blotting against full-length PCR amplicons of the five clag genes generated from Dd2 genomic DNA with the primers listed in Supplemental Table 2.

Genomic DNA was digested with the indicated restriction enzymes (New England Biolabs, Ipswich, MA), subjected to electrophoresis in 0.7% agarose gels, acid-depurinated, transferred, and crosslinked to nylon membranes. The blots were then hybridized overnight at 39°C in the aforementioned digoxigenin-labeled probe in DIG Easy Hyb solution (Roche Diagnostics) and were washed with low- and high-stringency buffers (2× saline-sodium citrate 0.1% SDS, at 23°C, followed by 1× saline-sodium citrate, 0.5% SDS, at 50°C) before digoxigenin immunodetection according to the manufacturer’s instructions.

**Mammalian Cytotoxicity Assays.** Inhibitor cytotoxicity was quantified by using human HeLa cells (CLL-2; American Type Culture Collection, Manassas, VA) seeded at 4000 cells/well in 96-well plates. Cultures were incubated with individual inhibitors for 72 h at 37°C in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum. Cell viability was quantified by using the vital stain 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), as described (Marshall et al., 1995).

**Results**

**Poor Growth Inhibition by ISPA-28 under Standard Culture Conditions.** ISPA-28 blocks PSAC on Dd2-infected cells with high affinity and has only weak activity against channels from HB3 parasites (K<sub>0.5</sub> values of 56 ± 5 nM and 43 ± 2 μM, respectively) (Nguitragool et al., 2011). If channel activity serves an essential role, then this small-molecule inhibitor should interfere with the propagation of Dd2 cultures but not HB3 cultures. Our initial in vitro parasite growth studies revealed an insignificant difference, with both parasite lines exhibiting sustained growth in RPMI 1640 medium despite high ISPA-28 concentrations (IC<sub>50</sub> values of >40 μM for both lines) (data not shown).

We considered possible explanations and determined that ISPA-28 efficacy against Dd2 channels was not compromised by metabolism of the inhibitor under in vitro culture conditions (Supplemental Fig. 1A). ISPA-28 was not significantly adsorbed by serum proteins or lipids (Supplemental Fig. 1, B–D), a phenomenon that is known to reduce the activity of some PSAC inhibitors and many therapeutic agents (Matsumata et al., 1987). Therefore, ISPA-28 has unexpectedly poor efficacy against the growth of Dd2 parasites.

One possibility is that channel activity is essential for malaria parasites but low transport levels remaining in the presence of inhibitor adequately meet parasite demands under standard in vitro culture conditions. Consistent with this idea, we noted sustained channel-mediated uptake in Dd2-infected erythrocytes even with high ISPA-28 concentrations; significantly less residual uptake was observed with compound 2, a broad-spectrum PSAC inhibitor with a comparable inhibitory K<sub>0.5</sub> value for Dd2 channels (P < 10<sup>-4</sup> for comparison of these inhibitors at 10 μM) (Supplemental Fig. 2). The unexpected difference in residual channel activities with these inhibitors may account for their different efficacies against in vitro parasite growth (IC<sub>50</sub> values of >40 and 4.7 μM, respectively) (Table 1).

Incomplete block with high ISPA-28 concentrations despite a low K<sub>0.5</sub> value for Dd2 channels suggests a complex mechanism of inhibition. Although dantrolene and furosemide dose-response data were adequately fitted with an equation that assumed 1:1 stoichiometry for inhibitor and channel molecules (Langmuir isotherm) (Supplemental Fig. 1, C and D), the ISPA-28 dose-response data were not well fitted (Supplemental Fig. 2). An improved fit was obtained with a two-component Langmuir equation. Because this two-component equation is consistent with several possible mechanisms, the stoichiometry and precise mode of channel block with ISPA-28 remain unknown.

**Selective Inhibition of Dd2 Growth by ISPA-28 When Nutrients Are Restricted.** If PSAC functions in nutrient acquisition for the intracellular parasite (Desai et al., 2000), then the complete inhibition by ISPA-28 may permit adequate nutrient uptake. This effect may be accentuated in cultures using RPMI 1640 medium, which contains high nutrient concentrations to support the growth of diverse cell lines (Sato and Kan, 2001). The large inward concentration gradients for nutrients in this medium may sustain parasite nutrient uptake despite nearly complete channel block.

To evaluate this possibility, we examined parasite requirements for isoleucine, an essential amino acid that the parasite cannot synthesize de novo (Istvan et al., 2011). Isoleucine is absent from human hemoglobin, which the parasite digests as a source of some amino acids; it must be acquired through uptake from serum (Liu et al., 2006; Martin and Kirk, 2007). We prepared RPMI 1640 media with a range of isoleucine concentrations and determined that Dd2 and HB3 parasites had quantitatively similar isoleucine requirements, with negligible growth when this amino acid is removed from the medium (EC<sub>50</sub> values of 10–16 μM isoleucine) (Fig. 1A). The addition of 15 μM ISPA-28 significantly increased Dd2 requirement for isoleucine (EC<sub>50</sub> = 34.6 ± 1.1 μM; P < 10<sup>-5</sup>, Student’s t test) but had no effect on HB3 parasites. This finding suggests an essential role for PSAC-mediated isoleucine uptake and is consistent with transport studies that showed this channel to be a major route of uptake after infection.

Because many nutritive solutes have significant PSAC permeability, we also examined parasite growth in RPMI 1640 medium after isolated removal of several other solutes, i.e., calcium panthothenate, cysteine, glutamic acid, glutamine, methionine, proline, and tryptophan. Hypoxanthine, a purine source that is not present in RPMI 1640 medium but is commonly added to parasite culture media, was also evaluated. Each of these solutes has documented PSAC permea-
<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>( K_{0.5} ) for PSAC Block</th>
<th>( IC_{50} ) for Growth Inhibition</th>
<th>( IC_{50} ) Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu M )</td>
<td>RPMI 1640 Medium</td>
<td>PGIM</td>
<td>RPMI 1640 Medium</td>
</tr>
<tr>
<td>ISPA-28</td>
<td></td>
<td>Dd2, 56;</td>
<td>Dd2, &gt;40;</td>
<td>Dd2, 0.66;</td>
</tr>
<tr>
<td></td>
<td>HB3, 43,000</td>
<td>HB3, &gt;40</td>
<td>HB3, &gt;50</td>
<td>HB3, N.D.</td>
</tr>
<tr>
<td>Furosemide</td>
<td></td>
<td>2700</td>
<td>&gt;200</td>
<td>21</td>
</tr>
<tr>
<td>Dantrolene</td>
<td></td>
<td>1200</td>
<td>42</td>
<td>3.8</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>87</td>
<td>23</td>
<td>0.27</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>33</td>
<td>15</td>
<td>0.17</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>6</td>
<td>18</td>
<td>0.23</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>84</td>
<td>4.7</td>
<td>0.41</td>
</tr>
<tr>
<td>TP-52</td>
<td></td>
<td>25</td>
<td>7.3</td>
<td>0.19</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>44</td>
<td>12.5</td>
<td>0.17</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>81</td>
<td>&gt;30</td>
<td>2.0</td>
</tr>
<tr>
<td>ISG-21</td>
<td></td>
<td>2.6</td>
<td>1.5</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Chloroquine   | Inactive | 0.22 | 0.34 | 0.67 |
Mefloquine    | Inactive | 0.022| 0.033| 0.66 |
Artemisinin   | Inactive | 0.018| 0.026| 0.66 |

N.D., not determined.
bility (Ginsburg et al., 1985; Gero and Wood, 1991; Asahi et al., 1996; Saliba et al., 1998; Lisk et al., 2006), but some also exhibit uptake by host erythrocyte transporters (Quashie et al., 2010; Winterberg et al., 2012). Microscopic examination of parasites propagated in these single-depletion experiments revealed the greatest detriment with removal of glutamine and hypoxanthine, but short-term growth studies revealed relatively modest effects (Fig. 1, B and C). The addition of ISPA-28 did not measurably increase Dd2 requirement for either solute in these experiments.

Because parasite growth reflects a complex interplay of multiple biochemical pathways and the availability of nutrients that feed into them, the effects of PSAC inhibition may be greater than predicted by deprivation studies restricted to single solutes. Therefore, we simultaneously varied the concentrations of isoleucine, glutamine, and hypoxanthine without manipulating other constituents in the RPMI 1640 medium. This approach revealed conditions that increased ISPA-28 potency against Dd2 parasites while preserving the negligible effect of the compound against HB3 parasites (Fig. 2A).

In the absence of transport inhibitors, this medium, termed PGIM, permitted continuous propagation of both Dd2 and HB3 parasites for >2 weeks, although at somewhat reduced rates (Fig. 2B). We noticed that cultures with low parasitemia levels grew well in PGIM but rates decreased with greater parasite burdens, which is consistent with nutrient limitation and competition among parasites in culture. To explore how these conditions might compare with in vivo expansion rates, we examined parasite propagation in pooled nonfasting serum collected from healthy donors. Human serum that was not diluted with synthetic medium also yielded relatively slow expansion of parasite cultures (Fig. 2B), which is consistent with the view that RPMI 1640 medium achieves high culture expansion rates through the use of supraphysiological nutrient levels.
We explored the role of the channel further by evaluating whether PSAC inhibitors and PGIM could be used to kill selectively parasites that express individual clag3 genes. Most laboratory parasite lines carry two clag3 genes on chromosome 3, but individual parasites appear to express only one allele at a time (Fig. 3A). Silencing of the other allele is thought to occur through epigenetic mechanisms (Cortés et al., 2007). This process, with relatively slow rates of switching between the two alleles, allows subsets of parasites to escape host immune responses against surface-exposed antigens.

ISPA-28 specifically inhibits channels associated with the Dd2 clag3.1 gene because it binds to a region near the C terminus of the encoded protein; it has little or no activity against channels linked to expression of Dd2 clag3.2 or either clag3 gene in unrelated parasite lines (Nguitragool et al., 2011). This inhibitor was used previously to select for Dd2 clag3.1 expression through osmotic lysis of infected cells with sorbitol, a sugar alcohol with high PSAC permeability. Sorbitol solutions containing ISPA-28 select for this allele because osmotic lysis eliminates infected cells whose channels are not blocked (Fig. 3B).

We hypothesized that ISPA-28 and PGIM might be useful in counter-selections for other clag3 alleles (Fig. 3B). Whereas sorbitol-induced osmotic lysis selects for expression of the ISPA-28-sensitive clag3.1 gene, growth inhibition in PGIM should favor cells expressing the resistant clag3.2 allele, because only parasites expressing unblocked channels could meet their nutrient demands and propagate successfully. We first examined the progeny clone 7C20, which carries the Dd2 clag3 locus and expresses both alleles in unselected cultures (Fig. 3, C–E). After selection through osmotic lysis with sorbitol and ISPA-28, surviving parasites had markedly reduced ISPA-28 affinity, which indicates that in vitro propagation with PSAC inhibitors can be used to select for rare
parasite subpopulations. Consistent with our predictions, RT-PCR assays confirmed strong negative selection against clag3.1, to yield parasites that preferentially expressed clag3.2. There were modest changes in the expression of clag genes on other chromosomes, which suggests that those paralogs may also contribute to PSAC activity. Importantly, the opposing effects of ISPA-28 on in vitro growth inhibition and on susceptibility to transport-induced osmotic lysis permitted purifying selections of either clag3 allele and revealed a strict correlation with channel phenotype.

Genomic Recombination to Overcome Defect in Epigenetic Switching. The parental Dd2 line retains exclusive expression of clag3.1 in unselected cultures, despite being isogenic with 7C20 at the clag3 locus. To explore possible mechanisms, we sought to select for Dd2 parasites expressing the clag3.2 allele. We first attempted transport selection using osmotic lysis with isolate-specific PSAC antagonist 43 (Supplemental Fig. 4A), an unrelated PSAC inhibitor with higher affinity for channels formed by expression of Dd2 clag3.2 than of clag3.1. Although this approach was used previously to select for 7C20 parasites expressing clag3.2 (Nguitragool et al., 2011), it proved insufficient to affect the channel phenotype in Dd2 parasites, despite repeated selections over 4 months (Supplemental Fig. 4, B and C).

We next attempted negative selection with growth inhibition in PGIM containing ISPA-28. After two cycles of drug pressure with 5 μM ISPA-28 for a total of 17 days, resistant cells were identified and characterized after limiting dilution to obtain the clone Dd2-PGIM28. Consistent with killing primarily through PSAC inhibition, transport studies with this resistant clone revealed a marked reduction in inhibitor affinity (Fig. 4A). Surprisingly, although the ISPA-28 dose-response relationship quantitatively matched that of 7C20 parasites after identical PGIM-based selection (Fig. 4A), full-length clag3.2 transcript was still undetectable (Fig. 4B). Because this observation excludes simple gene-switching, we considered spontaneous recombination between the two clag3 genes. We identified a chimeric clag3 transcript by using a forward clag3.1 primer and a reverse clag3.2 primer; PCR analyses confirmed that this chimera is present in the genome of the selected parasites but absent from the original Dd2 line (Fig. 4C). DNA sequencing Fig. 3. In vitro growth selection for clag3 alleles associated with inhibitor-resistant channels. A, schematic diagram showing the two clag3 genes of the 7C20 progeny clone. Arrow, active transcription of clag3.1. B, strategy for purifying selections for clag3.1 (blue) or clag3.2 (red) expression. Osmotic lysis of 7C20-infected cells with sorbitol and ISPA-28 spared cells that expressed clag3.1 (transport selection), whereas PGIM-based growth inhibition by ISPA-28 selected for clag3.2 expression. C, ISPA-28 dose-response curves (mean ± S.E.M.) for PSAC inhibition before selection, after transport selection of the 7C20 line, and after PGIM growth selection. Solid lines, best fits to the equation provided in Materials and Methods. D, expression ratios for the two clag3 alleles before and after selections, as determined with quantitative RT-PCR assays. High-affinity ISPA-28 block was associated with clag3.1 expression. Bars, mean ± S.E.M. of replicates from two to four separate trials each. E, expression (mean ± S.E.M.) of the five clag genes before and after each selection.

1110 Pillai et al.
indicated that the chimeric gene derives its 5’-untranslated region and the first ∼70% of the gene from clag3.1. After crossover between single-nucleotide polymorphisms 3680 and 3965 base pairs from the start codon, the gene carries the 3’-end of clag3.2. Therefore, the chimeric gene is driven by the clag3.1 promoter but encodes a protein with the C-terminal variable domain of clag3.2. This altered C terminus accounts for the reduced ISPA-28 efficacy against nutrient transport and thus the survival of this clone in our selection. Such homologous recombination also produces a parasite with a single clag3.2 gene and high ISPA-28 affinity, but that recombinant is not expected to survive growth inhibition selection in PGIM with ISPA-28.

We used quantitative RT-PCR assays to examine the transcription of clag genes in Dd2-PGIM28 and found that the chimeric gene is preferentially expressed (8.9 ± 1.3-fold greater than clag3.1; P < 0.002) (Fig. 4F). We then performed transport-based selection with sorbitol and ISPA-28 to examine whether Dd2-PGIM28 can undergo expression switching. As expected for an intact clag3.1 promoter and gene, this second selection yielded parasites that expressed native clag3.1 almost exclusively (PGIM-rev) (Fig. 4, F and G). Transport studies revealed an ISPA-28 dose-response relationship identical to that of the original Dd2 line (Fig. 4H). Therefore, the new chimeric clag3 gene can undergo epigenetic silencing and switching with clag3.1. It is not clear why the native clag3.2 gene of Dd2 parasites is refractory to transcription: DNA sequencing of the gene’s promoter region determined through DNA sequencing. The sites recognized by primers (in B and C) and the probe used for Southern blotting (in D) are indicated; vertical lines marked X and S represent XbaI and SphI restriction sites, respectively, as used in Southern blotting. F, expression (mean ± S.E.M.) of each clag gene in Dd2-PGIM28 before and after transport-based selection for clag3.1 with ISPA-28 (PGIM-rev). G, ratios quantifying the relative expression of clag3 and the chimeric gene, calculated from data in F and presented on a logarithmic scale. H, PSAC inhibition by ISPA-28 in the PGIM-rev line. Solid lines, from Fig. 3C.
diseases. They were only weakly effective against parasite growth in standard medium, but they exhibited significantly improved activity in PGIM. We also tested eight high-affinity PSAC inhibitors from five distinct scaffolds that were identified through high-throughput screening (Pillai et al., 2010). Each of the inhibitors was more potent when nutrient concentrations were reduced, strengthening the evidence for the channel's role in nutrient acquisition. The extent of improved efficacy was variable, but many compounds exhibited >100-fold improvements in efficacy with nutrient restriction (IC_{50} ratio) (Table 1). Factors such as the stoichiometry of inhibitor/channel interactions and resultant changes in the concentration dependence of channel block (Supplemental Fig. 2), compound stability in culture, and adsorption by serum may influence the magnitude of this ratio for individual compounds.

To explore therapeutic potential, we examined in vitro HeLa cell cytotoxicity. Several potent PSAC inhibitors were found to be nontoxic (Table 2), which suggests that they may be excellent starting points for medicinal chemical optimization to develop new antimalarial drugs that target parasite nutrient uptake.

Finally, we performed in vitro growth inhibition experiments with chloroquine, mefloquine, and artemisinin, approved antimalarial drugs that act at unrelated targets within intracellular parasites. Each of these drugs was modestly less active in PGIM than in standard medium (Table 1), providing evidence against nonspecific effects of our modified in vitro growth conditions. The improved efficacy of PSAC inhibitors with nutrient restriction is in contrast to the effect on existing antimalarial drugs and, therefore, implicates a distinct mechanism of action.

**Discussion**

Conservation of both PSAC activity and the recently implicated clag3 genes in rodent, avian, and primate malaria species suggests that this ion channel serves an important role for the intracellular parasite. Because the channel is absent from other apicomplexan parasites and higher organisms (Alkhalil et al., 2007), inhibitors may be suitable starting points for the development of highly specific antimalarial drugs. The biological role of PSAC was unclear, however, and concerns that the channel might be only an epiphenomenon related to infection hampered progress toward therapeutic agents (Staines et al., 2007). Here, we addressed this uncertainty through studies with isolate-specific and broad-spectrum PSAC inhibitors, linkage analysis in a genetic cross, molecular studies, and in vitro selections. These studies provided experimental evidence for an essential and targetable role of PSAC in nutrient acquisition for the intracellular parasite.

Previous growth inhibition studies with PSAC inhibitors have reported discrepancies between the concentrations required for transport inhibition and those needed to inhibit in vitro parasite growth (Staines et al., 2004; Pillai et al., 2010). In light of our findings, this discrepancy likely results from the use of RPMI 1640 medium, which was designed with high concentrations of many nutrients to maximize the growth of diverse cell types. Our examination of individual substrates revealed that isoleucine uptake through PSAC serves an essential role for the parasite. Plasma isoleucine concentrations in developed countries range between 60 and 95 μM (Armstrong and Stave, 1973; Milsom et al., 1979), with values likely to be lower in malaria-endemic countries (Sauders et al., 1967). These measured values are significantly lower than the 381 μM in RPMI 1640 medium. Furthermore, dose-response studies suggest that PSAC-mediated uptake is rate-limiting at these physiological concentrations (Fig. 1A). Although glutamine and hypoxanthine dose-response studies did not show similar effects alone, their combined restriction to produce PGIM yielded further improvements in PSAC inhibitor efficacy. Future refinements of PGIM to include other permeant nutrients with high concentrations in RPMI 1640 medium may yield further improvements in vitro PSAC inhibitor efficacy. Separate studies, such as in vivo efficacy studies with rodent malaria models, will be necessary to determine whether our findings can be translated into future antimalarial drugs.

Marked changes in inhibitor efficacy with relatively modest manipulations raise questions regarding the optimal conditions for high-throughput screens that use in vitro growth-inhibition assays to identify antimalarial drug leads; to date, such screens have used only standard RPMI 1640 medium (Plouffe et al., 2008; Gamo et al., 2010; Guiguemde et al., 2010). Although three existing antimalarial drugs exhibited unchanged efficacy with nutrient restriction (Table 1), it is important to recognize that in vitro parasite growth depends on many factors. We envision that compounds acting at some downstream sites involved in nutrient utilization or metabolism will also exhibit improved efficacy in more-physiological media such as PGIM. Such compounds might act synergistically with PSAC inhibitors. Future high-throughput screens that quantify parasite growth inhibition should consider the medium and other growth conditions carefully; conditions that most closely reproduce in vivo parasite development may be fruitful.

Although our studies provide additional evidence for the involvement of parasite clag3 genes in PSAC activity, there are still fundamental questions regarding the composition and structure of this unusual ion channel (Desai, 2012). The results of prior studies of these genes are largely consistent with our findings (Kaneko et al., 2001; Cortés et al., 2007; Vincensini et al., 2008), but there are some interesting discrepancies with a study that reported a clag3-knockdown parasite (Comeaux et al., 2011). That study used DNA transfection to disrupt clag3.2 and replace it with the selectable marker hDHFR; subsequent application of a dihydrofolate reductase inhibitor led to expression of hDHFR and silencing of clag3.1, presumably through the epigenetic mechanisms characterized here. Because both native clag3 genes exhibited undetectable expression in that study, the findings con-

**TABLE 2**

<table>
<thead>
<tr>
<th>PSAC Inhibitor</th>
<th>HeLa Cell CC_{50}</th>
<th>Specificity (HeLa Cell CC_{50}/Parasite PGIM IC_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>110</td>
</tr>
<tr>
<td>9</td>
<td>&gt;100</td>
<td>&gt;430</td>
</tr>
<tr>
<td>2</td>
<td>&gt;100</td>
<td>&gt;240</td>
</tr>
<tr>
<td>TP-52</td>
<td>&gt;100</td>
<td>&gt;530</td>
</tr>
<tr>
<td>5</td>
<td>&gt;100</td>
<td>&gt;50</td>
</tr>
<tr>
<td>ISG-21</td>
<td>86</td>
<td>43,000</td>
</tr>
</tbody>
</table>

CC_{50}, concentration of inhibitor that reduced conversion of MTS to formazan by 50%.
trast with the essential role in nutrient uptake proposed here. PSAC activity remains to be examined in the knockdown parasite; molecular studies are also required to explore ectopic recombination or other compensatory changes that might circumvent gene suppression (Fig. 4). Another possibility, i.e., that only some parasite lines require channel-mediated nutrient uptake, might reconcile our findings with the observation of a viable clag3-knockdown parasite. In that case, surveys of clinical isolates from patients with malaria might reveal novel PSAC-null parasite lines. Such studies should be pursued, because they are complementary to in vivo efficacy studies with rodent malaria species.

Allele-specific inhibitors, such as ISPA-28, represent useful reagents for examining epigenetic silencing mechanisms. In addition to the clag3 genes, several other parasite gene families exhibit regulated expression and switching to increase antigenic diversity and to achieve immune evasion (Scherf et al., 1998; Lavazec et al., 2007; Mok et al., 2007; Deitsch et al., 2009). ISPA-28 is especially useful because it can be used in both positive and negative selections for the Dd2 clag3.1 gene; our transport and growth inhibition strategies permit selection of channels that are either sensitive or resistant to this inhibitor. By combining these strategies, we identified a defect in clag3 gene-switching in the Dd2 line; intact switching in the 7C20 parasite, which is isogenic at the chromosome 3 locus, excludes defects in cis-regulatory elements such as the promoter and instead suggests other determinants, possibly transcription factors (Tonkin et al., 2009). Our selections also show that Dd2 parasites can overcome this switching defect through ectopic recombination between the two clag3 genes to produce a new chimeric gene. This chimeric gene is transcription-competent and encodes ISPA-28-resistant channels. Similar recombination events have been documented for other virulence genes within subtelomeric regions of the parasite genome (Kraemer and Smith, 2003); they likely account for the observed variations in clag3 copy numbers for common laboratory parasite lines (Chung et al., 2007).

Our studies suggest that nutrient uptake at the host membrane is rate-limiting. Although the composition of PSAC and the precise in vivo role of the channel are poorly understood, our findings support continued pursuit of antimalarial drug discovery targeting this novel ion channel. This target is especially attractive because some available PSAC inhibitors sterilize cultures at clinically achievable concentrations, with efficacies comparable to those of existing antimalarial drugs (Table 1). A legitimate concern relates to functional differences between channels of distinct parasite lines and associated polymorphisms in the clag3 product (Alkhalil et al., 2004, 2009; Nguiruago, 2011). Will such variability reduce clinical effectiveness against malaria or speed drug resistance? We do not think so. Critical channel properties, such as the list of permeant solutes and their relative transport rates, are strictly conserved in P. falciparum and other malaria parasite species (Lisk and Desai, 2005). In parallel with this observation, informatic analyses of the malaria parasite species (Lisk and Desai, 2005). In parallel with this observation, informatic analyses of the

Acknowledgments

We dedicate this article to the memory of Ian Bathurst, the Medicines for Malaria Venture project director for these studies.

Authorship Contributions

Participated in research design: Pillai, Nguiruago, Lyko, Dolinta, Butler, Nguyen, Peet, Bowlin, and Desai.

Conducted experiments: Pillai, Nguiruago, Lyko, Dolinta, Butler, Nguyen, Peet, Bowlin, and Desai.

Wrote or contributed to the writing of the manuscript: Pillai, Nguiruago, Lyko, Dolinta, Butler, Nguyen, Peet, Bowlin, and Desai.

References


Address correspondence to: Dr. Sanjay A. Desai, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12735 Twinbrook Parkway, Room 3W-01, Rockville, MD 20852. E-mail: sdesai@niaid.nih.gov
Solute restriction reveals an essential role for clag3-associated channels in malaria parasite nutrient acquisition


The Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD (A.D.P., W.N., B.L., K.D., S.A.D.) ; and Microbiotix, Inc., Worcester, MA (M.M.B., S.T.N., N.P.P., T.L.B.)
Supplemental Fig. 1. ISPA-28 is stable under culture conditions and is not adsorbed by serum. (A) Osmotic lysis kinetics for Dd2-infected cells in sorbitol without (black) or with 1 µM ISPA-28 (red and blue). The blue trace was recorded using a freshly prepared ISPA-28 solution whereas the red trace used ISPA-28 incubated with Dd2 cultures as described in the Materials and Methods. Identical slowing of sorbitol uptake reflects similar levels of PSAC inhibition and suggests that ISPA-28 is not inactivated through metabolism under culture conditions. (B-D) Structure and PSAC block dose responses for ISPA-28, dantrolene, and furosemide. Sorbitol-induced osmotic lysis kinetics with indicated concentrations of inhibitor were quantified without and with addition of 10% serum (black and red symbols, respectively). While block by ISPA-28 is not measurably affected, dantrolene and furosemide are significantly adsorbed by serum ($P < 0.01$ for 10 µM dantrolene; $P < 10^{-4}$ for 25 µM furosemide).
Supplemental Fig. 2. Residual PSAC-mediated uptake with high concentrations of ISPA-28. Plot shows inhibition dose responses for ISPA-28 and compound 2 for channels on the Dd2 parasite line; ordinate shows normalized permeability on a logarithmic scale. These inhibitors have similar $K_{0.5}$ values (dotted horizontal line), but have differing efficacies at high concentrations. Blue lines represent the best fits of the ISPA-28 dose response to Eq. 1 or to a single component Langmuir isotherm (solid and dashed lines, respectively).
Supplemental Fig. 3. Secondary scan for QTL associated with ISPA-28 parasite growth inhibition in the HB3 x Dd2 cross. Additional genomic loci were sought after controlling for the primary chromosome 3 locus. Dashed line represents the 0.05 significance threshold calculated from 1000 permutations.
Supplemental Fig. 4. Transport-based selection with ISPA-43 is insufficient to alter Dd2 channel phenotype. (A) Structure of ISPA-43, an inhibitor that preferentially blocks channels associated with clag3.2 expression in Dd2 and 7C20. (B) Timing of selections using osmotic lysis (blue and red horizontal dashes for 7C20 and Dd2, respectively). Selections were typically applied on 5 consecutive days by room temperature incubation in 5% sorbitol with 4 µM ISPA-43 for 30 min; cultures were then allowed to recover prior to examination of channel phenotype and repeated selection. Blue arrow indicates date when 7C20 selection was found to fully reverse channel phenotype; red arrows indicate dates of transport assays for Dd2 line before termination of selections. (C) ISPA-28 dose responses for PSAC inhibition after selections applied to 7C20 and Dd2 in panel B (blue and red circles, respectively; mean ± S.E.M.). Data for 7C20 are from (Nguitragool et al, 2011). Solid line reflects the dose-response for Dd2 prior to selection. Dd2 transport phenotype is not affected by transport-based selection with ISPA-43, but that of 7C20 is.
Supplemental Fig. 5. Southern blot showing specificity of *clag3* probe. Note that the probe recognizes full-length *clag3.1* and *clag3.2*, but not *clag* genes on other chromosomes.
**Supplemental Table 1.** *clag* gene specific primers used for RT-PCR experiments

<table>
<thead>
<tr>
<th>gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>clag2</em></td>
<td>CTCTTACTACTATTATATCCTCTCTCA</td>
<td>CCAGGCGTAGGCTCTTTAC</td>
</tr>
<tr>
<td><em>clag3.1</em></td>
<td>ACCCATAACTACATATTTCTAGTAATG</td>
<td>GACAAGTTCCAGAAGCATCCT</td>
</tr>
<tr>
<td><em>clag3.2</em></td>
<td>ACCCATAACTACATATTTCTAGTAATG</td>
<td>GATTTTAACTAGGAGCACTACATTTA</td>
</tr>
<tr>
<td><em>clag8</em></td>
<td>GTTACTACAACATCCCTGATTCAG</td>
<td>AATGAAAATATAAAAAATGCTGGGGGAT</td>
</tr>
<tr>
<td><em>clag9</em></td>
<td>TACCATTAGTGTATTATACACTTTAGG</td>
<td>CAAAAATATGGCAGTACTTGC</td>
</tr>
</tbody>
</table>

**Supplemental Table 2.** Primers used to amplify full-length *clag* genes from Dd2 genomic DNA.

<table>
<thead>
<tr>
<th>gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>clag2</em></td>
<td>ATGTACGTGTTAATATGACACTTTGAAG</td>
<td>TCATCGGTATTATCTGATTGAGTTTC</td>
</tr>
<tr>
<td><em>clag3.1</em></td>
<td>GTGCAATATATCAAAGTGTACATGCA</td>
<td>AAGAAAAATAATGAAAAAAGGTTAGA</td>
</tr>
<tr>
<td><em>clag3.2</em></td>
<td>GTTGAGTACGCACTAATATGTCAATTTG</td>
<td>AACCATAACATTATCATATATGTTAAATTACAC</td>
</tr>
<tr>
<td><em>clag8</em></td>
<td>CATTGGTATGAAATGTTCATGCAG</td>
<td>CAGGTATTTTGTAATCAGTGTC</td>
</tr>
<tr>
<td><em>clag9</em></td>
<td>ATGATAATTTGGTTTATCAACCAAC</td>
<td>AAAGGATCATAACGATAACGTTGC</td>
</tr>
</tbody>
</table>