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Altered plasmodial surface anion channel activity and *in vitro* resistance to permeating antimalarial compounds

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ABSTRACT

Erythrocytes infected with malaria parasites have increased permeability to various solutes. These changes may be mediated by an unusual small conductance ion channel known as the plasmodial surface anion channel (PSAC). While channel activity benefits the parasite by permitting nutrient acquisition, it can also be detrimental because water-soluble antimalarials may more readily access their parasite targets via this channel. Recently, two such toxins, blasticidin S and leupeptin, were used to select mutant parasites with altered PSAC activities, suggesting acquired resistance via reduced channel-mediated toxin uptake. Surprisingly, although these toxins have similar structures and charge, we now show that reduced permeability of one does not protect the intracellular parasite from the other. Leupeptin accumulation in the blasticidin S-resistant mutant was relatively preserved, consistent with retained *in vitro* susceptibility to leupeptin. Subsequent *in vitro* selection with both toxins generated a double mutant parasite having additional changes in PSAC, implicating an antimalarial resistance mechanism for water-soluble drugs requiring channel-mediated uptake at the erythrocyte membrane. Characterization of these mutants revealed a single conserved channel on each mutant, albeit with distinct gating properties. These findings are consistent with a shared channel that mediates uptake of ions, nutrients and toxins. This channel's gating and selectivity properties can be modified in response to *in vitro* selective pressure.

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1. Introduction

Invasion and growth within erythrocytes is a hallmark of plasmodium species; it permits these parasites to evade host immune responses and provides access to hemoglobin as a nutritive source. In the case of *Plasmodium falciparum*, the cause of the most severe form of human malaria, growth within erythrocytes also accounts for most of the clinical sequelae. Intracellular growth is associated with a dramatic remodeling of the host cytosol, with export of a large number of proteins to this compartment [1,2], generation of a specialized membranous network [3], and changes in erythrocyte membrane properties including increased permeability to various organic and inorganic solutes [4–6].

Electrophysiological studies have now established that the permeability changes result from one or more defined ion channels and cannot be attributed to non-specific membrane leaks [7]. While several other ion channels have been reported, a number of studies now point to the plasmodial surface anion channel (PSAC) as the primary uptake mechanism for most, if not all, small uncharged solutes and monovalent ions [8–10]. PSAC activity is conserved on erythrocytes infected with other malaria parasites [11] and is absent on cells infected with *Babesia divergens*, another apicomplexan parasite that invades human erythrocytes [12]. PSAC also has a number of unusual functional properties that distinguish it from known ion channels in higher organisms [13–15].

In vitro selection has recently been used to generate two separate parasite mutants that carry altered PSAC activity [9,16]. One mutant was generated after selection with blasticidin S, a peptidyl nucleoside antibiotic presumed to kill parasites by inhibiting protein translation on ribosomes within the intracellular parasite [17]. Although blasticidin S has been used in basic malaria research to select for transfected parasites expressing the deaminase *BSD* [18–20], a resistant mutant was spontaneously selected by continuous application of blasticidin S pressure to a specific parasite isolate (FCB) in the absence of the *BSD* resistance gene. The second channel mutant was selected with leupeptin, a cysteine and serine protease inhibitor that has multiple intracellular parasite targets [21–23]. This mutant did not have measurable changes in cellular protease activity or leupeptin sensitivity of parasite proteases; there were also no detectable

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changes in sequence or expression levels of key parasite protease genes.

What is the mechanism of acquired resistance in these two mutants? Because biochemical characterization revealed altered PSAC activity, both studies proposed that *in vitro* resistance results from changes in the channel that reduce toxin uptake at the host erythrocyte membrane and thereby prevent access to intracellular targets. However, parasite survival and expansion under selective pressure are complex and there are alternative explanations that deserve examination. Moreover, a number of other ion channels have been proposed for the infected erythrocyte membrane [7], further complicating interpretation of the macroscopic measurements in the previous reports.

To address these concerns, we have now undertaken more rigorous characterization of these mutants. First, we determined that resistance to either leupeptin or blasticidin S confers minimal protection against the other agent. We then used *in vitro* selection to generate a new parasite mutant resistant to both blasticidin S and leupeptin. Cell-attached patch-clamp revealed distinct changes in single channel gating and conductance that were strictly associated with each of the three mutant phenotypes, supporting the proposal of resistance acquired by selection of changes in a single ion channel type. Our study also provides insights into the PSAC's selectivity filter, which appears to have a surprising ability to allow permeation of a broad range of charged and uncharged solutes while maintaining the ability to distinguish between solutes of similar size, geometry, and charge.

2. Materials and methods

2.1. Growth inhibition assays

Isobologram analysis of growth inhibition by combinations of compound **2** with blasticidin S or leupeptin were performed using a SYBR green I-based fluorescence assay for parasite nucleic acid in 96-well format. Wild-type parasite cultures were synchronized in 5% D-sorbitol before seeding at 0.2 to 0.5% parasitemia and 5% hematocrit in RPMI 1640 supplemented with 25 mM HEPES, 2% serum, 50 mg/liter hypoxanthine, and defined dilutions of PSAC antagonist with toxin. Cultures were maintained for 3 days at 37 °C in 5% O_2–5% CO_2. The plates were then subjected to freezing–thawing before addition of SYBR green I at twice the manufacturer's recommended final concentration, incubation for 30 min, and measurement of fluorescence (excitation and emission wavelengths of 485 and 528 nm, respectively). Background fluorescence was subtracted by use of control cultures killed by 20 μM chloroquine. IC_{50} values for each ratio of PSAC antagonist and toxin were estimated by linear interpolation. Similar results were obtained with the Indo 1 and HB3 laboratory parasite lines.

2.2. In vitro selection of FCB-2mut

Mutant parasite lines resistant to blasticidin S or leupeptin were selected by continuous cultivation of wild-type parasites as described previously [9,16]. To select for a parasite resistant to both toxins, we challenged the single mutant lines *FCB-br1* and *HB3-leuR1* with 2.5 μg/mL blasticidin S and 50 μM leupeptin. After extended continuous cultivation (~6 months), a double mutant was generated from *FCB-br1* on both of two separate attempts. The clone, *FCB-2mut*, was then obtained by limiting-dilution cloning. We were unsuccessful in attempts to generate a double mutant from the *HB3-leuR1* line.

2.3. Leupeptin accumulation by intact cells

Leupeptin accumulation into erythrocytes infected with various parasite isolates were performed with a semi-quantitative assay that measures papain-mediated hydrolysis of Z-Phe-Arg-AMC and its inhibition by leupeptin from cell lysates [16]. We measured leupeptin content of intact cells after a 30 min preincubation with 40 μM leupeptin in PBS. This leupeptin concentration and preincubation duration optimally distinguishes between uptake by infected and uninfected cells, permitting sensitive detection of PSAC-mediated uptake. Measurement of uptake entailed rapid washes to reduce extracellular leupeptin to undetectable levels, lysis of the infected cell membrane with 0.05% saponin, addition of the released erythrocyte cytosolic contents to a buffered papain and Z-Phe-Arg-AMC mixture, and detection of fluorescence. Z-Phe-Arg-AMC is a peptide substrate that exhibits a marked fluorescence increase when digested by papain, a leupeptin-sensitive protease. Fluorescence measurements were normalized to % leupeptin content by comparison to matched controls incubated without leupeptin, which was defined as 0% content; complete inhibition of papain-mediated hydrolysis was set to 100% content. Control wild-type parasites were included in each experiment that examined parasite mutants, allowing direct comparison of accumulation.

2.4. Osmotic lysis kinetics

Continuous tracking of infected erythrocyte osmotic lysis kinetics in organic solutes was used to examine PSAC activity and was performed as described previously [24]. Trophozoite-infected erythrocytes were enriched by percoll-sorbitol separation, washed in PBS (150 mM NaCl, 20 mM Na_2HPO_4, pH 7.5), and resuspended at 37 °C and 0.15% hematocrit in permeant solutes (145 mM $PhTMA^+$ chloride or 280 mM sorbitol) buffered with 20 mM Na-HEPES, 0.1 mg/ml BSA, pH 7.4. Osmotic swelling and lysis were then continuously monitored by recording transmittance of 700 nm light through the cell suspension (DU640 spectrophotometer with Peltier temperature control, Beckman Coulter). The resulting transmittance recordings were normalized so that 100% osmotic lysis of infected cells corresponds to the steady-state transmittance measured after extended incubation (typically 2 h). The time to 50% lysis was estimated by interpolation. The dose–response for inhibition by compound **2** was determined using osmotic lysis experiments with sorbitol, as described [24].

2.5. Electrophysiology

Cell-attached patch-clamp recordings of trophozoite-stage infected erythrocytes were obtained as described previously [8]. All recordings used symmetric bath and pipette solutions of 1000 mM choline chloride, 115 mM NaCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 20 mM Na-HEPES, pH 7.4. This hypertonic solution increases the signal-to-noise ratio for single PSAC detection by increasing Cl^- flux through open channels and by reducing pipette electrical noise. Pipettes were fabricated from quartz capillaries and had resistances of 1 to 3 MΩ in the recording solution. Seal resistances were greater than 100 GΩ. Holding membrane potential was 0 mV; all recordings shown were obtained with pulses to −100 mV. We did not use perfusion of the bath. Voltage clamp recordings were obtained with an Axopatch 200B amplifier (Molecular Devices), filtered at 5 kHz, digitized at 100 kHz, and recorded with Clampex 9.0 software (Molecular Devices).

Dwell time distributions were determined from up to 75 s of single channel recordings for each isolate by using home-written code that detects mid-threshold crossings, uses linear interpolation of adjacent sample times, and corrects for a Gaussian filter risetime of 66.4 μs as described in detail previously [14]. Histogram ordinate values were normalized to percent of the total number of events detected under each condition. Histograms are displayed on square root-logarithmic plots because time constants for simple exponentially decaying processes are visible as maxima [25]. Spectral analyses were carried out as described previously [6,26]. These analyses used continuous