2008

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Antimicrobial Agents and Chemotherapy

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Antimicrob. Agents Chemother. **2008, 52(7):2346. DOI: 10.1128/AAC.00057-08.**
Published Ahead of Print 28 April 2008.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2008, p. 2346–2354 Vol. 52, No. 7
0066-4804/08/$08.00+0 doi:10.1128/AAC.00057-08

Changes in the Plasmodial Surface Anion Channel Reduce Leupeptin Uptake and Can Confer Drug Resistance in *Plasmodium falciparum*-Infected Erythrocytes▽

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Received 14 January 2008/Returned for modification 8 March 2008/Accepted 19 April 2008

Cysteine protease inhibitors kill malaria parasites and are being pursued for development as antimalarial agents. Because they have multiple targets within bloodstream-stage parasites, workers have assumed that resistance to these inhibitors would not be acquired easily. In the present study, we used in vitro selection to generate a parasite resistant to growth inhibition by leupeptin, a broad-profile cysteine and serine protease inhibitor. Resistance was not associated with upregulation of cysteine protease activity, reduced leupeptin sensitivity of this activity, or expression level changes for putative cysteine or serine proteases in the parasite genome. Instead, it was associated with marked changes in the plasmodial surface anion channel (PSAC), an ion channel on infected erythrocytes that functions in nutrient and bulky organic solute uptake. Osmotic fragility measurements, electrophysiological recordings, and leupeptin uptake studies revealed selective reductions in organic solute permeability via PSAC, altered single-channel gating, and reduced inhibitor affinity. These changes yielded significantly reduced leupeptin uptake and could fully account for the acquired resistance. PSAC represents a novel route for the uptake of bulky hydrophilic compounds acting against intraerythrocytic parasite targets. Drug development based on such compounds should proceed cautiously in light of possible resistance development though the selection of PSAC mutants.

Malaria is a leading cause of morbidity and mortality worldwide. Each year, up to 500 million humans are infected and 1 million to 2 million die as a result of infection with *Plasmodium falciparum*, the most virulent human parasite. Acquired parasite resistance to all available antimalarial drugs, combined with the absence of an approved vaccine, has created a desperate need to identify new parasite targets for drug development.

One group of parasite targets that has recently received much attention is the cysteine proteases. Several factors have contributed to this growing interest. A large number of putative cysteine proteases have been identified in the completed parasite genome sequence (22). A drug that inhibits several of these enzymes may be less susceptible to resistance because of its multiple sites of action. Moreover, cell biological studies have identified a divergent collection of essential activities mediated by these cysteine proteases. These include digestion of hemoglobin by intraerythrocytic parasites (24), timed erythrocyte rupture and release of invasive merozoites (7, 28, 38), host cell invasion (6), and processing of various preproteins. Finally, functional screens have identified a number of cysteine protease inhibitors with established in vitro and in vivo activities against malaria parasites (23, 26).

Important hurdles in translating these advances into a clinically useful antimalarial drug include toxicity due to inhibition of human proteases, the generally short half-lives of peptidomimetic agents in vivo, and unforeseen resistance mechanisms. The availability of protease enzymatic assays, libraries of known small-molecule protease inhibitors, and crystal structures of some parasite proteases have helped identify inhibitors with improved specificity and stability. Resistance mechanisms for cysteine protease inhibitors appear to be more complex and have not been as extensively studied. One study used in vitro selection with gradually escalating concentrations of a vinyl sulfone protease inhibitor to generate a highly resistant parasite isolate (31). Functional and genetic analyses of the selected mutant suggested that both upregulated expression of two falcipain cysteine proteases and reduced access to its targets in infected cells may contribute to resistance.

We now report the in vitro selection and characterization of a parasite resistant to leupeptin, a more readily available cysteine protease inhibitor. In vitro resistance was stable for many generations after removal of leupeptin pressure, suggesting changes at the level of the parasite genome. Surprisingly, our functional studies with this mutant revealed that basal cysteine protease activity was not upregulated and that its leupeptin sensitivity was unchanged. Instead, resistance was linked to marked changes in the plasmodial surface anion channel (PSAC), a parasite-induced ion channel on the host erythrocyte membrane that mediates the uptake of nutrients and various bulky organic solutes. These changes were associated with reduced leupeptin entry into infected erythrocytes, which appears to be the primary resistance mechanism. Our findings implicate an important role for PSAC in the uptake of water-

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▽ Published ahead of print on 28 April 2008.

soluble antimalarial drugs with low intrinsic membrane permeability. In addition, selection of PSAC mutants conferring reduced uptake represents a novel resistance mechanism that should be considered by antimalarial drug development programs. We present a straightforward test that can be used with any drug candidate to determine whether this type of resistance may arise.

MATERIALS AND METHODS

Reagents. Leupeptin hemisulfate and SYBR green I were obtained from Invitrogen (Carlsbad, CA); 2-butyl-5-imino-6-{[5-(4-nitrophenyl)-2-furyl]methylene}-5,6-dihydro-7*H*-[1,3,4]thiadiazolo[3,2-a]pyrimidin-7-one (NPF-1) was obtained from Chembridge Corp. (San Diego, CA); and *N*-α-benzyloxycarbonyl-L-phenylalanyl-L-arginine-(7-amino-4-methylcoumarin) (Z-Phe-Arg-AMC) was obtained from AnaSpec (San Jose, CA). Unless otherwise specified, all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Growth inhibition assays. Leupeptin growth inhibition dose-responses were determined using a SYBR green I-based fluorescence assay for parasite nucleic acid in 96-well format. 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 leupeptin at indicated concentrations. Cultures were maintained for 5 days at 37°C in 5% O_2–5% CO_2, with a medium change on day 3. The plates were then subjected to freezing-thawing before addition of SYBR green I at twice the manufacturer's recommended final concentration, incubation in the dark for 30 min, and measurement of fluorescence (excitation and emission at 485 and 528 nm, respectively). Growth inhibition studies with known antimalarials and other protease inhibitors were similarly performed, except that shorter incubation times (72 h) were used for blasticidin S, chloroquine, artemisinin, and quinine. For each inhibitor concentration, the mean of triplicate measurements was calculated after subtraction of background fluorescence from cultures killed by 20 μM chloroquine.

Incubations for 72 h were also used for growth inhibition studies that examined the effect of NPF-1, a specific PSAC antagonist (15), on leupeptin's toxic effect. These experiments used serial dilutions of various molar ratios of leupeptin and NPF-1. For each ratio, the concentrations required to produce 50% parasite killing (IC_{50}s) were determined by interpolation and are presented in a standard isobologram plot (18).

Transmission electron microscopy. Specimens were fixed overnight at 4°C with 2.5% glutaraldehyde–4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. They were then postfixed for 1 h with 0.5% osmium tetroxide–0.8% potassium ferricyanide, stained overnight with 1% uranyl acetate at 4°C, dehydrated with a graded ethanol series, and embedded in Spurr's resin. Thin sections were cut with an RMC MT-7000 ultramicrotome (Ventana, Tucson, AZ), stained with 1% uranyl acetate and Reynold's lead citrate, and visualized at 80 kV on a Philips CM-10 transmission electron microscope (FEI Company, Hillsboro, OR). Digital images were acquired with a digital camera system (Advanced Microscopy Techniques, Danvers, MA).

Measurement of hemozoin production. Beta-hematin, the principal pigment in parasite hemozoin, was measured as described previously (20). Briefly, parasites were synchronized, adjusted to 10% parasitemia, and cultured with 50 μM leupeptin for 18 h to allow maturation to the trophozoite stage and minimize the effects of leupeptin on later stages. Packed cells were harvested from leupeptin-treated parasites and untreated controls, lysed in 10 ml of ice-cold 5 mM Na_2HPO_4 (pH 7.6), and centrifuged (27,000 × *g*, 30 min). The pellet was washed and digested overnight at room temperature in 2 ml of 2.5% sodium dodecyl sulfate–25 mM Tris, pH 7.8. After centrifugation to remove soluble material, the remaining pellet was incubated in the same solution supplemented with 0.1 N NaOH to release the ferriprotoporphyrin IX incorporated in beta-hematin. The pigment released was detected with absorbance wavelength scans and was quantified at 400 nm.

Quantitative reverse transcription-PCR (RT-PCR). Total RNA was harvested from tightly synchronized mid-trophozoite-stage parasites for cDNA synthesis by using oligo(dT) and random hexamer primers with Superscript II reverse transcriptase (Invitrogen). Matched cDNA from wild-type and HB3-*leuR1* parasites was used as the template in PCR of small amplicons (100 to 150 bp) with specific primers for genes encoding cysteine, serine, and aspartyl proteases and the Quantitect SYBR green PCR kit (Qiagen, Valencia, CA). The reaction conditions were those recommended by the manufacturer. Amplification kinetics were followed in real time (iCycler iQ multicolor real-time PCR system; Bio-Rad, Hercules, CA). Transcript abundance for each gene was determined relative to that of 60S ribosomal subunit protein L18 (PlasmoDB accession number MAL13P1.209), an internal control used in each experiment.

Parasite protease activity and leupeptin sensitivity. Z-Phe-Arg-AMC is a peptidomimetic substrate for many cysteine and serine proteases. Protease-mediated hydrolysis yields increased fluorescence through liberation of the AMC group. We quantified parasite protease activity with this reagent using soluble parasite extracts prepared as described previously (25), except that trophozoite-infected erythrocytes were enriched to 95 to 99% parasitemia by Percoll-sorbitol separation (1). Extracts from HB3 and HB3 *leuR1* parasites were adjusted to 40 μg/ml (RC DC protein assay; Bio-Rad) in 0.1 M sodium acetate, pH 5.5, with 10 mM dithiothreitol and leupeptin, where indicated, and prewarmed to 37°C in a 96-well plate before addition of 50 μM Z-Phe-Arg-AMC. The fluorescence output was measured at 2-min intervals to estimate the rate of hydrolysis (excitation and emission wavelengths, 353 and 442 nm, respectively).

Erythrocyte leupeptin permeability assays. The Z-Phe-Arg-AMC protease assay was modified to detect and quantify leupeptin accumulation in erythrocyte cytosol. For this assay, uninfected or enriched infected erythrocytes were washed, incubated in phosphate-buffered saline (PBS) with leupeptin for indicated durations at room temperature, and washed four or five times in ice-cold PBS with 5% serum and 500 μM furosemide. Control experiments confirmed that these wash conditions remove all extracellular leupeptin with negligible loss of intracellular leupeptin. Cell number was then determined with a hemocytometer to standardize measurements between isolates. The erythrocytes were then suspended at 20% hematocrit in PBS with 0.05% saponin for 10 min before centrifugation (4,000 × *g*, 10 min, 4°C). This saponin treatment releases solutes in the erythrocyte compartment, but not those in the parasite compartment. Supernatant corresponding to the erythrocyte cytosol released from 10^8 cells was then resuspended to 1 ml in 0.1 M sodium acetate, pH 5.5, with 10 mM dithiothreitol–150 ng/ml papain (2.4×10^{-5} activity units/ml), incubated for 10 min, and mixed with 50 μM Z-Phe-Arg-AMC in 96-well plates before fluorescence measurements were obtained as described above.

While these fluorescence measurements resemble those described in the preceding section, they differ in that only the erythrocyte cytosolic compartment is used to evaluate and quantify leupeptin transport through channels on the erythrocyte membrane. Because this compartment has negligible protease activity, we added papain to catalyze Z-Phe-Arg-AMC hydrolysis. Control experiments revealed that leupeptin has a high affinity for inhibition of papain activity (IC_{50}, 5 nM), permitting high sensitivity in these measurements.

Osmotic lysis kinetics. Osmotic lysis experiments were performed as described previously (36). 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 0.5% hematocrit in 280 mM sorbitol, 20 mM sodium HEPES, 0.1 mg/ml bovine serum albumin, pH 7.4, supplemented with PSAC antagonists as indicated. 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, Fullerton, CA). This method yields permeability estimates that match those from radioisotope flux and patch-clamp recordings (36).

Electrophysiology. Cell-attached patch-clamp recordings of trophozoite-stage infected erythrocytes were obtained as described previously (2) in symmetric bath and pipette solutions of 1,000 mM choline chloride, 115 mM NaCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 20 mM sodium HEPES, pH 7.4. This hypertonic solution increases the signal-to-noise ratio for single PSAC detection by permitting higher rates of Cl^- flux through open channels and by reducing electrical noise contributed by the pipette. When phloridzin was used in single-channel recordings, it was present at identical concentrations in both pipette and bath compartments. Pipettes were fabricated from quartz capillaries by pulling to tip diameters of <0.5 μm and resistances of 1 to 3 MΩ in the recording solution. Seal resistances were >100 GΩ. Voltage clamp recordings were obtained with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 5 kHz with an eight-pole Bessel filter, digitized at 100 kHz, and recorded with Clampex 9.0 software (Molecular Devices). All single-channel analyses were carried out with locally developed code.

Statistical methods. Two-group comparisons used Student's *t* test except for those examining Z-Phe-Arg-AMC hydrolysis rates. For those hydrolysis rates, paired *t* test comparisons were used because we noticed day-to-day variations in the fluorescence signal for this reagent. Confidence intervals on the ratios used log transformations and *t* distributions with Student's degrees of freedom. In dose-response studies, concentrations producing 50% inhibition ($K_{0.5}$) were estimated by nonlinear least squares fitting to the equation $y = a/[1 + (x/K_{0.5})^b]$, where *a* and *b* are constants and *x* and *y* are the independent and dependent variables, respectively. *b* was set equal to 1 except in fits of phloridzin dose-

responses because this antagonist is known to have a complex stoichiometry of interaction with PSAC (9). The confidence interval and *P* value for the ratio of $K_{0.5}$ values in Fig. 2 were calculated by weighted least squares fitting of ratios and variances from individual experiments. These ratios and variances were calculated by a separate nonlinear least-squares fit for each experiment. Simulations show that this gives better coverage than combining the results of all experiments into one mixed-effects nonlinear model. All *P* values are two sided.

RESULTS

Selection of a leupeptin-resistant *P. falciparum* clone. Leupeptin (acetyl-leucyl-leucyl-arginal; molecular weight, 426.6) is a competitive, reversible inhibitor of serine and cysteine proteases. As reported previously (24), we found that 50 μM leupeptin sterilizes asexual-stage *P. falciparum* cultures, with no viable parasites detected by microscopic examination after 3 days. Upon continued culturing with leupeptin, however, resistant parasites were recovered within 8 weeks on five separate attempts. In these studies, several different laboratory isolates (3D7, HB3, FCB, Indo 1, and 7G8) were challenged with leupeptin; interestingly, leupeptin-resistant parasites were recovered only from the HB3 isolate, suggesting an effect of genetic differences between this and other laboratory isolates. A clonal population of leupeptin-resistant parasites, referred to here as HB3-*leuR1*, was obtained by limiting dilution cultures. Growth inhibition studies with this mutant revealed a fourfold increase in the leupeptin IC_{50} (74 ± 4 μM versus 18 ± 2 μM for the parental HB3 isolate; $n = 4$ trials each; $P < 10^{-4}$) (Fig. 1A). This relatively modest change in IC_{50} is sufficient to permit the continuous culture of HB3-*leuR1* in the presence of 50 μM leupeptin, a concentration that sterilizes cultures of wild-type isolates.

Leupeptin's toxic effects may be due to inhibition of proteases expressed at various parasite stages and intracellular sites; there are nearly 50 predicted cysteine or serine proteases in the parasite genome that may be targeted (39). To explore possible resistance mechanisms, we compared the microscopic effects of leupeptin on HB3 and HB3-*leuR1* parasites. As seen with other parasite isolates (11, 24), intraerythrocytic HB3 parasites exhibited enlarged digestive vacuoles after exposure to leupeptin; in contrast, HB3-*leuR1* parasites had a vacuole morphology indistinguishable from that of cells not exposed to leupeptin (data not shown). Transmission electron micrographs confirmed these observations and revealed reduced formation of hemozoin crystals in leupeptin-treated HB3 parasites, but crystals of normal size and number in HB3-*leuR1* parasites (Fig. 1B). These crystals are formed from the ferriprotoporphyrin IX released by protease-mediated hemoglobin digestion. We quantified this difference by measuring the production of sodium dodecyl sulfate-resistant hemozoin. When compared to matched controls (HB3 parasites without leupeptin treatment), leupeptin exposure significantly reduced production of hemozoin in HB3 parasites, but not in HB3-*leuR1* parasites (Fig. 1C and D). Because the selection of HB3-*leuR1* is associated with restoration of normal vacuole morphology and hemozoin formation, our studies support the prevailing view that inhibition of hemoglobin degradation contributes to leupeptin's toxic effect on wild-type parasites. HB3-*leuR1* may then have acquired resistance by upregulating vacuolar proteases, by altering their sensitivity to leupeptin, through the conversion of leupeptin into inactive metabolites (4), or by decreasing leupeptin access to these intraerythrocytic targets.

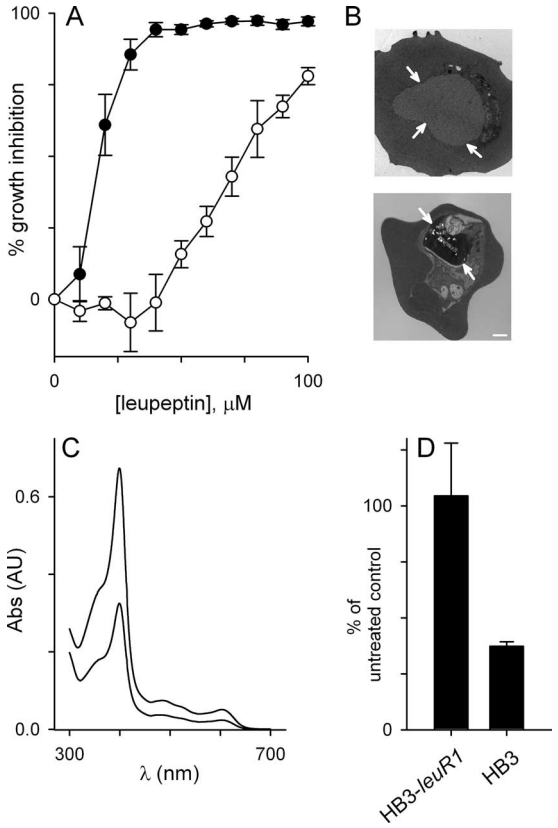

FIG. 1. Leupeptin sensitivity and parasite ultrastructure. (A) Leupeptin dose-response for in vitro growth inhibition of HB3 and HB3-*leuR1* parasites (filled and open circles, respectively). (B) Transmission electron micrographs of HB3- and HB3-*leuR1*-infected erythrocytes (upper and lower panels, respectively) after culturing with 100 μM leupeptin for 6 h. Parasite digestive vacuoles are demarcated with white arrows in both panels. While HB3 parasites exhibit swollen digestive vacuoles with a reduction in hemozoin crystals, HB3-*leuR1* parasites exhibit normal vacuolar morphology despite leupeptin exposure. The white scale bar in the lower panel represents 500 nm for both panels. (C) Absorbance (Abs) scan of solubilized hemozoin produced during an 18-h incubation with 50 μM leupeptin by HB3-*leuR1* and HB3 parasites (upper and lower traces, respectively). AU, arbitrary units. (D) Mean ± SEM absorbance at 400 nm from the scans in panel C normalized to 100% for HB3 parasites not exposed to leupeptin. Hemozoin production in the presence of leupeptin is significantly greater in HB3-*leuR1* ($P = 0.03$; $n = 4$ trials each).

Leupeptin resistance is not associated with changes in protease expression or inhibitor sensitivity. Although there are numerous proteases in the parasite digestive vacuole, two papain-family (clan CA) cysteine proteases, falcipain-2 and falcipain-3, are critical in hemoglobin digestion (29, 30). We used hydrolysis of the fluorogenic peptide substrate Z-Phe-Arg-AMC under buffered conditions that simulate the vacuolar milieu (25) and found essentially identical cysteine protease activities in HB3 and HB3-*leuR1* parasites (Fig. 2A) (95% confidence interval for the ratio of HB3-*leuR1* hydrolysis rate/HB3 hydrolysis rate, 0.89 to 1.10). Consistent with the known pharmacology of these proteases, addition of leupeptin slowed substrate hydrolysis by extracts from both isolates (Fig. 2B and

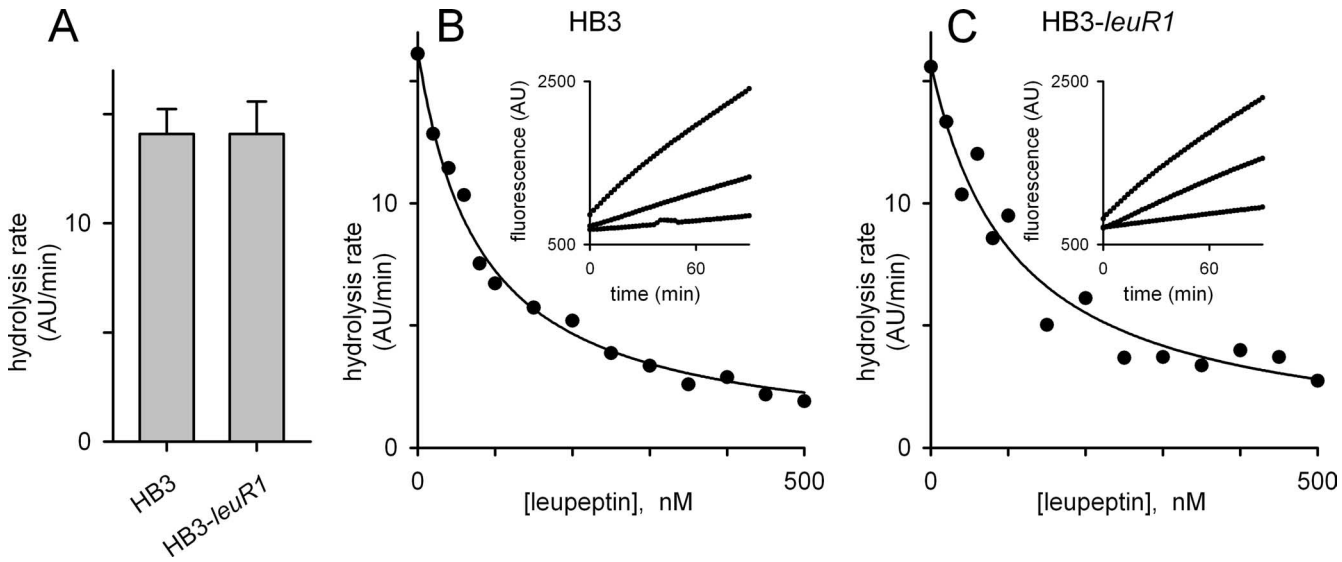

FIG. 2. Resistance is not associated with changes in protease activity or sensitivity to leupeptin. (A) Mean Z-Phe-Arg-AMC hydrolysis rates ± SEM as a measure of digestive vacuolar protease activity ($n = 5$ for each parasite analyzed as matched pairs; $P > 0.9$). (B and C) Leupeptin dose-responses for vacuolar proteases from wild-type and resistant parasites, respectively. Each symbol represents the mean of three rates determined by linear regression of fluorescence measurements between 10 and 90 min after addition of extract. The solid curve represents the best fit of the data to $y = a/[1 + (x/K_{0.5})]$. The insets in each panel show the primary fluorescence data for 0, 100, and 500 nM leupeptin (top to bottom, respectively) and demonstrate that the hydrolysis of Z-Phe-Arg-AMC proceeds with linear kinetics. AU, arbitrary units.

C). Leupeptin dose-responses, determined to evaluate possible changes in inhibitor affinity, were adequately fitted by an equation based on reversible inhibition in a 1:1 stoichiometry (see the legend to Fig. 2). The $K_{0.5}$ values for leupeptin inhibition were 87 ± 3.8 nM and 98 ± 6.5 nM for HB3 and HB3 *leuR1*, respectively ($n = 4$ dose-response experiments each). These values were not significantly different ($P = 0.433$; 95% confidence interval for the HB3-*leuR1* $K_{0.5}$ to HB3 $K_{0.5}$ ratio, 0.76 to 1.4).

Because functional assays may not detect changes in expression of some parasite proteases, we evaluated possible upregulation in the mutant by performing real-time two-step RT-PCR to quantify transcription of a large collection of cysteine, serine, and aspartyl proteases expressed during intraerythrocytic parasite stages (39). In contrast to the acquired resistance to another protease inhibitor (31), these experiments did not identify global upregulation of protease genes (Table 1). Some of these genes may have modest up- or downregulation that contributes to leupeptin resistance. More extensive studies will be necessary to determine reproducibility and possible biological significance of such changes. We also sequenced the falcipain-2 and falcipain-3 genes from both the HB3 and the HB3-*leuR1* parasites and found no mutations between these isolates (data not shown); there were also no polymorphisms relative to their sequences in the 3D7 isolate (PF11_0165 and PF11_0162, respectively). Thus, functional studies, expression profiles, and DNA sequencing do not implicate changes in parasite proteases as the primary basis of the selected leupeptin resistance.

Altered PSAC activity in HB3-*leuR1*. We next explored whether resistance is due to reduced leupeptin uptake by infected erythrocytes. The host erythrocyte membrane represents a major barrier for the uptake of serum solutes. Bulky organic solutes enter infected erythrocytes primarily via PSAC,

TABLE 1. Transcription of protease genes is not upregulated in HB3-*leuR1*[a]

Gene	Transcript ratio (HB3-*leuR1*/HB3)
PF08_0008	1.23 ± 0.39
PFC0310c	1.22 ± 0.17
PF11_0165	1.54 ± 0.38
PFE1355c	1.02 ± 0.04
PF11_0162	1.57 ± 0.26
PFE0355c	1.27 ± 0.39
PFB0340c	2.06 ± 0.37
PFB0350c	1.46 ± 0.34
PFB0330c	1.63 ± 0.35
PFB0345c	0.83 ± 0.12
PFB0335c	1.06 ± 0.13
PF11_0174	1.23 ± 0.36
PFE0340c	1.76 ± 0.24
PF11_0175	0.63 ± 0.19
PFA0220w	1.39 ± 0.42
PF11_0381	1.48 ± 0.49
PF14_0147	1.37 ± 0.27
MAL8P1.157	1.83 ± 0.37
PFI0225w	1.85 ± 0.34
PF14_0145	0.21 ± 0.08
PF14_0063	1.92 ± 0.74
PF13_0096	1.24 ± 0.37
PF11_0177	1.01 ± 0.20
PFL2290w	1.77 ± 0.68
PFD0230c	1.35 ± 0.62
PF14_0577	1.46 ± 0.21
PF13_0118	1.16 ± 0.15
PF14_0348	0.97 ± 0.22
MAL13P1.167	1.35 ± 0.45
PFI0135c	0.50 ± 0.08

[a] Genes encoding putative proteases were selected on the basis of expression during erythrocytic stages in a previous study (39). Values represent the means ± SEMs of the transcript abundance in HB3-*leuR1* relative to that in HB3, as determined by quantitative RT-PCR ($n = 3$ separate RNA harvests each).

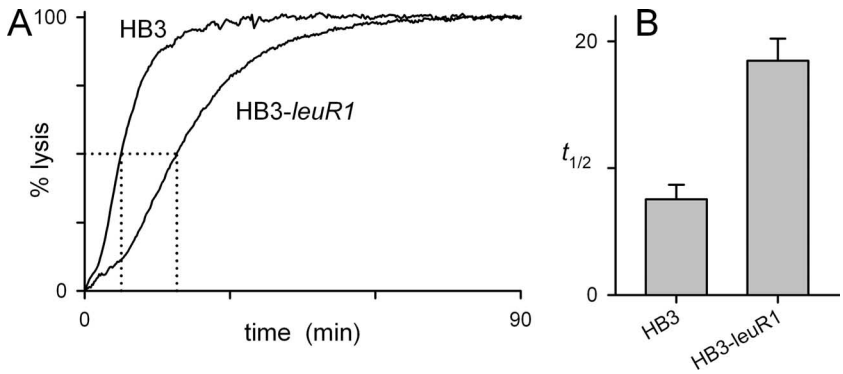

FIG. 3. Reduced organic solute permeability of HB3-*leuR1*-infected erythrocytes. (A) Osmotic lysis kinetics in isotonic sorbitol, determined by continuous monitoring of 700-nm light transmittance through suspensions of infected erythrocytes. Superimposed dotted lines are used to determine the time to 50% hemolysis and reveal the slower kinetics of sorbitol uptake by HB3-*leuR1*-infected erythrocytes. (B) Mean hemolysis half-times ($t_{1/2}$) ± SEM for each parasite.

an unusual broad-specificity ion channel on the host membrane (10). Important examples of these solutes include *N*-hydroxysulfosuccinimide esters (5) and pepstatin A (27), another peptidomimetic protease inhibitor. Because leupeptin may also enter infected erythrocytes via this channel, we evaluated PSAC activity on erythrocytes infected with HB3-*leuR1*.

Organic solute uptake via PSAC can be quantified with a transmittance-based light scattering assay (36). This assay has been used to estimate the absolute permeability coefficient for sorbitol and to determine the affinities of various PSAC antagonists (2, 9, 17, 36). Our survey of the functional properties of PSAC revealed significantly decreased sorbitol permeability in HB3-*leuR1*-infected erythrocytes (Fig. 3). We quantified this decrease with osmotic lysis half-time values of 7.5 ± 1.1 and 18.5 ± 1.8 min for HB3 and HB3-*leuR1*, respectively (mean ± standard error of the mean [SEM] for $n = 16$ determinations for each isolate). Because there is a reciprocal relationship between the lysis half-time and permeability, these values reflect a statistically significant decrease in sorbitol permeability in the HB3-*leuR1* parasite ($P < 10^{-5}$). At 50 μM, leupeptin had no measurable effect on sorbitol uptake with either parasite, excluding a direct effect of leupeptin on PSAC activity (data not shown).

We searched for other changes and identified altered affinities for two well-characterized PSAC antagonists. Channels on cells infected with HB3-*leuR1* exhibited a markedly reduced affinity for inhibition by phloridzin (Fig. 4A and B). Because phloridzin is not specific (9) and because other channels might also contribute to organic solute uptake (32), we obtained cell-attached patch-clamp recordings with and without phloridzin. Single PSAC recordings from HB3-*leuR1*-infected erythrocytes revealed markedly less effective inhibition by 100 μM phloridzin than those from HB3-infected erythrocytes (Fig. 4C). Concordant changes in the osmotic lysis assay and in single channel recordings are consistent with a primary role for PSAC in the uptake of diverse organic solutes, as suggested in various other studies (2, 5, 9, 10, 14, 15, 17).

We also identified a significant reduction in affinity for furosemide (Fig. 4E), a well-studied and somewhat more specific PSAC antagonist (2, 8). Because furosemide and phloridzin inhibit PSAC through binding sites on opposite membrane faces (9), this observation suggests global structural changes in PSAC on this mutant. The association between the selection of a mutant parasite and changes in PSAC is also consistent with the findings of previous studies that suggest that it is parasite encoded (2, 14).

Changes in PSAC confer drug resistance by reducing leupeptin uptake. The altered PSAC behavior of HB3-*leuR1* may contribute to resistance by reducing leupeptin uptake at the erythrocyte membrane. To test this hypothesis, we modified the Z-Phe-Arg-AMC hydrolysis protease assay to detect leupeptin accumulation in the erythrocyte cytosol. Infected erythrocytes were preincubated with leupeptin, washed to remove the extracellular protease inhibitor, and then treated with saponin to permeabilize the erythrocyte membrane without releasing parasite cytosolic components. Erythrocyte cytosol fractions prepared from infected cells significantly inhibited Z-Phe-Arg-AMC hydrolysis by papain, while those prepared from uninfected cells did not (Fig. 5A). Control experiments using preincubations without leupeptin did not show this difference (data not shown). Thus, trophozoite-infected erythrocytes have a measurably higher leupeptin permeability than uninfected erythrocytes. This parasite-induced permeability is mediated by PSAC because it was largely abolished by 50 μM NPF-1 (Fig. 5A), a specific PSAC antagonist (15).

Similar experiments with HB3-*leuR1*-infected erythrocytes indicated that they have a significantly lower leupeptin permeability than cells infected with HB3 (Fig. 5B and C; $n = 5$ for each parasite; $P = 2 \times 10^{-5}$). Thus, the altered PSAC contributes to resistance by selectively reducing passive leupeptin uptake across the erythrocyte membrane.

Various other parasite-induced ion channels on the erythrocyte surface have been reported by other workers (32). While it remains formally possible that changes in these channels may also contribute to reduced leupeptin uptake in HB3 *leuR1*, this is unlikely for several reasons. First, each of these other proposed channels is thought to represent a human ion channel activated by the parasite. In vitro selection of specifically engineered changes in human ion channels permitting reduced leupeptin entry but the sustained uptake of nutrients is difficult to envision, especially because erythrocytes lack heritable genetic material. Second, NPF-1 has no measurable ac-

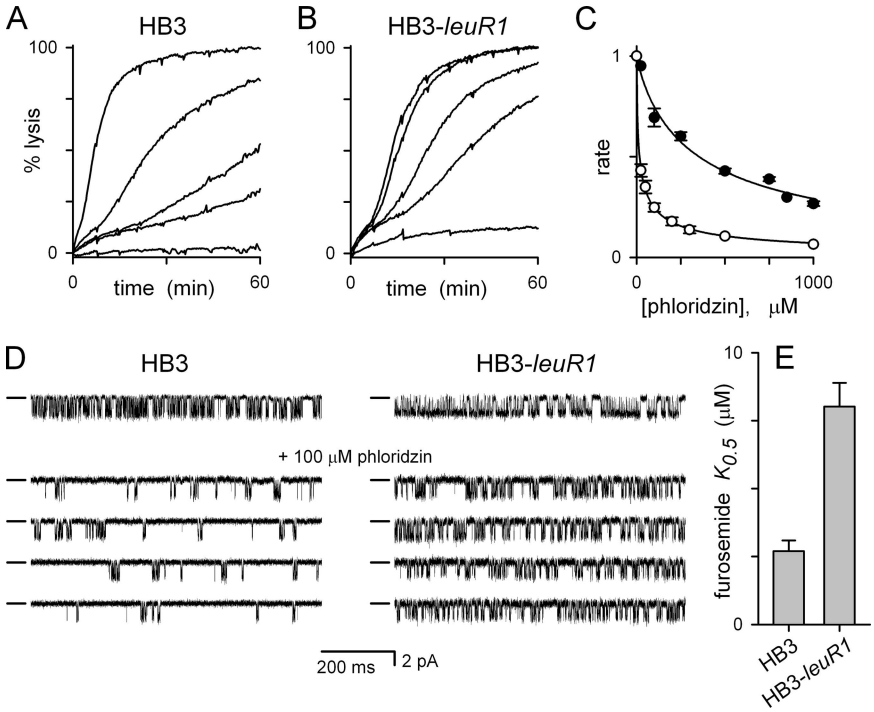

FIG. 4. Altered pharmacology links changes to PSAC. (A and B) Osmotic lysis kinetics in sorbitol for the indicated parasites with 0, 100, 500, 1,000, and 5,000 μM phloridzin (traces from top to bottom, respectively, in each panel). HB3-*leuR1* channels have reduced phloridzin affinity. (C) Phloridzin dose-responses for HB3 and HB3-*leuR1* (open and filled circles, respectively). Each symbol represents the mean ± SEM from up to seven osmotic lysis half-times after normalization to 1.0 without phloridzin. Solid lines represent best fits to $y = a/[1 + (x/K_{0.5})^b]$, with estimated $K_{0.5}$ values of 17 ± 0.9 and 340 ± 49 μM for HB3 and HB3-*leuR1*, respectively. (D) Single PSAC recordings without (top trace) or with (lower four traces) 100 μM phloridzin added to both the bath and the pipette. The left and right columns reflect recordings from erythrocytes infected with HB3 and HB3-*leuR1*, respectively; each column shows traces acquired from three separate patches. For all traces, the membrane potential was clamped at −100 mV. The dash to the left of each trace represents the closed channel level. Data are typical of five single channel patches recorded from each isolate. The inhibitor-free channel gating is also visibly altered in comparisons of the two top traces. This altered gating was seen in other patches and may reflect an additional change in the mutant PSAC. (E) Difference in $K_{0.5}$ values for inhibition of sorbitol-induced osmotic lysis by furosemide, estimated as described previously (36). $P = 2.4 \times 10^{-5}$ (Student's *t* test) determined from $n = 6$ trials each.

tivity against a battery of other ion channels in other systems (15, 17). To account for the reduced leupeptin uptake in HB3-*leuR1*, a channel unrelated to PSAC would need to be present on the erythrocyte membrane and also be sensitive to NPF-1, an unlikely coincidence. Third, it is difficult to account for the changes observed in single PSAC recordings (Fig. 4C) upon in vitro selection with leupeptin in scenarios in which PSAC is not involved in organic solute uptake.

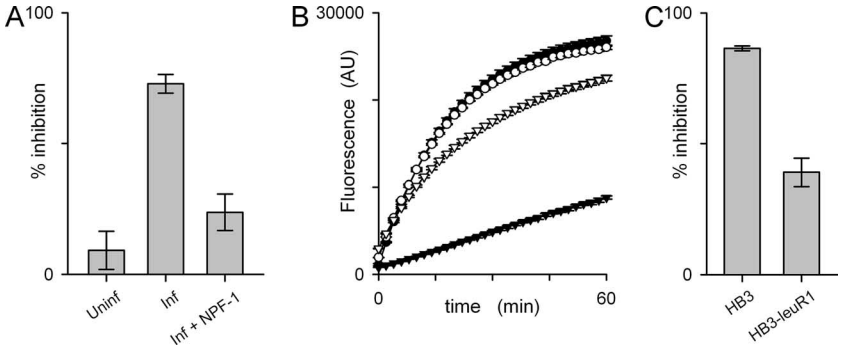

FIG. 5. Leupeptin uptake is mediated by PSAC and is decreased in the mutant. (A) Percent inhibition of papain-mediated Z-Phe-Arg-AMC hydrolysis by erythrocyte cytosol harvested from cells incubated with 40 μM leupeptin for 15 min at room temperature. Initial hydrolysis rates were determined by linear regression of measurements every 2 min for the first 10 min. Bars represent the means ± SEMs of four independent experiments with cytosolic fractions from uninfected cells, HB3-infected cells, or HB3-infected cells exposed to 50 μM NPF-1 during leupeptin loading (Uninf, Inf, and Inf + NPF-1, respectively). (B) Kinetics of papain-mediated Z-Phe-Arg-AMC hydrolysis in the presence of infected erythrocyte cytosolic fractions prepared before or after a 60-min incubation with 40 μM leupeptin at 37°C (circles and triangles, respectively). Symbols represent means ± SEMs of triplicate readings with erythrocyte cytosol from HB3- and HB3-*leuR1*-infected cells (black and white symbols, respectively). Sustained Z-Phe-Arg-AMC hydrolysis with HB3-*leuR1* fractions after leupeptin loading indicates reduced leupeptin uptake through the mutant PSAC. AU, arbitrary units. (C) Percent inhibition of papain-mediated Z-Phe-Arg-AMC hydrolysis, calculated from rates over first 10 min, measured as described for panel B.

TABLE 2. Leupeptin resistance does not confer resistance to other agents[a]

Compound	IC_{50}[b]	
	HB3	HB3-*leuR1*
Leupeptin	18 ± 2	74 ± 4
E-64	3.69 ± 0.13	5.88 ± 0.99
Pepstatin A	39.2 ± 13.2	29.36 ± 17.05
Blasticidin S	0.43 ± 0.15	1.15 ± 0.21
Chloroquine	10.41 ± 0.24	11.57 ± 1.80
Artemisinin	8.36 ± 1.98	7.71 ± 1.17
Quinine	77.13 ± 4.63	73.96 ± 1.76

[a] Growth inhibition studies with HB3 and HB3-*leuR1* were carried out as described in Materials and Methods.

[b] Mean IC_{50}s ± SEMs were determined from three or more dose-response experiments each and are presented in μM (leupeptin, E-64, pepstatin A, and blasticidin S) or nM (the other agents). There were no significant differences for agents other than leupeptin between the two parasites, but this may reflect our small sample sizes. The trend toward higher E-64 and blasticidin S IC_{50}s in the mutant may reflect partial cross-resistance with leupeptin.

Because resistance to vinyl sulfone protease inhibitors undergoes partial reversion upon removal of selective pressure (31), we maintained continuous cultures of HB3-*leuR1* without leupeptin pressure for 3 months. Subsequent challenge revealed that the progeny were still resistant to leupeptin's toxic effects. These parasites also exhibited quantitatively unchanged reductions in sorbitol permeability (osmotic lysis data not shown), indicating that the selected resistance is stable for many generations and suggesting one or more mutations in the genes encoding PSAC activity. In contrast to these findings, another recently identified functional PSAC mutant undergoes rapid reversion in the absence of selective pressure (14).

We also used in vitro growth inhibition studies to examine whether the selection of leupeptin resistance confers resistance to other protease inhibitors or to known antimalarial drugs. None of these other agents exhibited a significantly altered IC_{50} (Table 2). These compounds may enter infected cells primarily via other mechanisms, such as direct partitioning across membranes; alternatively, they may require uptake via PSAC but have relatively preserved permeabilities in this mutant. Blasticidin S was also tested because of its use to select for a distinct PSAC mutant (14). It appears to have some cross-resistance with leupeptin (Table 2), consistent with the proposal that it requires PSAC-mediated uptake to reach its parasite ribosomal target. Additional studies will be necessary to confirm this hypothesis.

Anticipating similar resistance to other compounds. Acquired resistance to antimalarial compounds via reduced PSAC-mediated uptake represents a new resistance mechanism that may be discouraging to drug discovery and development programs. Is it possible to predict which new drug candidates are most susceptible? To develop a suitable test, we evaluated the effects of NPF-1 on parasite killing by leupeptin. Because NPF-1 and other PSAC antagonists also inhibit parasite growth in vitro, we performed growth inhibition dose-response assays with various mixtures of leupeptin and NPF-1 and used an isobologram to examine possible interactions (Fig. 6). If parasite growth inhibition by NPF-1 and leupeptin involves noninteracting mechanisms, then the IC_{50} for each mixture should fall on the straight line connecting the effects of the inhibitors applied individually (Fig. 6, solid line). In contrast to this prediction, we observed a marked shift to the right of this line in our experiments, indicating that NPF-1 strongly antagonizes the toxicity of leupeptin. This antagonism is consistent with a critical role for PSAC in leupeptin uptake: NPF-1 presumably limits leupeptin's toxicity by blocking PSAC-mediated access to intracellular protease targets. We also performed growth inhibition studies with combinations of leupeptin and two other high-affinity, high-specificity PSAC antagonists (unpublished data). Both of these agents produced similar reductions in leupeptin toxicity (data not shown), excluding antagonism due to mechanisms other than reduced leupeptin uptake. Thus, in vitro antagonism of a drug candidate's toxicity by one or more PSAC antagonists can confirm a dependence on PSAC-mediated uptake. This simple test can be used with any new drug candidate to evaluate if resistance can arise through selection of reduced uptake via an altered PSAC.

DISCUSSION

Acquired resistance to antimalarial drugs may be the single greatest problem facing both prophylaxis and treatment of malaria worldwide. Indeed, prior to the development of chloroquine resistance, single-dose therapy was generally considered definitive treatment for *P. falciparum* malaria cases in all parts of the world. Over some 20 years, gradually increasing rates of parasite resistance to chloroquine led to the need for increases in the administered dose, the need for the use of multiple doses over 3 to 7 days, and outright treatment failure. Acquired chloroquine resistance had a devastating effect on malaria treatment and contributed significantly to abandonment of the global malaria eradication program launched by the World Health Organization (37).

Understanding the drug resistance mechanisms used by malaria parasites not only is an important step in attempts to restore the utility of existing antimalarials but also is critical to

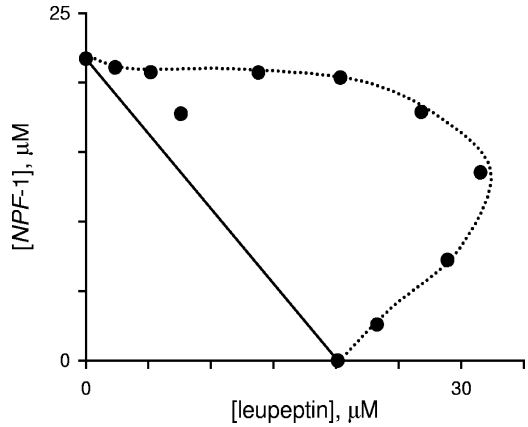

FIG. 6. Isobologram showing that NPF-1, a specific PSAC inhibitor, markedly antagonizes leupeptin's growth-inhibitory effect. Each symbol represents the concentrations of these two agents that together produce 50% parasite killing. The solid diagonal represents the line of additivity expected for noninteracting combinations. The results are typical of those for five independent experiments. The marked rightward shift of this profile reflects the higher concentrations of leupeptin required when PSAC is inhibited by NPF-1.

rational drug discovery and development programs. Molecular and biochemical studies have provided evidence for three distinct resistance mechanisms in malaria parasites to date. First, mutations in the parasitic target may reduce the drug's efficacy by lowering the affinity of the drug-target interaction or by sustaining the activity of the target in spite of drug binding. Important examples include sulfadoxine and pyrimethamine, which act synergistically to inhibit dihydropteroate synthase and dihydrofolate reductase, key enzymes in folate biosynthesis. Resistance to these drugs is primarily mediated by point mutations in both enzymes that reduce inhibitor affinity (21, 34). A second acknowledged, but less well characterized, mechanism is the upregulated expression of target enzymes through genome-level amplification or increased transcription (16, 31, 33); this strategy may sustain the needed parasite activity despite the presence of an inhibitor. The third acknowledged mechanism of resistance to antimalarials is through active extrusion of an unmetabolized drug, a mechanism that restores target activity by reducing the drug concentration at the target site. This may be the primary mechanism of resistance to chloroquine (13) and possibly also other antimalarial drugs (12).

Our study identifies a novel mechanism of antimalarial drug resistance. We generated in vitro leupeptin resistance and performed biochemical, molecular, and biophysical studies along with growth inhibition experiments to explore possible mechanisms. Our biochemical studies did not detect upregulated activity or reduced leupeptin sensitivity of digestive vacuolar proteases. There were also no point mutations in falcipain-2 or falcipain-3 or marked changes in the expression of blood-stage protease genes. Instead, significant changes in the erythrocyte membrane permeability of organic solutes were identified. Single-channel recordings confirmed that these changes resulted from an altered PSAC. We linked leupeptin resistance to these changes by determining that leupeptin entry into infected erythrocytes occurs primarily via PSAC and that resistant parasites exhibit markedly reduced leupeptin uptake. Thus, our studies implicate reduced leupeptin permeability via PSAC as the primary mechanism of resistance to leupeptin's growth-inhibitory effects. This proposal is consistent with leupeptin's net positive charge and bulky structure, which limit its transport across membranes in most other cell types (19). The large discrepancy between the required extracellular leupeptin concentration (IC_{50}, 18 μM for wild-type parasites; Fig. 1A) and its activity against extracts of parasite proteases ($K_{0.5}$, 87 nM; Fig. 2) is consistent with rate-limiting uptake at the erythrocyte membrane. In this context, mutations that result in reduced passive leupeptin uptake should readily permit the acquisition of resistance at the observed levels. Finally, this proposal is also consistent with studies demonstrating the increased permeability of other bulky solutes after infection with plasmodia (3, 5, 27). PSAC appears to mediate all of these changes.

In vitro resistance to blasticidin S by malaria parasites may also be due to reduced PSAC-mediated uptake (14); definitive evidence will require studies of blasticidin S transport across the erythrocyte membrane and examination of possible changes in its intracellular targets. In either case, the mutant described here exhibits less dramatic changes in channel properties, with higher sorbitol permeation and larger single-channel Cl^- currents than the blasticidin S-resistant mutant described previously. Consistent with this, we found that the growth rate of HB3-*leuR1* was indistinguishable from that of HB3 in the absence of leupeptin (data not shown), whereas a clear fitness cost was associated with blasticidin S resistance (14).

A separate study recently generated a parasite that exhibited in vitro resistance to morpholine urea-leucine-homophenylalanine-phenyl vinyl sulfone (31), a more specific cysteine protease inhibitor (35). In contrast to the findings of our studies with leupeptin, vinyl sulfone resistance was mediated by the combination of upregulated cysteine protease activity and decreased vinyl sulfone accumulation. The change in accumulation could have resulted from either reduced passive uptake or increased efflux across one or more membranes. In contrast to this uncertainty, our studies indicate that changes in leupeptin transport localize to the erythrocyte membrane instead of one or more parasite-associated membranes, because uptake into the erythrocyte cytosol was specifically measured (Fig. 5). This was achieved with 0.05% saponin to selectively permeabilize the erythrocyte membrane without compromising intracellular parasite membranes. Because parasite-induced transport across the erythrocyte membrane is generally accepted to be primarily passive (32), reduced leupeptin uptake probably represents reduced passive uptake and not increased efflux. Moreover, the effects of NPF-1 on leupeptin uptake (Fig. 5A) strongly suggest transport via PSAC, an ion channel that mediates only passive transport. Finally, vinyl sulfone resistance in the previous study was associated with only modest changes in leupeptin sensitivity, consistent with resistance via mechanisms distinct from the one described here.

The identification of a new resistance mechanism provides yet another challenge to groups working on the discovery and development of antimalarials. Fortunately, our findings suggest some obvious guidelines for reducing the risk of the acquisition of resistance via this mechanism. Drug leads that have high molecular weights, cigar-shaped structures, and net charge or high polarity are most likely to require PSAC-mediated uptake; indeed, both leupeptin and blasticidin S meet these criteria and have negative calculated log*P* values. Antimalarial leads that meet these criteria and that are nontoxic to mammalian cells lacking PSAC-like channels may be especially susceptible to this resistance mechanism. Antagonism of a candidate drug's in vitro growth-inhibitory effects by specific PSAC antagonists, as described here, would be highly suggestive and should result in increased vigilance.

ACKNOWLEDGMENTS

This research was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health; the Medicines for Malaria Venture (to S.A.D.); and NIH grant AI-47798 (to D.E.G.).

We are grateful to Elizabeth Fischer for preparation and analysis of the transmission electron micrographs and to David Hill for comments on the manuscript.

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