The plasmodial surface anion channel (PSAC) is an unusual ion channel induced on the human red blood cell membrane after infection with the malaria parasite, *Plasmodium falciparum*. Because PSAC is permeant to small metabolic precursors essential for parasite growth and is present on red blood cells infected with geographically divergent parasite isolates, it may be an ideal target for future antimalarial development. Here, we used chemically induced mutagenesis and known PSAC antagonists that inhibit in vitro parasite growth to examine whether resistance mutations in PSAC can be readily induced. Stable mutants resistant to phloridzin were generated and selected within 3 weeks after treatment with 1-methyl-3-nitro-1-nitrosoguanidine. These mutants were evaluated with osmotic lysis and electrophysiological transport assays, which indicate that PSAC inhibition by phloridzin is complex with at least two different modes of inhibition. Mutants resistant to the growth inhibitory effects of phloridzin expressed PSAC activity indistinguishable from that on sensitive parasites, indicating selection of resistance via mutations in one or more other parasite targets. Failure to induce mutations in PSAC activity is consistent with a highly constrained channel protein less susceptible to resistance mutations; whether this protein is parasite- or host-encoded remains to be determined.

We recently used the cell-attached and whole cell patch clamp methods to identify an unusual ion channel on the red blood cell (RBC)1 membrane of infected cells (1). This channel, the plasmodial surface anion channel (PSAC), has a number of unusual functional properties that distinguish it from known human channels. First, it accounts for increases in permeability to a range of solutes identified in many previous studies. PSAC is permeant to both anions (2) and organic solutes such as sugars (3), amino acids (4), purines (5), some vitamins (6), and a number of organic cations (7). Amazingly, although broadly permeant, it effectively excludes Na⁺ by >100,000-fold relative to Cl⁻. This combination, unparalleled in other known channels, permits osmotic stability of the infected RBC in serum while permitting the acquisition of diverse essential nutrients (8). PSAC achieves its stringent exclusion of Na⁺ through the combined effects of extracellular pore mouth cationic amines to repel extracellular cations and at least one weak binding site in its pore to favor bulky dehydrated ions over smaller ions (9).

Another unusual feature of PSAC is its small single channel conductance of ~20 pS in 1.15 molar Cl⁻ solutions (10); most other channels with broad selectivity profiles exhibit larger single channel conductances. A channel’s chord conductance, calculated from measured current amplitudes at a range of imposed membrane potentials, quantitatively corresponds to the number of ions passing through the open channel per unit time. In terms of Eyring rate theory, this channel’s small conductance reflects the one or more energetic barriers that each solute molecule must overcome to traverse the length of the channel pore. These barriers may function to additionally fine tune the selectivity properties of PSAC.

Finally, despite the nearly invariant membrane potential of human RBCs after infection, permeation through PSAC is voltage-dependent. Both whole cell and single channel recordings reveal significantly smaller absolute currents at membrane potentials (Vₘ) of +100 millivolts than of −100 millivolts despite equal but opposite driving forces for ion movement. Historically, channels exhibiting this phenomenon were often referred to as “inward rectifying” voltage-dependent channels. Although voltage-dependent channels are most well known as mediators of electrical signals on excitable cells, they are also present on many non-excitable cells (11). The voltage dependence of PSAC results from a reduced frequency and duration of openings at increasingly positive Vₘ values, presumably because of charged residues on the channel protein that move during transitions between the open and closed states. Although the physiological relevance of this voltage dependence is unknown, it represents an important functional marker of PSAC activity. There are other complex and unusual features of PSAC gating, the process of opening and closing, that suggest additional structural constraints on the protein (12).

Because of the unusual properties of PSAC and because of polymorphisms in channel gating apparent when RBCs from a single human donor are infected with genetically distinct parasite isolates (10), we proposed that PSAC is a parasite-encoded ion channel. Although several reports have suggested that PSAC activity results from the modification of host RBC proteins (13–15), none of these mutually exclusive proposals has been independently confirmed. In particular, we were unable to reproduce these reports despite intensive efforts (10).

Homology searches using known anion channel genes have not revealed obvious candidates for PSAC gene(s) in the completed *Plasmodium falciparum* genome sequence (16). It appears that PSAC genes differ sufficiently from known channels to render bioinformatic approaches ineffective. Here, we used an alternative approach with the goal of identifying these elusive genes. We used the potent mutagen 1-methyl-3-nitro-1-
Mutagen-induced Phloridzin Resistance in P. falciparum

nitrosoguanidine (MNNG) to increase the frequency of random mutations in parasite genes. We followed mutagenesis with the selection of in vitro resistance to growth inhibition by known PSAC antagonists. These antagonists are believed to kill parasites by preventing PSAC-mediated uptake of essential nutrients. This approach selected for unambiguous and stable resistance to growth inhibition by 100 μM phloridzin, a nonspecific antagonist of PSAC (17). Osmotic lysis and electrophysiological studies with phloridzin revealed that resistance did not result from either an altered mechanism or an affinity for PSAC inhibition. Effects of phloridzin on other parasite activities are implicated.

We also used single channel studies to determine that phloridzin inhibition of PSAC is complex, with at least two separate mechanisms. Comparison to inhibition by furosemide revealed additional important insights into the conformational changes required for PSAC gating. These detailed studies should help guide the design of novel and specific PSAC antagonists that may become future antimalarial drugs.

MATERIALS AND METHODS

Chemical Mutagenesis—Phloridzin-resistant mutants were generated and selected using a protocol based on that developed by Inselburg (18). Up to 2 × 10⁹ ring stage parasites (Indo 1 strain) were synchronized with the sorbitol lysis method (19) and cultured for 24 h in the presence of 150 μg/ml aphidicolin to produce a DNA replication block and to maximize the mutagenic effect of MNNG. After washing, these trophozoite-infected RBCs were incubated in culture medium (RPMI 1640) supplemented with 50 mg/liter hypoxanthine and 10% human serum) for 2 h before adding 0.5–1.5 Ci/ml [3H]hypoxanthine. Incorporation of [3H]hypoxanthine into parasite DNA and RNA, a direct measure of parasite viability (21), was measured in 96-well format using a plate reader and standard β-scintillation counting.

Osmotic Lysis Assays—The kinetics of infected RBC osmotic lysis in sorbitol solutions was followed as described previously (22). In brief, trophozoite-infected RBCs were incubated in standard culture medium, enriched to 95–99% by Percoll/sorbitol separation (23), washed in phosphate-buffered saline (150 mM NaCl, 20 mM sodium phosphate, pH 7.5), and resuspended to 0.5% hematocrit in sorbitol lysis solution (280 mM sorbitol, 20 mM sodium HEPES, and 0.1 mg/ml bovine serum albumin, pH 7.4) at 37 °C. The transmittance of 700-nm light through this cell suspension was followed continuously as a marker of osmotic swelling and lysis. This method produces estimates of permeation rates through PSAC that quantitatively match those obtained with both radioisotope flux and patch clamp (22). Because phloridzin inhibits host RBC membrane permeability through one or more binding sites on the intracellular face of PSAC (1), some experiments included a preincubation in the final phloridzin concentration before the addition of sorbitol lysis solution; the resulting levels of inhibition were comparable with those without pre-incubation, indicating that phloridzin uptake into intact infected RBCs is not rate-limiting.

Electrophysiology—Cell-attached patch clamp recordings of infected RBCs were obtained as described previously (1). Unless otherwise indicated, these experiments utilized symmetrical bath and pipette solutions of 100 mM choline chloride, 115 mM NaCl, 10 mM MgCl₂, 5 mM CaCl₂, and 20 mM sodium HEPES, pH 7.4. Pipettes with tip diameters of <0.5 μm and resistances of 1–4 MΩ were pulled from quartz glass. Recordings were filtered at 5 kHz with an eight-pole Bessel filter and digitized at 100 kHz. Seal resistances were typically >100 MΩ.

To determine mean single channel open probabilities (Pₒ) for wild-type and phloridzin-resistant mutants, we collected single channel molecular recordings from patches on RBCs infected with each parasite in the presence of 0, 25, or 100 μM phloridzin added both to bath and pipette. For each condition, we used a home-written code that identifies transitions between channel open and closed states by the 50% threshold mid-crossing technique and normalized the results to the measured Pₒ in the absence of phloridzin, 43 ± 2% (10).

RESULTS

Fig. 1A shows our algorithm for generating and selecting mutants resistant to PSAC antagonists. As reasoned previously (18), ring stage parasite cultures were synchronized by growth with aphidicolin, which blocks DNA replication and stalls parasites at the early trophozoite stage. When it is removed, DNA replication ensues with a more synchronized population of parasites. At this point, we added MNNG (Fig. 1A, green-shaded area). Because MNNG is unstable in aqueous solutions and may have other deleterious effects on cell cultures (26), timing its addition with the onset of DNA replication, achieved through the use of aphidicolin, should maximize its mutagenic potential. We varied the MNNG concentration to obtain ~95% killing of parasites, as judged by microscopic examination after 24 h. At this level there should be both a high frequency of introduced mutations and yet sufficient numbers of viable parasites to allow identification of mutants. Surviving parasites were then cultured for several days before applying selective pressure with 100 μM phloridzin (Fig. 1A, red-shaded areas). With this algorithm, resistant parasites that exhibited replication rates of 5–7-fold every 48 h in the continued presence of phloridzin were identified within 3 weeks.

We tested whether these mutant parasites represented stable genetic changes by culturing them under standard conditions without phloridzin for 5 weeks before performing the experiment in Fig. 1B. In the absence of phloridzin, growth rates of the selected mutants and the parent wild-type parasites were indistinguishable. In contrast, the addition of 100 μM phloridzin to the culture revealed a marked difference; within 3 days wild-type parasites were essentially eliminated, but the growth of the mutant continued unabated. The prolonged culture of these parasites in the absence of phloridzin before this experiment indicates that phloridzin resistance resulted from the acquisition of stable
Mutagen-induced Phloridzin Resistance in P. falciparum

We tallied our osmotic lysis experiments with a range of phloridzin concentrations and normalized them relative to the lysis rates without inhibitor (Fig. 2E, open symbols). We also collected between 94 and 254 s of single PSAC recordings at each phloridzin concentration on both wild-type and mutant parasites, determined channel open probability at a V_m of ~100 mV, and normalized these results to the open probability without phloridzin. When these independent transport measurements were plotted together, the effects of phloridzin on both sorbitol-mediated lysis and single PSAC recordings were concordant, consistent with a single channel for the uptake of diverse solutes (10). We also found that the dose response for the inhibition of phloridzin-resistant mutants was not measurably changed from that of wild-type parasites (Fig. 2E, compare triangles to circles); both were adequately fitted by a decaying Hill equation with a dissociation constant, K_{0.5}, of 24 μM. Thus, resistance to the growth inhibitory effects of phloridzin was mediated through mutations in one or more parasite genes not responsible for the expression of PSAC.

Previous whole cell patch clamp with phloridzin added to either the cytosolic or extracellular compartments revealed that inhibition by phloridzin occurs primarily at the intracellular face of PSAC (1). Here, we analyzed our single channel recordings to examine the mechanism of this inhibition. The addition of either 25 or 100 μM phloridzin to both the bath and pipette solutions did not affect the durations of PSAC openings (Fig. 3A), which are adequately described by a single exponentially decaying state with a mean duration of 0.2–0.4 ms (12). Instead, phloridzin altered the distribution of closed times by adding a population of closings with a mean duration of 46 ± 2 ms (Fig. 3B, smooth curve). The mean duration of these imposed closings was not affected by changes in phloridzin concentration. This collection of observations is conservatively explained by simple allosteric inhibition of PSAC through a single phloridzin binding site on the channel protein’s intracellular face.

Our analysis of dwell durations in the presence of phloridzin shows marked similarities to the recent characterization of PSAC inhibition by furosemide, which also produces allosteric inhibition at a single site with a mean block duration of 85 ms. Because furosemide has an ~10-fold higher affinity for PSAC inhibition (K_{0.5} = 2.7 μM) (10), we were surprised by the similar mean block durations for furosemide and phloridzin. It is generally assumed that access to binding sites on ion channels is diffusion-limited. If furosemide and phloridzin both satisfied this assumption, then the mean closed duration of phloridzin should have been some 10 times shorter than that of furosemide because of the direct inverse relationship between the K_{0.5} for an inhibitor and the measured mean closed duration in single channel recordings.

If the model of a single reversible binding site for each inhibitor is correct, the only explanation for this discrepancy would be that access to the binding site on PSAC is not diffusion-limited for at least one of the two inhibitors. Specifically, the rate constant for phloridzin binding would need to be significantly lower than that for furosemide binding to compensate for their similar unbinding rates. Visual examination of recordings in the presence of either 25 μM phloridzin or 25 μM furosemide (Fig. 4A, left and right columns, respectively) reveals that both compounds produce relatively long and similar periods of inhibition, apparent as regions devoid of downward channel openings. Although these compounds impose similar closed channel durations, channel openings in their presence differ markedly. Clusters of channel openings in the presence of phloridzin were visibly longer than those with the same concentration of furosemide. This observation, made in the

mutations in one or more parasite genes.

We then evaluated whether resistance resulted from a lowered phloridzin affinity for PSAC inhibition. We used a recently developed a light-scattering method that tracks the kinetics of osmotic lysis in sorbitol and produces quantitative estimates of PSAC inhibition (22). Fig. 2, A and B, shows the resulting lysis time courses for wild-type and mutant parasites, respectively. The rates of lysis without and with a range of phloridzin concentrations were not statistically different within the two populations of parasites.

Because there may be subtle changes in the gating, conductance, or interaction of PSAC with phloridzin that are not detectable with the macroscopic lysis assay, we performed cell-attached patch clamp of infected cells and obtained single PSAC recordings on both parasite isolates. We successfully identified single PSAC activity on mutant parasites (Fig. 2C); these PSAC molecules exhibited voltage-dependent gating and conductance resembling those seen previously on unmutagenized parasites (1). Based on the levels of inhibition seen in the osmotic lysis experiments (Fig. 2, A and B), we chose two phloridzin concentrations (25 and 100 μM) for inclusion in separate single channel recordings on both wild-type and mutant parasites (Fig. 2D, left and right columns, respectively). Phloridzin produced concentration-dependent reductions in PSAC gating on RBCs infected with each of these two parasites.
FIG. 2. **PSAC-mediated sorbitol and chloride transport measurements with and without phloridzin.** A and B, sorbitol-induced osmotic lysis assays with unmutagenized wild-type (WT) and phloridzin-resistant mutants in the presence of 0, 10, 50, or 300 μM phloridzin (top to bottom traces in each panel, respectively). % T, percentage of 700-nm light transmittance. C, single PSAC recording on a phloridzin-resistant mutant parasite without inhibitors. Imposed membrane potentials, $V_m$, were +100 mV and −100 mV (top and bottom traces, respectively). The significantly fewer openings at $V_m$ of +100 mV is consistent with the known voltage-dependent gating of PSAC (1). D, single channel recordings on wild-type (WT, left column of traces) and mutant parasites (right column) at a $V_m$ of −100 mV with the indicated phloridzin concentrations added to both bath and pipette compartments. Note that increasing phloridzin concentrations reduce the frequency of channel openings on both parasite isolates without altering their current amplitudes. In panels C and D, the closed channel levels are marked with dotted red lines. The vertical scale bar represents 2 pA in both C and D, the horizontal bar represents 33.6 ms (C) and 150 ms (D). E, dose responses for the inhibition of sorbitol-mediated lysis and single channel currents (open and filled symbols, respectively) using wild-type (circles) or phloridzin-resistant mutants (triangles). Lysis measurements represent the mean ± S.E. of up to nine trials for each parasite. Data were normalized to permit direct comparison of lysis results with those from patch clamp single channel open probability measurements. The *solid line* is the best fit of all measurements to $y = a(1 + (x/K_{0.5})^b)$ with estimates of 24 μM for $K_{0.5}$ and 0.77 for $b$. 

*Mutagen-induced Phloridzin Resistance in P. falciparum*
some 10-fold greater than the longest mean duration for intrinsic PSAC closings (12) and substantially less than the mean inhibition durations imposed by phloridzin or furosemide. Thus, this cutoff value minimizes the erroneous assignment of intrinsic and inhibitor-associated bursts. Fig. 4B shows the resulting distribution of burst durations; each of the 8,534 bursts tallied here results from measurable PSAC activity between consecutive inhibition events by phloridzin or furosemide (red and green histograms, respectively).

The simplest scheme for interaction between an inhibitor \( I \) and PSAC is shown in Reaction 1,

\[
I + P \rightleftharpoons IP \\
k_{on} P + k_{off} IP \\
\]

(Reaction 1)

where \( P \) represents the unbound channel and \( IP \) represents a bound and inhibited channel; \( k_{on} \) and \( k_{off} \) represent the forward and reverse rate constants for the binding process. If the interactions of the two inhibitors with PSAC obey this scheme, then the burst distributions shown in Fig. 4B should follow Equation 1,

\[
f(t) = \left( a \times \exp[\ln(t) - b]\right)^{0.5} \\
\]

(Eq. 1)

in which \( t \) represents the duration of individual bursts and \( a \) and \( b \) are constants. The distribution’s mean value is given by \( \exp(b) \) and is visible as a peak on this plot (12, 24). We found that the burst distribution for 25 \( \mu \)M phloridzin was well fitted by Equation 1 (Fig. 4B, smooth red line) with a mean value of 21 \( \pm \) 0.6 ms. The forward rate constant, \( k_{on} \), is then inversely related to this mean value.

With 25 \( \mu \)M furosemide, our algorithm for identifying bursts between consecutive inhibition events produced a distribution peaking at substantially shorter durations (Fig. 4B, green histogram). Here, the best fit to Equation 1 (Fig. 4B, smooth green line) yielded a mean burst duration of 2.9 ms. Because this estimate is nearly 10-fold less than that for phloridzin, access to their respective binding sites predominantly accounts for the differing affinities of furosemide and phloridzin. In contrast, the mean durations of inhibitor-induced closings (Fig. 3B), a direct measure of \( k_{on} \), plays a relatively minor role in determining the relative affinities of these two agents. Our model for interactions between PSAC and these two inhibitors, which incorporates open and closed durations in single channel recordings as well as previous whole cell measurements, is illustrated in Fig. 5.

Interestingly, the bursts of activity seen with 25 \( \mu \)M furosemide were not well fitted by Equation 1 (Fig. 4B, green histogram and smooth curve); the bursts instead required two or more on-rate constants with values too similar to one another to be easily

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**Fig. 4. Burst analyses with phloridzin and furosemide.** A, single PSAC recordings with either 25 \( \mu \)M phloridzin or 25 \( \mu \)M furosemide added to both bath and pipette (left and right columns, respectively). Vm for all traces was clamped at −100 mV; red dashes represent the closed channel levels. Scale bars represent 2 pA (vertical) and 200 ms (horizontal). B, durations of open channel bursts with 25 \( \mu \)M phloridzin or 25 \( \mu \)M furosemide (red and green histograms, respectively). Smooth lines represent the best fits to Equation 1. Notice that the phloridzin distribution peaks at a significantly greater duration, indicating that phloridzin’s lower affinity for PSAC inhibition results from a lower on-rate constant.

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The context of the identical concentrations of the two inhibitors, is qualitatively consistent with slower binding rates for PSAC-phloridzin interactions than for PSAC-furosemide interactions.

We next quantified this observation to test whether it can account for the differing macroscopic affinities of phloridzin and furosemide. We defined bursts of PSAC activity as clusters of openings flanked by closings of ≈10 ms. This cutoff value is
resolved (not shown). Because unbinding is adequately fitted by a single $k_{\text{off}}$ value (12), these separate but similar forward rate constants for binding presumably represent independent routes by which furosemide can reach a single pocket on the channel protein to achieve its inhibitory effect.

Fig. 6 shows a separate complex effect of phloridzin on PSAC activity. We found that continued recording in the presence of either 25 or 100 $\mu$M phloridzin invariably leads to an altered single channel phenotype. The already small conductance of PSAC was abruptly reduced by $\sim$65% (Fig. 6). This clearly different activity typically lasted for seconds to 1–2 min before complete and irreversible loss of all channel activity from these patches. The possibility that this activity represents a channel distinct from PSAC was excluded by the tight link between its appearance and the disappearance of typical PSAC gating and conductance, its PSAC-like inward-rectifying voltage-dependence, and its transitory nature. Additional experimentation will be needed to characterize this behavior.

DISCUSSION

The killing of parasites by nearly every known PSAC antagonist supports an essential role for this unique parasite-induced channel. In light of the high permeability of PSAC to diverse solutes needed for parasite growth, the most appealing proposal is that this channel serves as the first step in a sequential diffusive pathway for nutrient acquisition by the intracellular parasite (1). In this model, waste products such as amino acids, purines, and vitamins would be needed to characterize this behavior.

Alternative proposals for PSAC function also exist. In one study (30), a role in facilitating parasite egress from the RBC at the end of its 48-h intracellular cycle was proposed based on mathematical modeling of PSAC-mediated Na$^+$ uptake, which eventually leads to swelling and osmotic lysis of the infected RBC. Using several assumptions, this study calculated that the low but finite Na$^+$ permeability of PSAC would produce osmotic lysis at 48 h, matching the duration of the intraerythrocytic parasite cycle. In this model, the physiological role of PSAC would be to promote parasite egress from the RBC via a narrowly defined Na$^+$ permeability; its permeability to nutrient solutes such as amino acids, purines, and vitamins would then be merely an epiphenomenon. In addition to other problems with this proposal, various studies of rupture and merozoite release indicate that proteases are required and that parasitophorous vacuolar membrane lysis precedes lysis of the host RBC membrane (31, 32), findings that are inconsistent with a role for Na$^+$-mediated osmotic lysis.

Although a PSAC role in nutrient acquisition seems the most reasonable (1), there is still no direct evidence for any proposal. Here, we used chemical mutagenesis and selection of resistance to PSAC antagonists to search for such evidence. Although we attempted to find mutants resistant to four separate PSAC antagonists, only experiments with phloridzin have produced successful selections to date. Because these mutants exhibited PSAC activity with unaffected gating, conductance, and affinity for phloridzin, in vitro killing of parasites by phloridzin is likely mediated by one or more other targets within the infected RBC complex.

We consider phloridizin to be the least specific of the commonly studied PSAC antagonists. Not only is it or its congeners, phloretin, known to inhibit a diverse collection of channels and carriers, but these compounds induce unambiguous changes in artificial lipid bilayer permeability due to membrane dipole effects and disordering of lipid tail groups (33). With this level of poor specificity, it is not surprising that phloridzin has other essential targets within the infected RBC.

Our study raises some important caveats about the interpretation of parasite growth inhibition studies. Prior to these findings, workers in the PSAC field accepted that parasite killing by phloridzin was primarily mediated via its actions on PSAC; there was concordance between its $K_{0.5}$ for channel inhibition (Fig. 2E) and its $IC_{50}$ for parasite growth inhibition (17). Because the selected mutants had growth rates unaffected by 100 $\mu$M phloridzin despite an unchanged $K_{0.5}$ for PSAC inhibition, this concordance appears to be only coincidental. In light of the nonlinear relationship between nutrient uptake and the successful production of viable progeny, the quantitative concordance of these half-maximal inhibitory values should have been considered weak evidence even without our studies.

Random mutagenesis, as performed here, represents an important check on the drug development concept that a given compound lacks off-target effects. In principal, it can also be used to gauge how easily important parasite targets can acquire resistance mutations. For example, resistance to chloroquine, a safe and inexpensive antimalarial drug, can be primarily attributed to mutations in PfCRT, a membrane protein of unknown function. Mutations in a single locus of
this protein have been successfully generated by selection with lethal doses of chloroquine applied to \textit{in vitro} cultures (34); mutations at this locus (Lys-76) proved sufficient to convert at least one chloroquine-sensitive parasite into iso-
lates resistant to clinically achievable doses. Targets such as PSAC that consistently fail to be mutated with this approach may have highly constrained structures and be more desir-
able to drug development programs.

Our inability, to date, to generate mutants with chemical mutagenesis could also be explained if PSAC activity results from a parasite-activated human protein on the RBC mem-
brane. Although the unusual functional properties of PSAC and the presence of biophysical polymorphisms linked to par-

Our chemical mutagenesis algorithm offers a powerful way to study parasite-encoded phenotypes in the infected RBC com-
plex. Because the mature human RBC lacks DNA and is not affected by MNNG, mutagenesis should be able to dissect the relative contributions of host and parasite factors to a pheno-
type. It may have more potential utility in \textit{P. falciparum} than in other model organisms for two important reasons. First, gene-specific transformations remain difficult in \textit{P. falciparum} despite extensive efforts by various groups (35). Reasons for this include the parasite’s relatively slow replication rate (44–48 h) and the inacu-

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