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Irreversible effect of cysteine protease inhibitors on the release of malaria parasites from infected erythrocytes

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Abstract

By studying the inactivation of malaria parasite culture by cysteine protease inhibition using confocal microscopy of living cells, and electron microscopy of high-pressure frozen and freeze-substituted cells, we report the precise step in the release of malaria parasites from erythrocytes that is likely regulated by cysteine proteases: the opening of the erythrocyte membrane, liberating parasites for the next round of infection. Inhibition of cysteine proteases within the last few minutes of cycle does not affect rupture of the parasitophorus vacuole but irreversibly blocks the subsequent rupture of the host cell membrane, locking in resident parasites, which die within a few hours of captivity. This irreversible inactivation of mature parasites inside host cells makes plasmodial cysteine proteases attractive targets for anti-malarials, as parasite-specific cysteine protease inhibitors may significantly augment multi-target drug cocktails.

Introduction

Since there is no effective vaccine for malaria, and because the causal agent *Plasmodium falciparum* is resistant to many drugs, there is interest in developing inhibitors that target cysteine and serine proteases. These drugs are known to interfere with the parasitic asexual life cycle by trapping maturing parasites in clusters and diminishing *de novo* infection of erythrocytes (Lyon and Haynes, 1986; Salmon et al., 2001; Wickham et al., 2003; O'Donnell and Blackman, 2005). A deeper understanding of the precise role proteases play in the pathophysiology of this disease is critically needed, since despite decades of research there remains controversy about their function in the malarial erythrocyte cycle, particularly in parasite release (Rosenthal, 2004; Pandey et al., 2006; Blackman, 2008). Two recent reports identified two parasite proteases that may mediate the cascade of final cycle events: cysteine protease dipeptidyl peptidase 3 (DPAP3) (Arastu-Kapur et al., 2008) and subtilisin-family serine protease PfSUB1 (Arastu-Kapur et al., 2008; Yeoh et al., 2007), but the precise time and place of their action within the release process remains enigmatic.

The release of parasites appears to be a two step process. Parasites are first released from the parasitophorous vacuole within the erythrocyte, and afterwards released from the erythrocyte (Wickham et al., 2003). After the parasitophorous vacuolar membrane and the erythrocyte membrane are breached, the parasites are free to invade fresh erythrocytes. Although the release

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of parasites is crucial for the life cycle of the parasite, its mechanism is unknown and only limited technologies exist for its analysis (Cowman and Crabb, 2006). Imaging of the release process *in vitro* (Glushakova et al., 2005) shows that it is fast and very sensitive to a variety of conditions, thus limiting possible experimentation on the end of the erythrocyte cycle in living cells. The cycle, however, is blocked by wide spectrum cysteine and cysteine/serine protease inhibitors such as E-64, bADA and leupeptin (the last inhibitor was used alone or in combination with chymostatin and antipain). Such treatment does not interfere with parasite maturation and allows one to analyze the accumulated parasite clusters (Lyon and Haynes, 1986; Delplace et al., 1988; Salmon et al., 2001; Soni et al., 2005; Gelhaus et al., 2005). Treatment blocks rupture of one of the two membranes surrounding parasites, but the origin of the limiting membrane that preserves the remaining cluster is a subject of disagreement (reviewed in Blackman, 2008). As a consequence, the order of membrane rupture during parasite release is controversial as well.

Pursuing the dual goal of deciphering the mechanism of malaria parasite release from erythrocytes and the role of proteases in this process, and taking into account the extreme fragility of late stage infected erythrocytes we developed a new approach for the differential labeling of the membrane of live infected erythrocytes (Glushakova et al., 2005) and a quantitative parasite release assay coupled with the morphological analysis of live infected cells undergoing a cycle transition (Glushakova et al., 2007). We now show that the broad-spectrum cysteine protease inhibitor E-64 as well as cysteine/serine inhibitors leupeptin and calpeptin act during the last few minutes of the infection cycle by inhibiting the final step in parasite release process – the opening of the erythrocyte membrane. The breakdown of the vacuolar membrane is not blocked. In contrast to the findings of Salmon and colleagues (Salmon et al., 2001), clustered parasites are irreversibly inactivated by both reversible and irreversible cysteine protease inhibitors.

Results

Microscopy of the parasite cycle arrest by broad-spectrum cysteine protease inhibitor E-64

Laser scanning confocal microscopy of live schizonts approaching the end of the erythrocyte cycle was performed to compare parasite cluster formation in E-64-containing medium and the normal release process (Glushakova et al., 2005). We confirmed the inhibitory effect of E-64 on schizont rupture (Salmon et al., 2001) and recorded the products of cycle arrest induced by this drug. Schizonts approaching the end of the cycle showed the same progression of major morphological transformations in control and in drug-containing medium (Movies S1–S2). Specifically, late schizonts first were transformed into the “flower” forms, a morphologically transient stage that lasts only for a few final minutes of the parasite's life within an erythrocyte (Glushakova et al., 2005). This form is limited by the erythrocyte plasma membrane and is characterized by a rounded shape, the absence of a visible space between parasite and erythrocyte membrane, and a symmetrical distribution of parasites around the food vacuole (Fig. 1A). Next, after a short swelling period, an individual flower either ruptured to release parasites (in control medium; Movie S1) or was transformed into a cluster of parasites (Fig. 1 B) (in the presence of E-64; Movie S2). No membrane fragments were observed in the space that surrounded *de novo* produced clustered parasites in E-64 medium, while membrane fragments remained as expected (Glushakova et al., 2005) stuck to glass at the sites of parasite release in control medium. *De novo* formed clusters are morphologically different than flowers from which they appear to originate (Fig. 1 A and 1 B). In particular, parasites in clusters were less tightly packed than in schizonts, and they were arranged without the symmetry typical for the flower stage of schizont maturation. Food vacuoles in clusters were distant from the central position characteristic of the flowers. Parasites in clusters moved relative to each other in directions that indicated their separation from one another (Movie S2). Furthermore, parasites

occupied the entire space within the limiting membrane, and their cell bodies were visible in DIC microscopy with strong contrasting dark and light bands that were close to those of released parasites, suggesting a large gradient in refractive index between parasite and surrounding environment.

Most release recordings in normal medium show a striking similarity between the immediate pre-release images of flowers (that exist for only a few seconds before erythrocyte rupture) and E-64-induced parasite clusters (Movies S1–S2). Neither the timing of schizont transformation into flower nor the final flower shape were significantly different from those of controls. Thus, the "cluster-like flower form" is very likely the final pre-release stage of the parasite cycle. We observed, however, one distinct feature in the morphology of schizonts undergoing transformation into the flower form in the presence of the E-64: the erythrocyte membrane was intensively ruffled (compare Movie S1 and S2 as well as representative images from these recordings in Fig. 1 C and Fig. 1 D). This ruffling may reflect as-yet unknown differences in the erythrocyte cytoskeleton of drug-treated schizonts in comparison with the control schizonts. Parasite clusters observed in live culture share some similarity to the clusters described in early works with cysteine or cysteine/serine protease inhibitors (Lyon and Haynes, 1986; Delplace et al., 1988; Salmon et al., 2001; Gelhaus et al., 2005) but the direct comparison of their morphology is difficult because of the invasive fixation procedures employed in other studies. All these works describe the production of clusters limited by a single membrane, the origin of this membrane is a matter of controversy.

Parasite clusters are limited by erythrocyte plasma membrane

To determine the origin (erythrocyte membrane vs. parasitophorous vacuolar membrane, PVM) of the outermost membrane surrounding these clusters of parasites, we labeled live cells with various fluorescent probes. We used probes that do not require cell washing before microscopic examination, in order to minimize artifact. To mark the erythrocyte membrane, we detected the main erythrocyte glycoprotein, glycophorin A, or all surface proteins if erythrocyte membrane was biotinylated at the beginning of the parasite cycle. All clusters produced by E-64 treatment were limited by erythrocyte plasma membrane, regardless of labeling method or probe (Fig. 1 F, 1 H). Electron microscopy confirmed that parasite clusters have one limiting membrane (Fig. 1 I–J, black arrowheads). Multiple membrane fragments, which likely originate from the ruptured vacuolar membrane, were located inside cluster in between parasites (Fig. 1 J, white arrowhead). It is worth mentioning that knobs have almost disappeared from the cluster-limiting erythrocyte membrane and those that were preserved have a much less prominent architecture (S.Glushakova, unpubl. obs.). This observation may suggest an unexpected target for non-cysteine proteases in schizonts undergoing the preparation for parasite release. Some clusters (but not schizonts and normal RBC) had defects in their limiting membrane (Fig. 1 J, asterisk), indicating either an increased fragility of the cluster membrane or the presence of small defects in the cluster membrane before its processing for electron microscopy. The latter interpretation is supported by Lyon and Haynes report (1986) of detection of internal vacuolar membrane antigens in clusters using fluorescent antibodies in the wet cell suspensions without cell permeabilization (Lyon and Haynes, 1986).

Cysteine proteases act at the end of the erythrocyte cycle

The findings that cysteine protease inhibitor affects only the very end of the parasite cycle does not exclude the possibility that cysteine proteases act early in the cycle. Indeed, an extended treatment time (6 –12 h) with inhibitors was used in all previously reported research that demonstrate a blockage of parasite release. Using a new assay to quantify parasite release (Glushakova et al., 2007) we were able to analyze the kinetics of the inhibitory effect of E-64 on the asexual parasite cycle. Addition of 10 μM E-64 to synchronized cultures of

P.falciparum for even 30 min of culture blocked the release process with an efficiency of 90.23 ± 1.51% (mean ± s.e.), n=4. Longer treatment did not increase its effect (n=18; p>0.3; Fig. 2 A). The percentage of schizonts transformed into clusters after a 30 min treatment was equal to the percentage of ruptured schizonts after the same period in control cultures (mean ± s.e. 102.56 ± 6.6%, n=4), suggesting the normal kinetics of schizont maturation upon short drug treatment. Data from live recording of cluster-formation in both treated and untreated cultures supports the conclusion that cysteine proteases act only at the very end of the cycle, before infected erythrocytes rupture and release parasites. At the earliest times after the addition of inhibitors (~10 min, due to technical considerations), confocal recordings of apparently normal schizonts shows cluster-formation and the presence of parasite clusters. Schizonts and parasite clusters in cultures treated with E-64 for long times had swollen food vacuoles (Fig. 2 B–C; compare DIC images of control schizont (B) and cluster (C), suggesting the accumulation of undigested hemoglobin in this organelle (Rosenthal et al., 1988). There was no obvious increase of the size of food vacuoles clusters and schizonts in cultures treated for short times (Fig. 1 A–B). The earliest detected increase in food vacuole size was observed after 4 h of schizont treatment with E-64 (Table S1) suggesting that the inhibition of parasite release does not require prolonged inhibition of hemoglobin degradation.

In summary, treatment of cultures with the irreversible broad-spectrum cysteine protease inhibitor E-64, which does not affect serine proteases, had multiple effects on *P. falciparum* culture: 1) blocked parasite release, acting within the last minutes of the cycle, 2) induced formation of parasite clusters, limited by a single erythrocyte membrane; and 3) interfered with hemoglobin degradation in food vacuoles during prolonged treatment.

All three of these effects were also seen with protease inhibitors of various specificities: leupeptin and calpeptin (Aoyagi et al., 1969; Tsujinaka et al., 1988) at concentrations 10 μg/ml and 1 μM, respectively (Fig. S1). A moderate but specific inhibition of parasite release (40.3 ±5.1 %, mean ± s.e., n=4) and cluster formation was observed at 100 nM concentration of calpeptin. This potent inhibitor is likely not acting through erythrocyte calpain, because its specific inhibitor calpastatin peptide (CS Peptide) did not induce cluster formation and had no inhibitory effect on parasite release (mean ± s.e. 102.5± 1.8% of control value at 5 μM, n=3), but we could not check for its permeation into cells. In contrast to leupeptin and calpeptin, the broad spectrum caspase inhibitor Z-VAD-FMK and the more specific caspase 3 (Z-DEVD-FMK) and caspase 8 (Z-IETD-FMK) inhibitors did not induce formation of parasite clusters and had only a partial inhibition effect upon release (29.5%, 43.6%, and 49.5%, respectively, mean values of 2–5 independent experiments), even at high concentrations of 100 μM. Thus, the inhibitory effect of cysteine protease inhibitors is likely not acting through caspases.

E-64 irreversibly inactivates parasites and terminates infection in culture

Several experimental approaches were used to study the reversibility of the E-64-induced disruption of the parasite erythrocyte cycle. We analyzed the kinetics of parasite release from infected cells after drug withdrawal, using a standard release assay or modified assays that allow us to follow the fate of clusters and schizonts individually. We also tested the viability of clustered parasites using propidium iodide (PI; detection of necrotic cells) and YO-PRO-1 (detection of apoptotic cells), and we assessed the infectivity of isolated clusters in the standard replication assay. Finally, we treated regular parasite culture with the drug for 3 days and then followed the culture for 3 more days after drug withdrawal. As shown below, all data support the generalization that the drug irreversibly affects *only* infected cells undergoing the cycle transition. The membranes of E-64-induced parasite clusters did not rupture, and resident parasites died within a few hours. However, treated schizonts harbored live parasites and did successfully release them when drug was removed before the end of the cycle. Specifically, withdrawal of protease inhibitor from the culture medium after short incubation times restores

parasite release rates within the first 2–3 h, judged from the standard release assay (Fig. 3 A). Restoration of parasite release after longer treatments was much less efficient (Fig. 3 B), suggesting an additional effect of this inhibitor on schizont maturation. The release assay also defined the source of the *de novo*-released parasites in treated cultures after drug withdrawal: the increase in the number of newly ruptured cells upon withdrawal of E-64 is equal to the decrease in the number of schizonts (Fig. 3 C; see legend). Cluster numbers were preserved. Taken together, these data indicate that schizonts, but not parasite clusters, release infectious parasites.

To confirm the irreversible effect of E-64 on parasite release from the clusters, the fate of individual clusters and late schizonts was followed directly using light microscopy of live cells for up to 4.5 hours after the withdrawal of inhibitor. Optical fields showing both late schizonts and clusters in the same field were monitored continuously up through the time of release of parasites from late schizonts. Not one event of cluster rupture was observed in three independent experiments with 54 clusters and 20 late schizonts while all late schizonts successfully finished the cycle (Fig. 3 D–E). Thus, parasites could not be released from clusters, indicating that drug treatment irreversibly disrupted a crucial step in the release process.

Parasites in our experiments were viable in schizonts at the moment of cluster production (Fig. 4; 10.3-min frame), judging from the absence of labeling both with YO-PRO-1 (detection of apoptotic cells) and with PI (detection of necrotic cells) staining. These dyes permeate the cluster membrane (Lyon and Haynes, 1986) and permit detection of dead parasites and testing for early signs of E-64 toxicity to parasites. Double labeled parasites (YO-PRO-1 and PI) appeared in parasite clusters at a different time, starting from approximately 20 min after cluster formation (Fig. 4). The simultaneous labeling of parasites with both dyes argues for a necrotic type of parasite cell death. In 2 to 3 h, most of the clusters harbored dead parasites in drug-treated cultures (72%; n=60, means of two independent experiments). More than 90% of clusters had dead cells by 8 h of drug treatment, and the majority of clusters (87%; n=90, mean of two independent experiments) had multiple PI-positive cells. The kinetics of the accumulation of dead parasites in clusters was similar to the kinetics of the accumulation of dead parasites in the sites of parasite release in control medium (our unpublished observation). Thus, individual parasites captured in clusters have a low viability like that of parasites released *in vitro* (O'Donnell and Blackman, 2005). Correspondingly, clusters that we isolated according to the original protocol of Salmon and coworkers (Salmon et al., 2001) were not infectious in our hands (Fig. S2) and harbored dead merozoites (Fig. S3). Finally, synchronized parasite cultures treated for 3 days with 10 μM E-64, starting from the ring or late schizont stages, did not show parasite replication during the following 3 days in the standard *in vitro* replication assay (Fig. S4).

We were also interested in determining whether the inhibition of proteases by reversible cysteine/serine protease inhibitors leupeptin and calpeptin at critical times during parasite release would reverse. Therefore, we used the approaches described above. Once leupeptin-induced or calpeptin-induced clusters were formed, they did not release parasites and did not restore infection (Fig. S5A, effect of leupeptin; calpeptin has the same effect on parasite release, as shown in Fig. S5B, one of two independent experiments). Like E-64, reversible cysteine/serine protease inhibitors did not affect schizont maturation upon treatment of short duration (Fig. S5C), despite the different membrane permeating properties of these inhibitors: calpeptin is a membrane permeating inhibitor, whereas leupeptin, and possibly E-64, may enter erythrocyte cytoplasm through a parasite-induced channel in the erythrocyte membrane (Lisk et al., 2008). Thus either the proteases of interest for release are in an inhibitor-inaccessible compartment (perhaps the vacuole?) or proteases for release can be synthesized very quickly after inhibitor removal. Taken together, our results suggest that once parasite clusters are produced by inhibition of proteolysis, the end of the cycle is irreversibly ruined, with no chance

of parasite release, regardless of whether protease activity is restored or not. Thus, cysteine protease inhibitors-induced clusters of malaria parasites are surrounded by a membrane of erythrocyte origin and are incapable of sustaining the infectious cell cycle.

Discussion

At the end of the erythrocyte cycle of the human malaria parasite *Plasmodium falciparum*, parasites are released from infected erythrocytes in a process that includes the sequential rupture of parasitophorous and erythrocyte membrane. Our study demonstrates that cysteine proteases act at the very last step -- the rupture of the erythrocyte plasma membrane. Most importantly, inhibition of only one of the two classes of proteases that mediate parasite release from the host cells (Arastu-Kapur et al., 2008; Yeoh et al., 2007; Blackman, 2008) can *irreversibly* block the cycle and inactivate parasites by arresting them inside erythrocytes and abolishing replication in culture, suggesting the hypothesis that such inhibition will potentially block parasite dissemination *in vivo*.

A large number of putative proteases have been identified in the *P.falciparum* genome, among them 33 cysteine and 16 serine proteases (Wu et al., 2003). One of the best studied proteolytic cascades in the parasite's erythrocyte cycle is the degradation of hemoglobin. It requires two classes of proteases: aspartic proteases (four plasmepsins) and papain-like cysteine proteases (three falcipains) (for review see Rosenthal, 2002). Both hemoglobin digestion and parasite release could be inhibited with cysteine protease inhibitors, however there is no direct data implicating falcipains in parasite release (for review see Blackman, 2008). A model of sequential protease-depended events leading to the initiation of parasite release is now proposed that includes both cysteine and serine proteases as well as possible unidentified players (Arastu-Kapur et al., 2008; Yeoh et al., 2007). Cysteine protease DPAP3 (Arastu-Kapur et al., 2008) could be the first acting protease to act during the maturation of serine protease PfSUB1. The latter protease is ultimately discharged from a subset of dense granules, the exonemes, at the end of the erythrocyte cycle (Yeoh et al., 2007) and hydrolyzed papain-like SERAs located in the parasitophorus vacuole. Downstream events and substrates for proteases involved in parasite release have not yet been identified but likely require the activity of at least one more cysteine protease for SERA protein processing (Li et al., 2002). An intricate interplay between several proteases appears to form the core of the initiation of the parasite release process. E-64, a broad-spectrum inhibitor of cysteine proteases that is not active against serine proteases, may block some of the described proteolytic events and capture mature parasites inside erythrocytes. We were able to separate the observed effect of E-64 on hemoglobin degradation and parasite release and to decouple these processes by showing that the drug acts as early as a few minutes after its application, by blocking the rupture of mature schizonts without any obvious effect on parasite morphology. This result is supported by the weak anti-hemoglobinase activity in SAK1, a specific inhibitor of cysteine protease DPAP3 that is involved in parasite release (Arastu-Kapur et al., 2008).

Our data suggests that cysteine proteases are not required for the rupture of the vacuolar membrane. Indeed, electron microscopy revealed the presence of one limiting membrane around loosely associated parasites in both high-pressure frozen and chemically fixed clusters. The multiple membrane fragments, likely derived from the ruptured parasitophorous vacuole and visible only in the chemically fixed clusters, were located randomly between and around parasites inside clusters. Good structural preservation of biological membranes in high-pressure freezing technique is often linked to a high proportion of membrane proteins, because membrane lipids are often lost during high pressure freezing and freeze-substitution procedure (Walther and Ziegler, 2002). If this is true, then our data is consistent with the idea that the cluster-limiting membrane originates from the protein-rich erythrocyte membrane, and the internal membrane fragments originate from the protein-poor vacuolar membrane that excludes

major erythrocyte membrane proteins such as glycophorin A and C, and band 3 (Lauer et al. 2000) and lacks cytoskeleton proteins (Atkinson et al., 1988). Electron and fluorescent microscopy data are in agreement with the data of Gelhaus et al. (Gelhaus et al., 2005). These authors showed that a new inhibitor of cysteine proteases, bADA, which penetrates to the erythrocyte cytoplasm, block parasite release and produce parasite clusters limited by a single membrane of erythrocyte origin. Two inhibitors we tested, E-64 and leupeptin, are non-membrane penetrating inhibitors. However, it was shown that leupeptin and possibly E-64 permeates erythrocyte membrane through a parasite-induced channel (Lisk et al., 2008). It is not known yet whether these inhibitors can reach the parasitophorous vacuolar space. Calpeptin, a membrane-penetrating derivative of leupeptin, demonstrates the same effect on parasite release (and on hemoglobin degradation) as leupeptin and E-64 but at much lower concentrations (1 μM). However, subcellular compartmentalization of calpeptin in infected erythrocytes is also unknown. This uncertainty make it difficult to form conclusions as to the exact place of proteases/inhibitors action. It is clear, however, that the kinetics of inhibitor accumulation in this compartment is quick enough to execute their effect within few minutes of the drug application.

Thus, several tested inhibitors that affect cysteine proteases (E-64, bADA, leupeptin and calpeptin) all interfere with the rupture of erythrocyte membrane. These results are inconsistent with the original observation that the cysteine protease inhibitor E-64 liberates parasite clusters that are limited by the parasitophorus vacuole membrane (Salmon et al., 2001) and with a contradictory results of Soni and colleagues (2005) that cysteine/serine protease inhibitor leupeptin preserves vacuolar but not host cell membrane. However, we confirmed effect of leupeptin described in the early works of Lyon and Haynes,1986; Delplace et al., 1988; Wickham et al., 2003. Thus, a previous uncontested conclusion from the early studies that the rupture of the two membranes required for parasite release is differentially regulated by different proteases is revised. The controversy could originate from the different techniques used for the analysis of the extremely fragile parasite clusters: we employed a live cell observation without intervention of the physical and chemical treatments required for the traditional methods of cell isolation and the histochemical analysis of fixed cells. We believe that extremely gentle handling of parasite clusters is critical for analysis of their morphology, and any manipulation with clusters-containing cultures may interfere with structure preservation and produce artifactual results. Previously, we showed (Glushakova et al., 2007) that treating infected cells with PBS or serum-free medium induces cell swelling and hemolysis. As a result, the release of parasites from these cells is inhibited, and cultures accumulate damaged late-stage infected cells, some of which extrude vacuole-enclosed parasites from the erythrocyte membrane. A technique combining repetitive cell washing in PBS with subsequent smear preparation, used in research reported in some previous papers (Salmon et al., 2001; Soni et al., 2005), could shear off the erythrocyte plasma membrane, producing a significant number of schizonts without membranes that had not yet reached the end of the cycle. Indeed, in a majority of published images, fixed/dried and immunolabeled parasite clusters have an irregular shape (Salmon et al., 2001; Soni et al., 2005; Wickham et al., 2003; Gelhaus et al., 2005) suggesting disruption and possible absence of their limiting membrane. As we show here, parasite clusters in live cultures are perfectly rounded objects because they are swelled erythrocyte ghosts harboring mature parasites, food vacuoles, and fragmented parasite-derived internal membranes. Another factor that could lead to artifact is the permeability of the cluster's membrane for proteins, as demonstrated by Lyon and Haynes (1986). Thus, a limiting membrane could be permeable for soluble GFP-targeted proteins used by Wickham and colleagues (2003), who concluded that vacuole membrane is preserved in E-64-treated parasite clusters. Indeed, our pilot experiments show no GFP fluorescence in clustered parasites that have originated from GFP-positive infected cells (S.Glushakova, unpubl. obs.).

In summary, treating live cells with inhibitors of cysteine proteases prevents parasite release from infected erythrocytes at the end of the cycle, probably because cysteine proteases suddenly enter the erythrocyte cytoplasm once the parasitophorus vacuole membrane is disrupted. Once active in the compartment facing the plasma membrane of the erythrocyte, these proteases act to disrupt it. In the presence of protease inhibitor, parasites are degrade and die within clusters. It is unlikely that E-64 has a direct toxic effect on parasites. Our finding that clustered parasites locked inside erythrocytes in E-64-containing medium have approximately the same life span as released parasites that did not invade red cells in normal medium *in vitro* is argues against this scenario. Our working hypothesis is that E-64 "toxicity" is executed through a fatal prolongation of the otherwise normal transient "cluster stage", when separated parasites enter erythrocyte cytoplasm as a result of the breaking of the vacuolar membrane. Combined with the short life span of mature parasites, this cluster stage prolongation leads to the irreversible blockage of the plasmodial erythrocyte cycle. The withdrawal of cysteine protease inhibitors (irreversible and reversible) does not restart parasite release from clusters as previously suggested (Salmon et al., 2001), but permits schizonts to release parasites at future times. Thus, a scheduling of dosage informed by these studies may be important for testing protease inhibitors as antimalarial drugs. Hopes for antimalarial protease inhibitor drugs are augmented by the finding that relatively low concentrations of a reversible inhibitor, calpeptin, had a potent inhibitory effect on parasite release.

Experimental procedures

Culture of *Plasmodium falciparum* and a synchronization procedure

Plasmodium falciparum strain 3D7 (ATCC, Manassas, VA) was cultured according to the Trager-Jensen method (Trager and Jensen, 1976) in human erythrocytes in RPMI 1640 medium (Gibco) supplemented with 25 mM Hepes (Gibco), 4.5 mg/mL glucose (Sigma), 0.1 mM hypoxanthine (Gibco), 25 μg/mL gentamicin (Gibco), and 0.5% AlbuMax (Gibco). A combination of Percoll-enrichment and sorbitol-treatment procedures were used to obtain a culture of *P. falciparum* with a 3- to 4-h span of synchronization (detailed description in (Lambros and Vanderberd, 1979; Druzewski et al., 1984). Only freshly drawn human erythrocytes or those dated no more than 48 hours after phlebotomy were used to initiate synchronized cultures. While erythrocytes that were stored longer than 48 hours after phlebotomy supported parasite replication, cultures using them had a significant fraction of defective schizonts, and many of the drug-induced parasite clusters were structurally damaged.

Parasite replication assay

To test the effect of E-64 on parasite replication, a synchronized parasite culture was maintained according to the Trager-Jensen method (Trager and Jensen, 1976) at 0.5% hematocrit. To avoid aspiration of parasite clusters formed in drug-containing cultures, we started cultures at low parasitemia and maintained cultures in the normal medium or medium supplemented with drug for the first 3 days without medium change. After 72 h, cells were washed by centrifugation (1,900 × g, 5 min at room temperature), resuspended in the normal medium, and returned to culture for 3 more days with a daily medium change. Daily aliquots of infected cells were taken in order to assess parasite replication by staining parasitized cells with acridine orange (Molecular Probes, Eugene, OR).

Parasite Release Assay

To quantify the parasite release process, we used our recently developed method (Glushakova et al., 2007). Briefly, a highly synchronized parasite culture was used for drug treatment, starting 42–50 h after culture initiation, depending on the length of the drug treatment (as much as 8 h or as little as 30 min). The end of the drug treatment time always coincided with the end of the parasite erythrocyte cycle. Treatment was performed in medium supplemented with

Albumax at 37°C. The treatment time included the last 2-h period in chambers for microscopy (for accumulation of release sites from the release events) before the quantification of parasite release. Cells were treated with drug in chambers for microscopy only in short-time experiments. To analyze the reversibility of drug effect on parasite release we diluted drug-treated cultures at least 200 × with the normal medium. This "drug-removal" procedure was used to avoid cell centrifugation and resuspension, which affected the morphology of parasite clusters. After this procedure, cells were immediately injected into chambers for microscopy and incubated for 1 to 3 h at 37°C to assess parasite release. Control cultures were treated the same way but in the normal medium.

We analyzed a live culture of *P.falciparum* using differential interference contrast (DIC) microscopy (confocal microscope LSM 510, Zeiss, 100× or 63× oil 1.4 NA objectives). We tested the cysteine protease inhibitor E-64 (Sigma, St. Louis, MO) at 10 μM concentration, the cysteine/serine protease inhibitors leupeptin (Sigma, St. Louis, MO) at 10 μg/ml concentration and calpeptin (Calbiochem, San Diego, CA) at 0.1 – 1 μM concentrations, the caspase inhibitors Z-VAD-FMK, Z-DEVD-FMK, and Z-IETD-FMK (Calbiochem, San Diego, CA) each at a 100 μM concentration, and calpastatin peptide (CS Peptide; Calbiochem, San Diego, CA) at 5 μM concentration.

Confocal recording of live infected erythrocytes

Confocal microscopy was performed at 37°C using a laser scanning confocal microscope (LSM 510, Zeiss) with a 100× or 63× 1.4 NA oil objective. Laser excitation at 633 nm with an intensity below 10 μW was used to avoid photo-induced cellular damage.

Assessment of the food vacuole size in parasites in live infected erythrocytes

We performed DIC microscopy of infected cells as described above and analyzed digital images using a software package (Image Browser, Zeiss). Specifically, the area of the spot that represents the food vacuole was measured in 10 randomly selected cells. This value is presented as mean ± s.e (μm^2).

Labeling of erythrocyte membrane with fluorescent markers

Two different labeling procedures were used to determine the origin of the clusters' limiting membrane. Neither approach required cell washing before microscopic analysis. Late-infected schizonts were not subjected either to centrifugation or to any other procedure of isolation or purification. To detect multiple proteins on the erythrocyte surface, cells were biotinylated in the early ring stages of infected cultures. Biotinylated cells were labeled with 20 nM Quantum-Dots 525 conjugated with streptavidin (Quantum Dots, Hayward, CA) at the end of the cycle just before microscopy (for details of procedure see reference (Glushakova et al., 2005). Quantum Dots 525 were excited with a 488-nm laser. To detect the most abundant glycoprotein of erythrocyte membranes, glycophorin A, we labeled cells with 2 μg/ml Allophycocyanin-(APC-) antibody to glycophorin A (anti-CD235a) (BD Biosciences, San Jose, CA) just before microscopic analysis. APC was excited with a 514-nm laser. Because of high concentrations of detected antigens on the cell surface and because of the use of the primary-labeled antibodies to glycophorin A or Quantum Dots-conjugated streptavidin, the signal/noise ratio was high enough to avoid cell washing before microscopy. In control experiments (labeling of parasitophorus vacuoles extruded from erythrocytes by hypotonic shock), we confirmed that the membrane of the parasitophorus vacuole does not have glycophorin A. In addition, we also confirmed that it is not biotinylated and that it could not be labeled with Quantum Dots-conjugated streptavidin. Labeled cells were injected into chambers (HybriWell ™ HBW20; Grace Bio-Labs, Bend, OR) at hematocrit ~ 0.2%, sealed, and used for microscopy for all methods described above.

Electron microscopy

Cells were pre-fixed with 0.2% paraformaldehyde to render the sample non-infectious for subsequent handling and then pelleted at 1,900 × g for 5 min. Both high-pressure-frozen and chemically-fixed samples were prepared. For high-pressure freezing, the sample was placed in the cavity of a slot grid that was sandwiched between two flat-bottom planchettes immediately before freezing using an HPM10 (Bal-Tec, Balzers, Liechtenstein). Freeze-substitution occurred in acetone containing 2% osmium tetroxide for 72 h at −90°C, followed by linear warming to room temperature over 24 h in an EM-AFS freeze-substitution system (Leica Microsystems, Wetzlar, Germany). For chemical fixation, the sample were treated with 1.5% glutaraldehyde and 1.5% osmium tetroxide for 45 min at room temperature. The cells were then transferred to acetone in a succession of PBS-acetone solutions (25%,50%,75%, and 100% acetate) for 15–20 minutes each. High pressure-frozen and chemically-fixed samples were washed 3 times in acetone for 10–15 minutes and then infiltrated in 25%, 50%, 75%, and 100% Spurr's resin (Spurr, 1969) for 8–12 h each. A final infiltration at 100% for 12h was followed by the polymerization of the samples at 65°C for 48 h. We sectioned polymerized blocks to 60–70 nm using a diamond knife on a Ultracut microtome (Leica, Microsystems, Wetzlar, Germany) and picked them up on Formvar-coated slot grids. These sections were then stained with 1% uranyl acetate (Stempak and Ward, 1964) for 5 min and lead citrate (Reynolds, 1963) for 5 min. Images were collected with a 2k × 2k UltraScan (Gatan, Pleasanton, CA) digital camera on a Technai F30 (FEI, Hillsboro, OR) microscope operated at 300 kV at the indicated magnification.

Parasite viability assay

We detected apoptotic/necrotic parasites using labeling with a combination of fluorescent dyes, YO-PRO-1 (100 nM) and PI (1 μg/ml) (Molecular Probes, Eugene, OR). Dyes were added into the culture medium, and individual infected cells were monitored for the appearance of parasites with labeled nuclei. Alternatively, 30 min after addition of dyes clusters with labeled parasites were counted. No labeling was detected in morphologically normal schizonts during several hours of cell observation in the presence of these dyes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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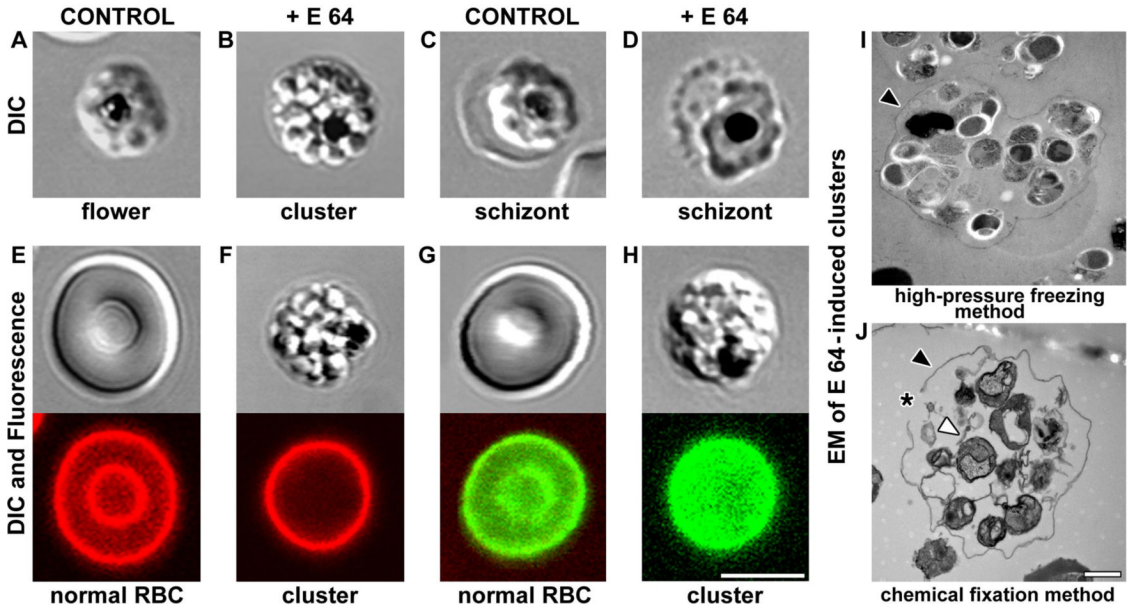

Figure 1. E-64, an inhibitor of cysteine proteases, produces parasite clusters limited by erythrocyte plasma membrane

A–D: DIC images of control schizont at "flower" stage (**A**), drug-induced parasite cluster (**B**), and late schizonts in control (**C**) or E-64-containing medium (**D**). Note the relaxing or ruffled shape of erythrocyte membrane in the last two images.

E–H: Detection of the antigens on the limiting membrane of parasite clusters with fluorescent light microscopy. Glycophorin A detection (red color) on the surface of a normal erythrocyte (**E**) and a parasite cluster (**F**); detection of entire erythrocyte membrane proteins (green color) on the surface of a normal erythrocyte (**G**) and a parasite cluster (**H**). Labeling methods are described in the "Experimental procedures" section. Fluorescent images of cells are supplemented with DIC images of the same cells. Note that the erythrocyte membrane is preserved in the long-treated infected cell with the swelled food vacuole (**H**).

I–J: Detection of the cluster-limiting membranes with electron microscopy using a high-pressure freezing technique (**I**) or a chemical fixation procedure (**J**). Note that the erythrocyte outer membrane (black arrowheads in **I** and **J**) is well preserved and the inner-cellular membranes (white arrowhead in **J**) are extracted during high pressure freezing, while both membrane systems are well preserved under chemical fixation. Different levels of membrane preservation in high pressure freezing is thought to be due to membrane characteristics (see Discussion). Asterisk (**J**): defect in the limiting membrane. Scale bar = 5 μm **(A–H)** or 1 μm **(I–J)**.

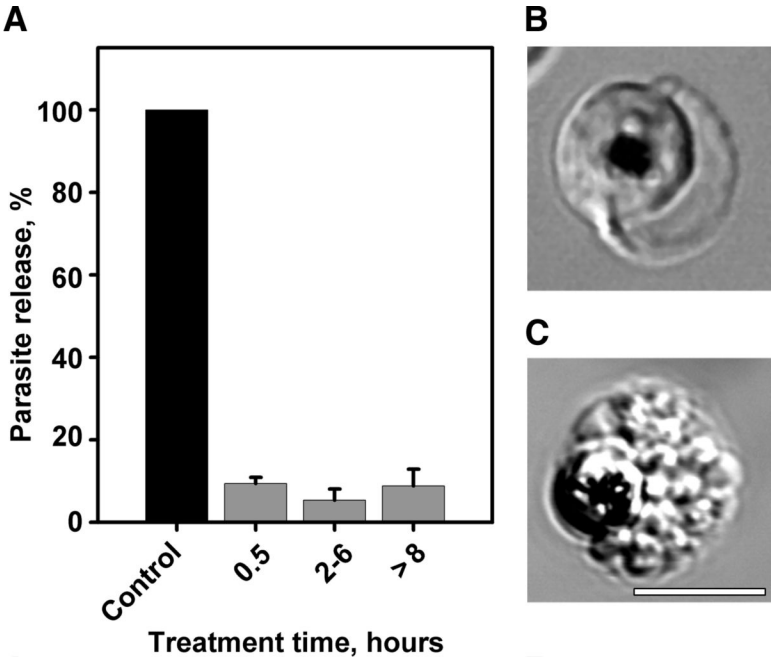

Figure 2. E-64 blocks parasite release from infected erythrocytes within a few minutes of drug application and interferes with hemoglobin degradation in food vacuoles upon prolonged drug treatment

Inhibition of parasite release upon different periods of drug-treatment (**A**). Synchronized culture of *P.falciparum* was treated with E-64 for 30 min or longer at the time of cycle transition, and the effect of treatment, in comparison with untreated cultures, was assessed in a release assay. The result is presented as mean value ± s.e. (n = 4, 7, and 11 for treatments of 30 min, 2–6 h, and 8 or more hours, respectively). **DIC images**: control schizont (**B**) and drug-induced cluster (**C**) with enlarged parasite food vacuoles in the long-term drug-treated cultures. Scale bar = 5 μm.

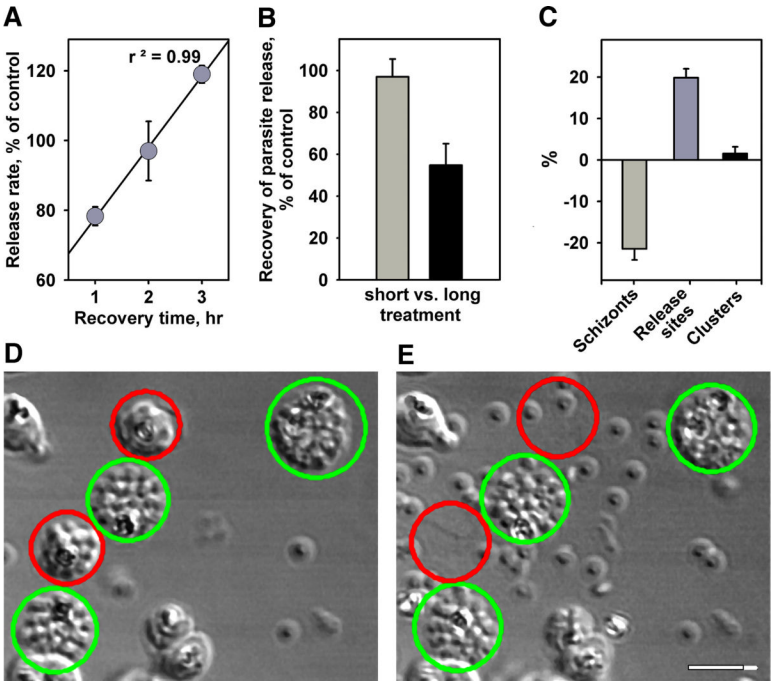

Figure 3. Inhibition of cysteine proteases blocks parasite release from drug-induced parasite clusters but not from schizonts upon drug withdrawal

A. Kinetics of parasite release in E-64-treated cultures (10 μM) upon drug withdrawal (treatment time 0.5–1 h; mean value ± s.e., n=3). **B**. Recovery of parasite release after 2 h of post-drug withdrawal in cultures treated for different time intervals with drug (0.5–1 h or "short treatment" vs. 8 h or "long treatment"; mean value ± s.e., n = 3–4). **C**. Evidence that sites of release originate from schizonts upon drug withdrawal from treated cultures. Cultures were treated with 10 μM E-64 (40 min to 8 h) and after drug withdrawal cells were injected into the chambers and the proportion of schizonts, clusters, and the sites of parasite release were assessed before and after 1–3 h of parasite release recovery (mean ± s.e.; n = 11). Note that the increase in the number of newly ruptured cells upon withdrawal of drug is equal to the decrease in the number of schizonts; the number of clusters did not changed. **D – E**. Direct observation of schizont but not cluster rupture in the culture after drug withdrawal. Culture treated with E-64 (10 μM, 4 h) were injected into the chambers after drug withdrawal and optical fields harboring both late schizonts and clusters **(D)** were monitored until the release of parasites from late schizonts **(E)**. Note that two late schizonts (in red circles) ruptured and released parasites while three clusters (in green circles) were preserved. A representative field (total n=17) from a representative experiment (n=3). Scale bar = 5 μm.

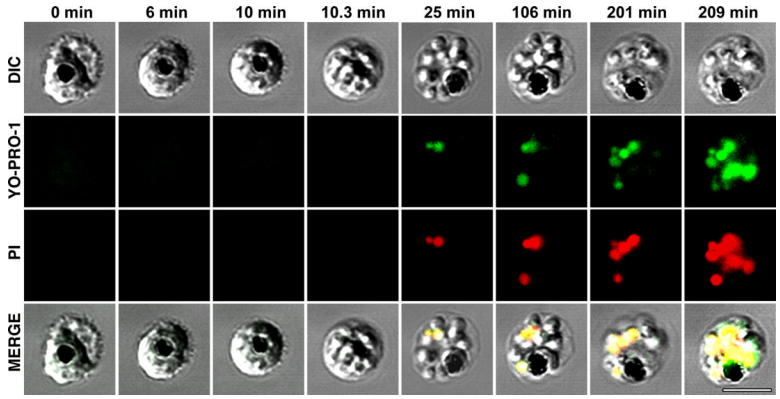

Figure 4. Clustered parasites die within the few hours of captivation

Kinetics of dead parasite accumulation in the E-64-induced clusters. Culture in medium containing E-64 (10 μM), PI (1 μg/ml), and YO-PRO-1 (100 nM) was placed into the chamber, and the appearance of the YO-PRO-1-positive parasites (apoptotic, green color) or PI-positive parasites (necrotic, red color) was monitored in the individual schizont as the end of the cycle approached. Transformation into flower: 10 min frame; cluster formation: 10.3 min frame. A representative infected erythrocyte. Note the flattening of dead parasites. Scale bar = 5 μm.