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Sphingolipid synthesis as a target for chemotherapy against malaria parasites

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ABSTRACT **The human malaria parasite *Plasmodium falciparum* contains sphingomyelin synthase in its Golgi apparatus and in a network of tubovesicular membranes in the cytoplasm of the infected erythrocyte. Palmitoyl and decanoyl analogues of 1-phenyl-2-acylamino-3-morpholino-1-propanol inhibit the enzyme activity in infected erythrocytes. An average of 35% of the activity is extremely sensitive to these drugs and undergoes a rapid, linear decrease at drug concentrations of 0.05–1 μM. The remaining 65% suffers a slower linear inhibition at drug concentrations ranging from 25 to 500 μM. Evidence is presented that inhibition of the sensitive fraction alone selectively disrupts the appearance of the interconnected tubular network in the host cell cytoplasm, without blocking secretory development at the parasite plasma membrane or in organelles within the parasite, such as the Golgi and the digestive food vacuole. This inhibition also blocks parasite proliferation in culture, indicating that the sensitive sphingomyelin synthase activity as well as the tubovesicular network may provide rational targets for drugs against malaria.**

Malaria is a major infectious disease which afflicts over 200 million people world wide; a million children die of the disease each year. It is caused by protozoan parasites of the genus *Plasmodium*. *Plasmodium falciparum*, one of four species infectious to humans, causes the most severe and fatal forms of the disease. The growing threat of drug-resistant forms has created an urgent requirement for new drugs against the disease. Targeting features of the parasite not found in host cells provides one approach to new drug development.

The blood stage infection, which is entirely responsible for the symptoms of malaria, begins with the entry of a merozoite into the red cell. The intraerythrocytic parasite develops in a parasitophorous vacuole through morphologically distinct ring (0–24 h) and trophozoite (24–36 h) stages to schizogony (36–48 h), where mitosis occurs and 10–16 daughter merozoites are assembled. At the end of schizogony, the infected erythrocyte ruptures, and the released merozoites reinvade red cells to maintain the asexual cycle.

The mature erythrocyte is enucleated and has no intracellular organelles. A secretory feature of asexual parasite development is the formation of a tubovesicular membrane network (TVM) in the infected-erythrocyte cytoplasm (1–3). The TVM extends from the periphery of the parasite into the cytoplasm of the erythrocyte, as detected in cells labeled with the fluorescently labeled lipid 1-phenyl-2-[*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-5-indacene-3-pentanoyl)]-D-*erythro*-sphingosine (C_5-DMB-ceramide) and examined by high resolution laser confocal microscopy (1, 3). Scanning electron micrographs of parasites freed from their host cell membranes also indicate the presence of continuous tubovesicular elements emerging from the parasitophorous vacuolar membrane (2). The budding of these interconnected tubovesicular structures occurs by a process apparently independent of coated-vesicle formation (3). Because the TVM develops beyond the parasite plasma membrane and is obligatory for intraerythrocytic development, biochemical activities of the organelle are of interest with respect to defining eukaryotic secretory processes, as well as drug targets, in the infected red cell. Our previous results have indicated that a striking biochemical feature of this network is the presence of the Golgi enzyme sphingomyelin synthase (3).

The lack of incorporation of appreciable amounts of choline or free fatty acid into sphingomyelin in infected erythrocytes led to an earlier suggestion that sphingomyelin is not synthesized by the parasite (4). However, subsequent studies have demonstrated that both radiolabeled and fluorescent ceramides are efficiently converted to sphingomyelin in infected but not uninfected erythrocytes (3, 5–7), confirming the presence of a parasite synthase. The ceramide substrate for this enzyme is not obtained from *de novo* sphingolipid synthesis but probably from the hydrolysis of host sphingomyelin (S.A.L. and K.H., unpublished data), and phosphatidylcholine internalized from the host cell membrane (8–11) may be an efficient phosphocholine donor. In eukaryotes, sphingomyelin synthase is a conserved activity of the secretory pathway. The most recent evidence indicates that in mammalian cells it resides in the cis-Golgi (12, 13), a compartment which can be reorganized to the endoplasmic reticulum (ER) by the drug brefeldin A and contains additional markers such as ERD2, a receptor for protein retention in the ER (14–16). In contrast, in *P. falciparum*, ERD2 (PfERD2) and sphingomyelin synthase are localized in distinct compartments of the Golgi (6). Unlike PfERD2, the sphingomyelin synthase site is not reorganized by brefeldin A, indicating that its dynamics are altered in the parasite (6). Furthermore, although the enzymatic activity lies entirely within the parasite at the merozoite stage, in rings and trophozoites a fraction is exported to the TVM, indicating a developmental reorganization of the parasite's Golgi between the merozoite and ring stages (3). These data suggested that molecular properties of the plasmodial sphingomyelin synthase may be distinct from its mammalian counterpart and underlie the secretory development of the TVM beyond the parasite plasma membrane.

To further characterize these differences, we investigated the sphingolipid analogues *dl-threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) and *dl-threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) for their effects in *P. falciparum*. These compounds are known to inhibit the synthesis of sphingomyelin in chinese hamster ovary (CHO) cells

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Abbreviations: C_6-NBD-ceramide, *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl-D-*erythro*-sphingosine; C_5-DMB-ceramide, 1-phenyl-2-[*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-5-indacene-3-pentanoyl)]-D-*erythro*-sphingosine; C_6-NBD-sphingomyelin, *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl-D-*erythro*-sphingomyelin; PPMP, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; TVM, tubovesicular membrane network; SSS, sensitive sphingomyelin synthase; ISS, insensitive sphingomyelin synthase.

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and also inhibit the synthesis of glucosylceramides in a large variety of mammalian cells (17–20). We report a feature in the inhibition of plasmodial sphingomyelin synthase activity and its specific effect on tubular development of the TVM, as well as intraerythrocytic parasite survival.

MATERIALS AND METHODS

Materials. RPMI medium 1640 was obtained from GIBCO/BRL. A+ human serum was from Gemini Biological Products (Calabasas, CA). *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl-D-*erythro*-sphingosine (C_6-NBD-ceramide) and C_5-DMB-ceramide were from Molecular Probes. *dl-threo*-PPMP, *dl-threo*-, and *dl-erythro*-PDMP were from Matreya (Pleasant Gap, PA). Imipramine, stearylamine, and *dl-erythro*-dihydrosphingosine were obtained from Sigma. U-18666A and sphingosine were from Biomol (Plymouth Meeting, PA). [G-^{3}H]Hypoxanthine was from Amersham.

Culturing of Parasites and Inhibition of Growth. *P. falciparum* FCB, FCR-3/A_2, HB-3, and Itg2F6-C32p21 strains were cultured *in vitro* by a modification of the method of Trager and Jensen (21, 22). Parasites were synchronized by incubating schizonts (36–48 h) isolated from a 65% Percoll gradient with a 20-fold excess of uninfected red cells for 12, 24, 36, or 48 h to obtain young rings (0–12 h), late rings (12–24 h), trophozoites (24–36 h), and schizonts (36–48 h), respectively.

Inhibition of parasite growth was measured by incubating synchronized cultures at 0.5% parasitemia with 0–20 μM of *dl-threo*-PPMP for 24 h and then following the incorporation of [G-^{3}H]hypoxanthine for another 24 h (23). Alternatively, the cultures were incubated with 0–20 μM *dl-threo*-PPMP or 5 μM *dl-threo*-PDMP, *dl-erythro*-PDMP, and the indicated lipophilic amines, and the morphological development of intraerythrocytic maturation was monitored every 24 h in Giemsa-stained slides. Both methods yielded equivalent results.

Inhibition of Sphingomyelin Synthase in Intact and Lysed Infected Erythrocytes. Short-chain, fluorescent analogues of ceramide have been used to detect the presence of sphingomyelin biosynthetic activity in cells (24). When C_6-NBD-ceramide is delivered to *P. falciparum*-infected erythrocytes the only fluorescent product detected is *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl-D-*erythro*-sphingomyelin (C_6-NBD-sphingomyelin) (3, 6, 7). Glycosylceramides are not detected, and there is no evidence for their synthesis by *Plasmodium* (3, 5–7). In the presence of excess C_6-NBD-ceramide the amount of C_6-NBD-sphingomyelin formed by infected erythrocytes is a direct and quantitative measure of sphingomyelin synthase (3, 7). To assay for the inhibition of sphingomyelin synthase in intact cells (1×10^8 parasites per ml in RPMI medium 1640) and fractions from lysed cells, samples were incubated with 0–500 μM *dl-threo*-PPMP, *dl-threo*-PDMP, or *dl-erythro*-PDMP for 30 min at 37°C and then mixed with 10 μM C_6-NBD-ceramide for another 30 min at 37°C. The extracted lipids were separated, and the formed C_6-NBD-sphingomyelin was quantitated (3, 7). Inhibition of sphingomyelin synthase by other lipophilic amines was also determined in the presence of 5 μM imipramine, stearylamine, U-18666A, *dl-erythro*-dihydrosphingosine, or sphingosine.

RESULTS AND DISCUSSION

***dl-threo*-PPMP Inhibits Two Distinct Forms of Sphingomyelin Synthase Activity in Infected Erythrocytes.** Since no antibodies or gene sequence are available for the enzyme, identification of sphingomyelin synthase must be done by direct analysis of the enzymatic activity. Uninfected erythrocytes do not contain sphingomyelin biosynthetic activity, but infected erythrocytes actively convert exogenously added ceramide substrates to sphingomyelin (3, 5–7). As shown in Fig. 1, the addition of 0.05–0.5 μM *dl-threo*-PPMP to late ring stage infected erythrocytes (12–24 h) of the *P. falciparum* FCB strain led to an initial, rapid decrease in the sphingomyelin synthase activity. The slope of this inhibition tapers off with an average of 35% inhibition observed at 1–5 μM *dl-threo*-PPMP. However, at concentrations >25 μM, the enzymatic activity further decreased with ≈80–90% of the total cellular enzyme being inhibited at 500 μM *dl-threo*-PPMP (Fig. 1 *Inset*). This biphasic inhibition curve indicates that infected erythrocytes contain two pools of sphingomyelin synthase with respect to their inhibition by the sphingolipid analogue. One pool, comprising 35% of the total activity, is very sensitive to the drug and is completely blocked at concentrations of 0.05–5 μM. The second pool comprises 65% of the total enzyme activity, is only inhibited at concentrations greater than 25 μM, and complete inhibition is observed at 500 μM.

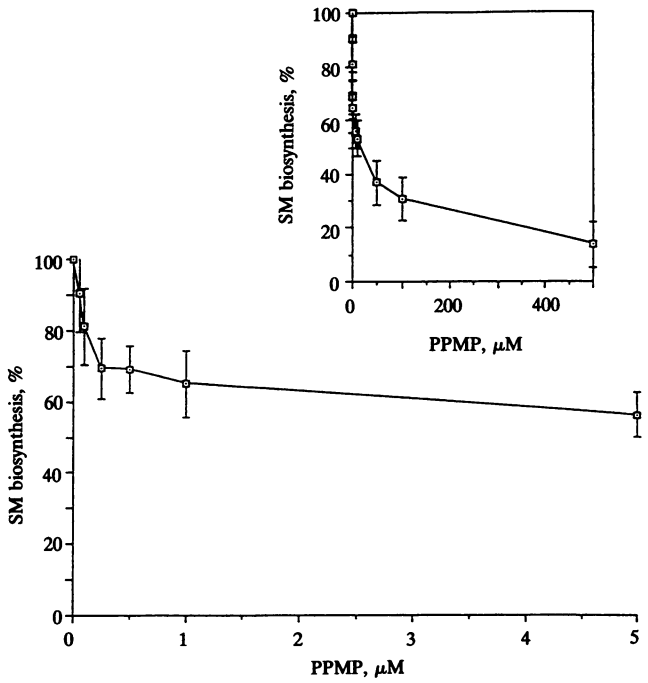

FIG. 1. Effects of *dl-threo*-PPMP on sphingomyelin (SM) synthesis in ring-stage infected erythrocytes. Rings at 12–24 h in the cycle were incubated with 0–500 μM *dl-threo*-PPMP (PPMP) and assayed for enzyme inhibition.

When the cells were broken by hypotonic lysis and treated with 5 μM *dl-threo*-PPMP, 33% of sphingomyelin synthase continued to be inhibited compared to 35% inhibition in intact

Table 1. Inhibition of sphingomyelin (SM) synthesis by 5 μM *dl-threo*-PPMP in intact and lysed, infected erythrocytes

Sample	Glutamate dehydrogenase activity, %	SM synthesis, %	Inhibition of SM synthesis, %
Intact cells	—	100	35
Lysed cells	100	97	33
Supernatant	98	2	3
Pellet	2	95	30

Ring-stage-infected (12–24 h) erythrocytes were lysed in 10 volumes of hypotonic buffer (10 mM NaCl/0.3 mM KCl/1 mM Na_2HPO_4/0.2 mM KH_2PO_4, pH 7.2) and subjected to ultracentrifugation (10 min; 100,000 × *g*) to separate supernatant and pelleted membrane fractions. These fractions (adjusted to a final concentration of 1× PBS) and intact cells were assayed for sphingomyelin (SM) biosynthesis and its inhibition by 5 μM *dl-threo*-PPMP. Cell lysis was confirmed by assaying for release of the parasite cytosolic glutamate dehydrogenase activity (25).

cells (Table 1). If access of the drug is a limiting factor in intact cells, the curve (Fig. 1) would predict that all of the sphingomyelin biosynthetic activity in lysed cells should be inhibited by 5 μM *dl-threo*-PPMP. However, since 33% of the activity is inhibited in lysed cells compared with 35% inhibition in intact cells, the two pools of enzyme detected must reflect the existence of two distinct forms of parasite sphingomyelin synthase activity in ring-stage infected erythrocytes, rather than differential access of the drug to the enzyme in the TVM and the Golgi. The inhibition of the second, insensitive sphingomyelin synthase (ISS) of infected erythrocytes at concentrations >25 μM *dl-threo*-PPMP is similar to the effects of these sphingolipid analogues in inhibiting mammalian sphingomyelin synthase (17–20). In contrast, the sensitive sphingomyelin synthase (SSS) activity of infected erythrocytes inhibited at low (<5 μM) concentrations of *dl-threo*-PPMP appears to be prominent only in the parasite system.

***dl-threo*-PPMP and *dl-threo*-PDMP Block Intraerythrocytic Growth by Specifically Inhibiting the SSS at Ring/Trophozoite Stages.** To determine whether inhibition of the SSS had any effect on parasite growth, we measured the incorporation of [G-^{3}H]hypoxanthine (23) into infected cells in the presence of various concentrations of *dl-threo*-PPMP. As shown in Fig. 2*A*, growth was reduced by 15% at 0.1 μM *dl-threo*-PPMP and by 100% at >3 μM, and the IC_{50} was found to be 0.85 μM. A comparison of the data in Fig. 2*A* with those in Fig. 1 indicates that the inhibition of parasite growth effected by *dl-threo*-PPMP closely parallels the inhibition of the SSS, suggesting that the two might be linked.

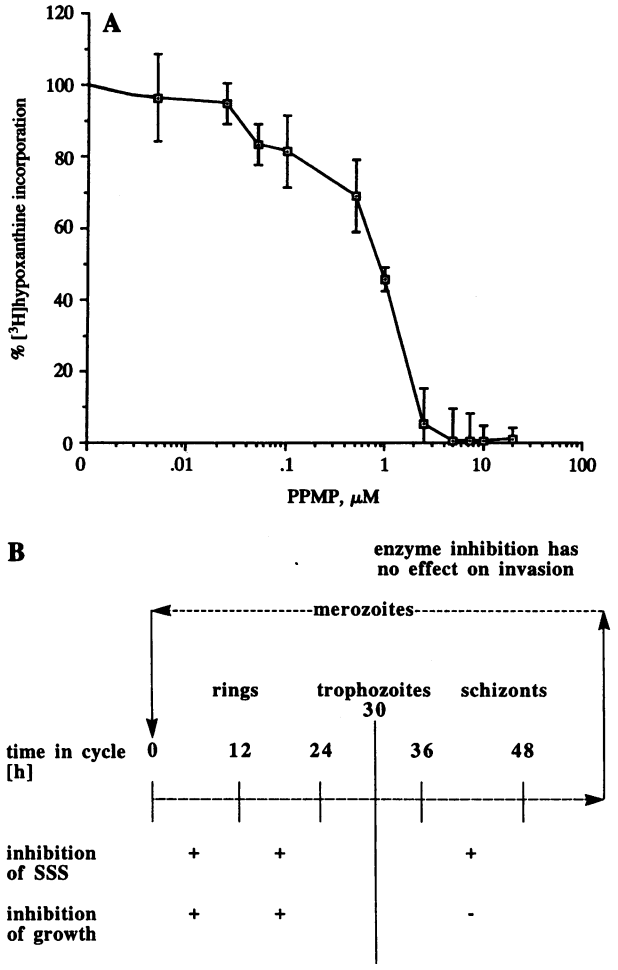

FIG. 2. (*A*) Inhibition of *in vitro* growth of *P. falciparum* FCB ring-stage-infected erythrocytes by *dl-threo*-PPMP (PPMP) (0.005–20 μM). The incorporation of [^{3}H]hypoxanthine at a given drug concentration is expressed as a percentage of that seen in the absence of drug and plotted against the drug concentration. Each data point represents an average of three independent measurements taken in triplicate. No incorporation of radiolabel was seen with uninfected erythrocytes (data not shown). The IC_{50} was 0.85 μM. (*B*) Summary of the effects of 5 μM *dl-threo*-PPMP on inhibition of the SSS, growth of rings, trophozoites, and schizonts, as well as merozoite entry into red cells.

Since the IC_{50} for PPMP is 0.85 μM, and complete inhibition of SSS activity, as well as parasite growth, is detected at 3–5 μM *dl-threo*-PPMP, we investigated the effects of 5 μM concentrations of stereoisomers of a shorter chain analogue and additional lipophilic amines on both inhibition of SSS and parasite growth. As shown in Table 2, as with *dl-threo*-PPMP, incubation of late ring-stage infected cells (12–24 h) with 5 μM *dl-threo*-PDMP also inhibits ≈35% of the sphingomyelin synthase activity in infected erythrocytes. Enzyme inhibition was not due to the drugs being lipophilic amines since five distinct molecules, including imipramine, stearylamine, U-18666A, *dl-erythro*-dihydrosphingosine, and sphingosine, had no effect on the enzyme activity (Table 2). Furthermore, the inhibition was stereospecific, could be completely reversed by washing the drugs out of the cell, and neither *dl-threo*-PPMP nor *dl-threo*-PDMP was inhibitory to either DNA or protein synthesis (Table 2). Thus, 5 μM *dl-threo*-PPMP or -PDMP specifically inhibits the SSS activity in late ring-infected erythrocytes and this inhibition appears to be linked to a block in late ring-stage growth (Table 2). Extracellular merozoites, early ring (12–24 h), trophozoite (24–36 h), and schizont (36–48 h) stages also contain the SSS activity (data not shown), but unlike rings, enzyme inhibition in schizonts has no effect on intraerythrocytic growth or release of invasive daughter merozoites (summarized in Fig. 2*B*). Trophozoite populations at 24–36 h display a growth response intermediate to that between rings and schizonts, suggesting that the enzyme regulates an essential secretory function required in the first 30 h of development but not at schizogony or merogony (Fig. 2*B*). Inhibition of SSS and parasite growth by *dl-threo*-PPMP were detected in several strains of *P. falciparum*, including the FCB, FCR-3, HB-3, and Itg strains (data not shown), indicating that the drugs are not restricted to a specific parasite strain and strongly suggesting that the SSS is a conserved, essential, secretory activity in malarial parasites.

Inhibition of the SSS Selectively Disrupts Secretory Maturation in the TVM. Since the TVM is a secretory organelle which contains sphingomyelin synthase and develops in ring-stage parasites, we investigated whether inhibition of the SSS activity influences its development in the red cell. To ensure that the cells were inhibited in the enzyme activity from the onset of intraerythrocytic development, rings were prepared by incubating 36- to 48-h schizonts with uninfected red cells

Table 2. *dl-threo*-PPMP and *dl-threo*-PDMP specifically inhibit the SSS and block growth of rings

Treatment	Inhibition, %		
	SM synthesis	SSS	Growth
No treatment	0	0	0
dl-threo-PPMP*	36	100	100
dl-threo-PDMP*	31	86	100
dl-erythro-PDMP	3	0.1	5
Imipramine	0	0	7
Stearylamine	0	0	0
U-18666A	0	0	1
dl-erythro-Dihydrosphingosine	4	0.1	8
Sphingosine	0	0	0

Rings (12–24 h) incubated with 5 μM concentrations of the indicated compounds were analyzed for inhibition of sphingomyelin (SM) synthase activity and effects on parasite growth.
*Treatment had no effect on protein or DNA synthesis, and removal of drug by washing cells restores normal development.

and allowing merozoite release and invasion to occur in the presence of 5 μM *dl-threo*-PPMP. TVM structures labeled in control rings (formed in the absence of the drug) indicate the presence of a characteristic, small tubular structure (long thin arrow) extending from the body of the parasite (Fig. 3*Ai*). The vesicle (indicated by a small arrow), although apparently free in these sections, connects back to the body of the parasite in preceding sections (data not shown). Rings formed in the presence of *dl-threo*-PPMP contain no tubular structures. Instead, small intraerythrocytic structures are seen as apparently discrete labeled domains separated from each other (Fig. 3*Aii*). An examination of the 20 optical sections (of which the three shown are a part) taken through the cell shown in Fig. 3*Aii* also failed to reveal any connections between the isolated structures. A total of 240 optical sections through 12 cells selected at random from the control and *dl-threo*-PPMP-treated groups confirmed the presence of a single, interconnected TVM network in the former and an average of four or five disconnected "spots" of fluorescence markedly devoid of labeled tubular connections in the latter. Thus, inhibition of the SSS appears to alter the interconnected "tubovesicular morphology" of the TVM into punctate intraerythrocytic compartments or "vesicles" in ring-stage parasites.

When the young rings in the cell shown in Fig. 3*Aii* are incubated in the continued presence of *dl-threo*-PPMP for another 24 h, they remain blocked in tubular development in the erythrocyte cytoplasm (compare labeled elements in Fig. 3*Bii* with the long tubular structure marked by an arrow in Fig. 3*Bi*). They do, however, form a large food vacuole (Fig. 3*C*), express merozoite surface protein 1 (a marker of the parasite plasma membrane synthesized >30 h in the cycle; Fig. 3*D*) and show two to four sites of PfERD2 staining in the Golgi (Fig. 3*E*), all of which are characteristics induced in trophozoites 24–36 h in the cycle (6, 26, 27). Thus, inhibition of the SSS activity does not block maturation of rings to trophozoites with respect to secretory development at or within the plasma membrane of the organism, but selectively affects the TVM. Hence, the observed retardation in parasite size (compare Fig. 3*Bii* with Fig. 3*Bi*) may be due to inhibition of the TVM, suggesting that proper assembly of the network is essential for growth. Remarkably, despite the prolonged incubations, removal of the drug restores the structures seen in Fig. 3*Bi*, indicating that (*i*) the dispersed vesicular appearance of the network in the cells shown in Fig. 3*Aii* and *Bii* is not due to its nonspecific degeneration in the cell and that (*ii*) although inhibition of the SSS inhibits the *de novo* assembly of tubular elements, it does not fragment a preexisting tubule (data not shown). Predictably, mature trophozoites and schizonts that contain a fully extended tubular network are not affected by the drug.

Concluding Remarks. In mammalian cells, low concentrations (1–5 μM) of *dl-threo*-PPMP or -PDMP have no effect on sphingomyelin synthase but can inhibit the synthesis of glucosylceramides (17–20). As indicated earlier, glucosylceramide

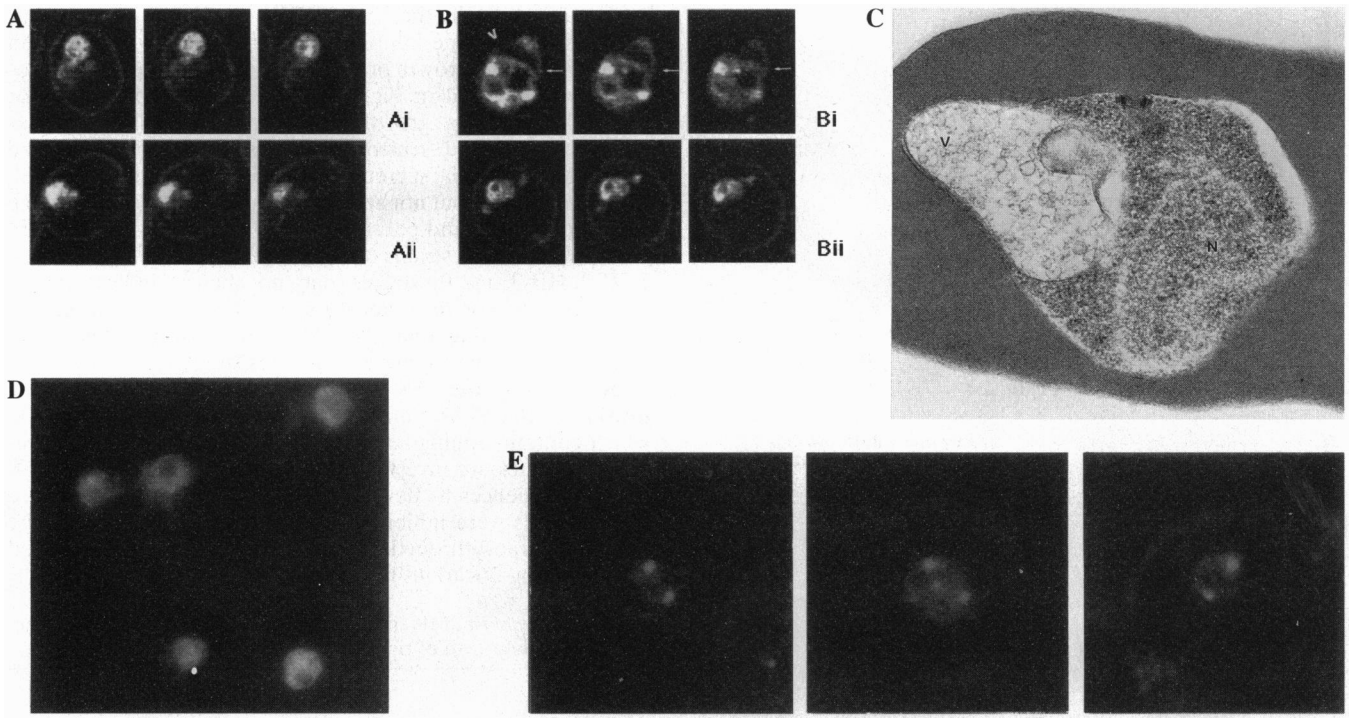

FIG. 3. Effects of inhibition of the SSS on secretory development in infected red cells. Purified schizonts were incubated with uninfected erythrocytes with or without 5 μM *dl-threo*-PPMP for 12 h (*A*) or 36 h (*B–E*). (*A* and *B*) Effects of enzyme inhibition on the TVM. Control (*Ai* and *Bi*) and drug-treated cells (*Aii* and *Bii*) were labeled with 20 μM C_5-DMB-ceramide and viewed on a custom-made laser confocal microscope (built by Stephen J. Smith, Department of Molecular and Cellular Physiology, Stanford University). Images were taken at 400-nm intervals along the *z* axis (3). Raw confocal data were processed by using ADOBE PHOTOSHOP software. Three of 20 consecutive optical sections are shown for each series. The prominently labeled periphery in *Ai, Aii*, and *Bii* is the red cell membrane. The amount of lipid label detected at the red cell membrane (indicated by arrowhead in the first section) of a mature trophozoite/early schizont shown in *Bi* is consistently lower (9). The diameter of the red cell is 7 μm. (*C–E*) Effects on secretory expression at and within the parasite plasma membrane. (*C*) Parasites treated with *dl-threo*-PPMP for 36 h were fixed, processed for electron microscopy (15), thin-sectioned (Polybed), and examined in the electron microscope. The digestive vacuole (V) and the nucleus (N) are marked. (×10,000.) Control cells also display a prominent vacuole, but the size of the parasite is much larger (data not shown). (*D* and *E*) Blood smears of 36-h drug-treated cells were subjected to indirect immunofluorescence assays with antibody to merozoite surface protein 1 (MSP-1) (*D*) or PfERD2 (*E*) and FITC-conjugated goat anti-rabbit antibodies. In *E*, the cells were stained and photographed to detect the periphery of the red cell and the parasite as faint outlines. The punctate bright dots are indicative of PfERD2 staining (6). Parasites that develop in the absence of drug also stained with these antibodies, indicating (as expected) the synthesis of MSP-1 and multiplication of PfERD2 sites in the Golgi at the trophozoite stage (6, 26). No detectable fluorescence signal was seen in samples probed with preimmune serum (data not shown). (Scale: 1 cm = 2.5 μm.) All samples were analyzed blind.

synthase is not detected in *P. falciparum*, an organism known to be limited in many glycosylation functions (1, 3, 7, 28). Hence, the target of these drugs in malarial parasites is expected to be sphingomyelin synthase, and the toxic effects could be due to depleting bulk levels of sphingomyelin or influencing second messenger pathways by depleting diacylglycerol and/or increasing levels of ceramide. It is not known whether parasite sphingomyelin is structurally distinct from host sphingomyelin in either the chain length or the degree of saturation. The concentration of *dl-threo*-PDMP effective against the plasmodial SSS is comparable with that required to inhibit glucosylceramide synthase in mammalian cells. Thus, levels of this compound required to control parasite proliferation may also be harmful to the host, and prolonged treatment with *dl-threo*-PDMP can lead to poor growth of kidneys and liver (29). The IC_{50} of PPMP is 0.85 μM, which is twice that for quinine (a drug used to treat chloroquine-resistant malaria in resistant field strains) and one-fourth to one-half that of primaquine (23). When we monitored cultures with parasitemias ranging from 0.5–10%, we found that *dl-threo*-PPMP can inhibit parasite growth at concentrations as low as 0.05 μM (data not shown). The drug has no significant toxic effect on the *in vitro* long-term growth of cultured mammalian cells up to 20 μM (S.A.L. and K.H., unpublished data), suggesting that it may be the preferred compound to test *in vivo* as an antimalarial treatment.

It is not yet known whether sphingomyelin synthase detected in the Golgi and the plasma membrane of mammalian cells is due to the presence of more than one isoform of the enzyme in the cell (30). Our results suggest that two distinct activities are present in the lower eukaryote *P. falciparum*, one of which (the SSS) is selectively required for the development of the TVM in the cytoplasm of infected erythrocytes. This activity is not prominent in the mammalian system: its physiological substrates may be distinct (possibly with respect to the lipid chains of ceramide), but this specificity remains undetected while using synthetic short chain substrates such as C_6-NBD- or C_5-DMB-ceramide. Its selective action on the TVM suggests that it may reside and directly inhibit sphingomyelin synthesis there. However, it is formally possible that the activity resides within the parasite and regulates secretory export, including that of the ISS, to the TVM by a pathway independent of secretion to the digestive vacuole and the parasite plasma membrane. Although inhibition of the SSS markedly inhibits tubular morphologies, it does not entirely abrogate the appearance of membrane structures in the erythrocyte cytoplasm. This suggests that in addition to sphingomyelin biosynthesis other secretory activities are also required for the assembly of the network in the red cell cytoplasm. We have identified one which could function as an effective target for drugs. However, since the TVM appears to be essential to the intraerythrocytic survival of the parasite, additional components that regulate its organization and function may also be targets for future antimalarial treatments.

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