CMLS, Cell. Mol. Life Sci. 60 (2003) 577–587
1420-682X/03/030577-11

CMLS Cellular and Molecular Life Sciences

Research Article

Ceramide mediates growth inhibition of the *Plasmodium falciparum* parasite

I. Pankova-Kholmyansky [a], A. Dagan [b], D. Gold [a], Z. Zaslavsky [c], E. Skutelsky [d], S. Gatt [b] and E. Flescher [a,*]

[a] Department of Human Microbiology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978 (Israel), Fax: + 972 3 640 9160, e-mail: flascher@post.tau.ac.il
[b] Department of Biochemistry, Hebrew University-Hadassah School of Medicine, Jerusalem 91120 (Israel)
[c] Interdepartmental Core Facility, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978 (Israel)
[d] Department of Pathology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978 (Israel)

Received 11 December 2002; received after revision 19 January 2003; accepted 23 Januray 2003

Abstract. In mammalian cells, ceramide mediates death by chemotherapeutic drugs. We analysed, for the first time, the role of ceramide in inhibiting growth of the malaria-causing parasite *Plasmodium falciparum*. Added exogenously, ceramide significantly decreased the number of parasites, and this effect was abolished by sphingosine-1-phosphate, a biological antagonist of ceramide action. Ceramide can induce death of cancer cells by decreasing glutathione levels, and in our work it induced dose- and time-dependent depletion of glutathione in *P. falciparum* parasites. N-acetylcysteine, a precursor of glutathione, abrogated the cytotoxic effect of ceramide. Thus, ceramide can mediate growth inhibition of *P. falciparum* parasites by decreasing glutathione levels. The antimalarial drugs artemisinin and mefloquine induced the death of *P. falciparum* parasites by sphingomyelinase-generated ceramide and by decreasing parasite glutathione levels. Altogether, ceramide was identified as a signalling molecule capable of inducing growth inhibition of *P. falciparum* malarial parasites.

Key words. Malaria; ceramide; glutathione; parasite growth; artemisinin; mefloquine.

Malaria is one of the major infectious diseases worldwide. Three to five hundred million people are infected with plasmodia parasites [1, 2]. The most virulent species of *Plasmodium* is *Plasmodium falciparum* which kills approximately 1.5–2 million people each year, mostly children. In endemic areas, the disease is managed by chemotherapy and chemoprophylaxis. One of the main problems with developing more efficient chemotherapeutic agents is that cellular mechanisms mediating *P. falciparum* death are still insufficiently understood. Understanding these mechanisms is essential for the development of novel rationally designed drugs.

In mammalian cells, ceramide has been shown to mediate the therapeutic effects of anticancer chemotherapy and radiation [3], to play a pivotal role in a variety of cellular processes [4] and to participate in the regulation of growth, proliferation, differentiation and apoptotic death of cells [5]. Ceramide can be generated from sphingomyelin (SM) via action of various sphingomyelinases (SMases) or via de novo synthesis [6, 7]. Recently, a *P. falciparum* neutral SMase (PfNSM) gene encording a 46-kDa protein identical to that of bacterial SMases was identified [8]. In the present work, we investigated the role of ceramide in the death of *P. falciparum* parasites.

Different extracellular stimuli cause hydrolysis of SM, generating ceramide and cell death that is accompanied by a decrease in glutathione levels in tumour cells [9]. Recent reports demonstrate that a decrease in intracellular glutathione mediates ceramide-induced oncotic necrosis [9] and apoptosis [10].

* Corresponding author.

In this study, we demonstrated that ceramide has a malaricidal effect. Furthermore, artemisinin and mefloquine, drugs whose mechanism of action is not completely understood, induce the generation of ceramide via SMase, thereby inhibiting the growth of *P. falciparum* parasites. Finally, ceramide-induced inhibition of *P. falciparum* growth is mediated by a decrease in parasite glutathione levels.

Materials and methods

Materials

Artemisinin, N-acetyl cysteine (NAC), reduced glutathione (GSH), 5,5′-dithio-bis(2-nitrobenzoicacid) (DTNB), glutathione reductase from baker's yeast (EC 1.6.4.2), β-NADPH and SMase from *Staphylococcus aureus* were obtained from Sigma (St. Louis, Mo.). Mefloquine was a kind gift from Hoffmann-La Roche (Basel, Switzerland). Artemisinin was dissolved in ethyl acetate. A membrane-permeable analogue of ceramide C_6 was obtained from Calbiochem-Novabiochem (La Jolla, Calif.). BODIPY FL C12-sphingomyelin was obtained from Molecular Probes (Eugene, Ore.). (2-isopropyl-1-((4-(3-N-methyl-N-(3,4-dimethoxy-β-phenethyl)amino) propyloxy)benzenesulfonyl))-indolizine (SR 33557) was a kind gift from Sanofi Recherche (Montpellier, France). Streptolysin O (SLO) was supplied by Dr. S. Bhakdi (Mainz, Germany). N-butyl sphingosyl phosphoryl choline (AD 2482) was synthesized as follows: condensation of sphingosyl phosphoryl choline and n-butyraldehyde in methanol : water, 8 : 12 v : v, followed by reduction for 12 h with sodium borohydride. Purification was performed on a silica gel column. The compound was loaded in dichloromethane and eluted with increasing ratios of methanol and dichloromethane. The compound was viewed by thin layer chromatography and determined to be of at least 95% purity.

Parasite culturing and cytotoxicity assay

The FCR-3 strain of *P. falciparum* parasites was cultured asynchronously in vitro as described by Trager and Jensen [11]. Parasites were grown in RPMI-1640 with 10% human plasma, 25 mM $NaHCO_3$ and 25 mM HEPES, displaying a haematocrit of 2–4% [12], under an atmosphere of 3.5% CO_2, 3.5% O_2 and 93% N_2 [13–15]. Parasite growth and drug cytotoxicity were followed for 6 h and were determined by percentage of infected erythrocytes (parasitaemia) as monitored by observation of Giemsa-stained smears [16–18]. The parasitaemia in control (untreated) parasites was 15–30%. This technique allows the detection of parasite growth inhibition within a few hours. This short-term culture system enabled focusing on early biochemical processes leading to parasite growth inhibition.

Enzyme assays

P. falciparum-infected red blood cells (iRBCs), 2×10^8/ml, were exposed for 5–60 min to artemisinin or mefloquine, each at 100 nM. In the assay for neutral SMase [19], each reaction tube contained 25 µl BODIPY FL C_{12}-SM (0.08 nmol/µl, fluorescent SM) in 10 mM $MgCl_2$ and 0.5% Triton X-100 (v/v). After exposure to drugs, iRBCs were lysed in 10 vol of hypotonic buffer (10 mM NaCl/0.3 mM KCl/1 mM Na_2HPO_4/0.2 mM KH_2PO_4, pH 7.2) [20] and centrifuged at 20,000 g for 5 min. The pellet was dissolved in 25 µl of 250 mM HEPES/NaOH buffer, pH 7.5, and 25 µl of BODIPY FL C_{12}-SM and incubated overnight at 37°C. Fifty microlitres of chloroform and 50 µl of methanol were added, and the reaction mixture vortexed vigorously and centrifuged at 1500 g for 5 min at room temperature. The lower phase was transferred to a new tube and dried under N_2. Twenty microlitres of chloroform was added to the lipid film, vortexed and loaded on a lane of a TLC plate, LK6D silica gel 60 A (Whatman, Clifton, N. J.). TLC plates were run using a solvent system of chloroform:methanol (9 : 1). The TLC plates were viewed under UV illumination and band net intensity (in arbitrary units) was determined with the aid of Kodak Digital Science 1D software (Kodak, Rochester, N. Y.). The assay is based on the generation of fluorescent ceramide from fluorescent SM, as a result of SMase activity.

For assay of acid SMase [19], each reaction tube contained 25 µl BODIPY FL C_{12}-SM (0.08 nmol/µl) in 0.2 mM buffer acetate, pH 4.5, and 1% Triton X-100 (v/v). SLO was used to selectively permeabilise the plasma membrane of iRBCs while leaving the parasitophorous vacuolar membrane (PVM) intact [21]. Following exposure to drugs, 2×10^8 iRBCs/ml were resuspended in 200 µl RPMI and 24 µl of 90 ng/µl SLO, and mixed immediately. The mixture was incubated for 10 min at 37°C, shaken gently and centrifuged for 4 min at 3200 g. The pellet was washed with RPMI containing a protease inhibitor cocktail and centrifuged for 4 min at 3200 g. Twenty-five microlitres of PBS and 25 µl of BODIPY FL C_{12}-SM solution were added to the pellet and incubated overnight at 37°C. Fifty microlitres of chloroform and 50 µl of methanol were added to the reaction mixture, and then the assay was performed as described for neutral SMase.

Ceramide determination [22]

iRBCs, 2×10^8/ml, were exposed to artemisinin or mefloquine, at 100 nM each, for 5 min – 2 h. Following exposure, iRBCs were lysed in 10 vol hypotonic buffer (10 mM NaCl/0.3 mM KCl/1 mM Na_2HPO_4/0.2 mM KH_2PO_4, pH 7.2) [20] and centrifuged at 1500 g for 5 min. The pellet was resuspended in 1 ml 'kill solution' ($CHCl_3$/MeOH/1N HCl, 500 : 500 : 5), 270 µl BSS (135 mM NaCl/4.5 mM KCl/1.5 mM $CaCl_2$/0.5 mM $MgCl_2$/6.5 mg

glucose/10 mM HEPES, pH 7.2) and 30 µl of 100 mM EDTA, vortexed, and centrifuged at 450 g for 5 min at room temperature. Four hundred microlitres of the lower organic phase was transferred to a new glass tube and dried under N_2. Five hundred microlitres of 0.1 M KOH/MeOH was added to the lipid film and the mixture was incubated for 1 h at 37 °C, then shaken and covered with parafilm. Ceramide standards were included from this step onwards. Five hundred microlitres of $CHCl_3$, 270 µl BSS and 30 µl 100 mM EDTA was added to each tube, then vortexed and centrifuged at 450 g for 5 min. Five hundred microlitres of the lower organic phase was transferred to a new glass tube and dried under N_2. The following mixture, per sample, was prepared: 26 µl of cardiolipin (25 mg/ml) and DETAPAC (1 mM), 6.2 µl of octyl-β-D-glucopyranoside (825 mM/ddH_2O), 50 µl of 2 × reaction buffer (100 mM NaCl, 100 mM imidazole, 2 mM EDTA, 25 mM $MgCl_2$, pH 6.5), 8 µl of imidazole/DETAPAC (10 mM/1 mM), 2 µl of 100 mM DTT, 1 µl of 100 mM ATP, 3.3 µl of ddH_2O and 3.5 µl of 1 mg/ml DG kinase (Calbiochem). The mixture was then vortexed and incubated at room temperature for 30 min. One microlitre of [γ-^{32}P]ATP (NEN, Boston, Mass.) was added, vortexed and 100 µl of the mixture was added to each tube and incubated for 30 min at room temperature. The reaction was stopped and ceramide-1-phosphate was extracted by adding 1 ml 'kill solution', 170 µl BSS and 30 µl of 100 mM EDTA. Tubes were vortexed and centrifuged at 450 g. Five hundred to 550 µl of the lower phase was transferred to a new glass tube and dried under N_2. Lipid films were resuspended in 50 µl $CHCl_3$:MeOH (1:1) and 40 µl of the suspension was analysed by TLC. TLC plates were run using a solvent system of $CHCl_3$, MeOH and HAc (65:15:5). The plates were exposed and radioactive bands corresponding to ceramide-1-phosphate were analysed by phosphorimager (Molecular Dynamics, Piscataway, N. J.). The level of ceramide was determined by comparison with a standard curve.

Determination of parasite glutathione levels [23]

iRBCs, 2 × 10^8/ml, were exposed to ceramide, at 10–80 µM, and to artemisinin and mefloquine at 0.01–10,000 nM for 30 min to 3 h. Following exposure, parasites were isolated by suspending iRBCs in a 20-fold volume of lysis buffer (7 mM K_2HPO_4/1 mM NaH_2PO_4/11 mM $NaHCO_3$/58 mM KCl/56 mM NaCl/1 mM $MgCl_2$/14 mM glucose/0.02% saponin) for 10 min at 37 °C. The pellet was washed three times and resuspended in 150 µl of the same lysis buffer and disrupted by three cycles of freezing and thawing [24]. Protein concentration of the supernatant was determined by BioRad protein dye assay. For the glutathione determination, 40 µl of supernatant was deproteinised with 2 vol 5% sulphosalicylic acid. The mixture was vortexed and centrifuged at 10,000 g for 20 min at 4 °C. The glutathione content was measured by the glutathione reductase-coupled DTNB-GSH-recycling assay [23].

Methods for detection of apoptosis

iRBCs were exposed to antimalarial drugs (100 nM) and ceramide (40 µM), and the following assays were performed.

Terminal deoxynucleotidyl transferase DNA fragmentation assay

All procedures were performed according to the Oncogene Research Products (Cambridge, Mass.) protocol [25].

Gel electrophoresis for detection of DNA laddering

This technique was modified according to Higuchi et al. [26]. iRBCs were lysed by suspension in 0.013% saponin to yield free parasites. These were resuspended in digestion buffer (100 mM NaCl/10 mM Tris, pH 8/25 mM EDTA/0.5% SDS). After incubation with 2.5 µl proteinase K (20 mg/ml) at 55 °C for 2 h, 2.5 µl proteinase K were added and incubation continued overnight at 37 °C. TE buffer (1M Tris-HCl/0.5M EDTA, pH 8) was added to the DNA for an incubation period of 1 h at 37 °C. The extracted DNA was resolved on a 1.2% agarose gel.

Determination of apoptosis by electron microscopy [27]

iRBCs were washed twice in PBS, resuspended in Karnovsky's fixative (1% glutaraldehyde/3% paraformaldehyde in cacodylate buffer, pH 7.4) for 1 h at room temperature, and washed in PBS. Fixed cells were diluted in PBS, centrifuged for 5 min at 1000 g and the upper phase was discarded. The pellet was resuspended in 2% OsO_4 and 0.2 M cacodylate buffer (1:1 v/v) and post-fixed for an h at 4 °C. Samples were washed twice for 10 min in cacodylate buffer and dehydrated by sequential incubation, for 10 min each, in 30, 50, 70 and 90% ethanol, and twice for 10 min each in 100% ethanol. After additional incubation for 10 min in propylene oxide, the samples were embedded in Araldite. Thin sections were prepared by using a LKB Ultratome, stained by 5% oranyl acetate and lead acetate, and examined in a JEON-100 CX transmission electron microscope at 80 KV.

Caspase 3 assay [28, 29]

All procedures were performed according to the PharMingen (San Diego, Calif.) protocol.

Statistical analysis

The statistical significance of the results was determined, where appropriate, by two-sided Student's t test. Results are presented as means ± SD of three replicated experiments.

Results

Inhibition of *P. falciparum* growth by ceramide

Ceramide can mediate the biological effects of anticancer chemotherapy and radiation [3]. Cell-permeable short-chain ceramide (C_6) was used to investigate the role of ceramide in *P. falciparum* death. Exogenously added ceramide at concentrations of 10–80 µM exhibited a dose-dependent cytotoxic effect (expressed as growth inhibition) on *P. falciparum* (fig. 1).

Inhibition of *P. falciparum* growth by SMase

The enzyme responsible for ceramide generation from sphingomyelin is SMase [6]. SMase from *S. aureus* was added exogenously and exhibited a dose-dependent growth inhibition effect on *P. falciparum* (fig. 2). These results suggest that the exogenous SMase degraded membrane SM, and the ceramide thus produced initiated a signalling pathway culminating in the inhibition of *P. falciparum* parasite growth. The concentrations of SMase employed in these experiments did not cause erythrocyte haemolysis.

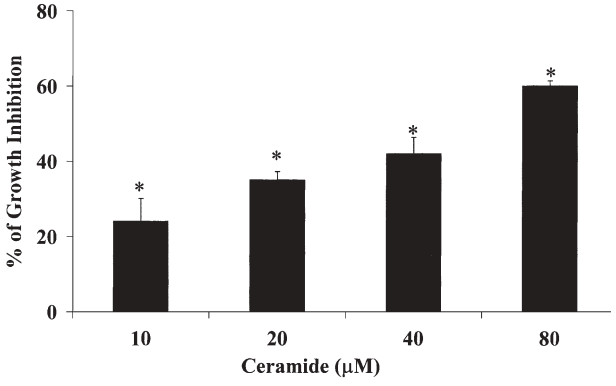

Figure 1. Effect of exogenously added ceramide on *P. falciparum*. *P. falciparum* parasites were exposed to ceramide (C_6) for 6 h. Ceramide exhibited a dose-dependent antimalarial effect, significant at *p < 0.05. Parasitemia in control (untreated) cultures was 27%.

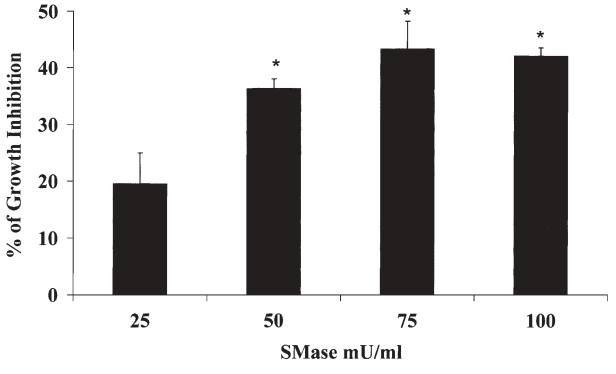

Figure 2. Effect of exogenously added SMase on *P. falciparum. P. falciparum* parasites were incubated with bacterial SMase at the indicated concentrations. SMase exhibited an antimalarial effect, significant at *p < 0.05. Parasitemia in control (untreated) cultures was 20%.

Prevention of ceramide-induced toxicity for *P. falciparum* parasites by sphingosine-1-phosphate

Sphingosine-1-phosphate (SPP), a metabolite of the sphingomyelin pathway and a biological antagonist of ceramide action, prevents ceramide-induced death in mammalian cells [30]. To determine whether SPP can prevent the killing effect of ceramide, *P. falciparum* parasites were exposed to ceramide with or without SPP at 10 µM (a non-toxic concentration for the parasites). The cytotoxic effect of ceramide (40% growth inhibition) was significantly reduced by SPP to approximately 10% growth inhibition ($p < 0.05$).

Decrease in *P. falciparum* glutathione levels mediates ceramide-induced parasite death

Hydrolysis of SM induced by extracellular stimuli results in a decrease in glutathione levels and death of cancer cells [9, 10]. In the present study, we found that ceramide added exogenously induced a dose- (fig. 3A) and time- (fig. 3B) dependent decrease in glutathione levels in *P. falciparum* parasites. NAC a precursor of glutathione and an antioxidant, at 1 mM (non-toxic for the parasites) significantly reduced the growth inhibition effect of ceramide. NAC alone had no effect on *P. falciparum* growth. Parasites were incubated with NAC for 2 days be-

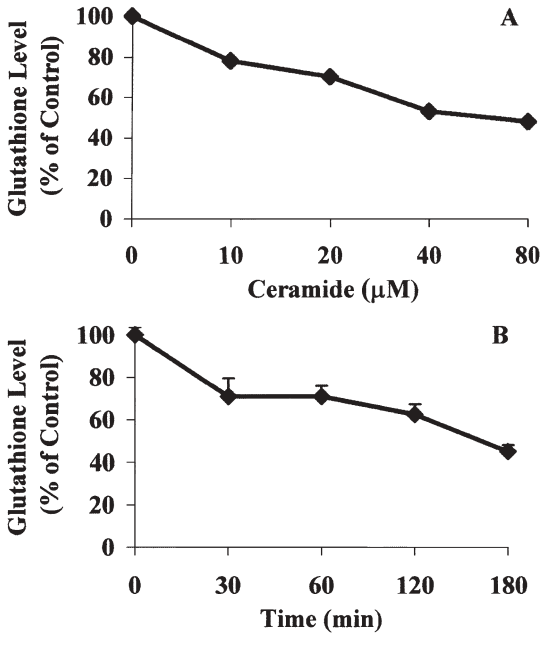

Figure 3. Effect of ceramide on parasite glutathione levels. (*A*) Exogenously added ceramide, from 10 to 80 µM, induced a dose-dependent decrease in glutathione levels after 3 h incubation. (*B*) Ceramide was added at 40 µM and produced a time-dependent depletion of glutathione. The basal level of glutathione in untreated parasites was 12.8 nmol/mg protein.

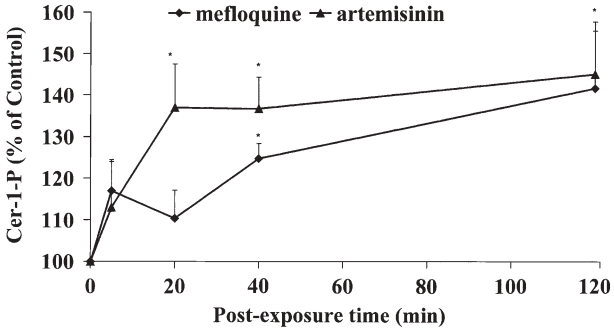

Figure 4. Intracellular ceramide levels in *P. falciparum* parasites exposed to artemisinin and mefloquine. *P. falciparum* parasites were treated with artemisinin or mefloquine at 100 nM each for the indicated times. Drug treatment induced a significant rise in cellular levels of ceramide in treated (*$p < 0.05$) versus untreated parasites. The basal level of ceramide in *P. falciparum* parasites was 650 pmol/2 × 10^8 iRBCs.

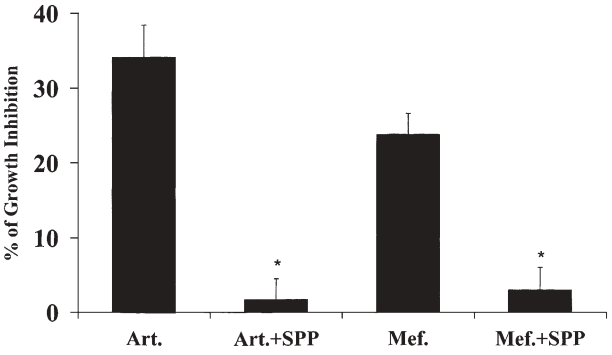

Figure 5. Effect of SPP on artemisinin- and mefloquine-induced death of *P. falciparum*. SPP (10 µM) was added 2 h before the antimalarial drugs in a serum-free medium. *P. falciparum* parasites were cultured with artemisinin (Art.) or mefloquine (Mef.) at 100 nM for 6 h. SPP prevented the killing effect of the antimalarials (*$p < 0.05$). Parasitaemia in control (untreated) cultures was 27%.

fore incubating with ceramide (6 h) and NAC was replenished daily because of its lability [31, 32]. The cytotoxic effect of ceramide (36% growth inhibition) was significantly reduced by NAC to approximately 5% growth inhibition ($p < 0.005$).

These results show for the first time that ceramide can induce growth inhibition of *P. falciparum*, the causative agent of pernicious malaria.

Next, we evaluated the importance of ceramide in the inhibition of *P. falciparum* growth induced by antimalarial drugs.

Induction of elevated ceramide levels in *P. falciparum* parasites by artemisinin and mefloquine

Two antimalarial drugs, artemisinin and mefloquine (at 100 nM, a non-haemolytic concentration) were used in this study as models to analyse whether ceramide mediates drug-induced cytotoxic effects. This concentration of antimalarial drugs produced approximately 30–35% cytotoxicity in the parasites. These levels of cytotoxicity of antimalarials are in line with those reported by other investigators [33]. Parasites were treated with antimalarial drugs and the intracellular levels of ceramide were measured. Artemisinin and mefloquine induced a significant rise in cellular ceramide levels ($p < 0.05$) (fig. 4).

Inhibition of the antimalarial effect of artemisinin and mefloquine by SPP

To find out whether the ceramide antagonist SPP can inhibit the killing effect of antimalarial drugs, *P. falciparum* parasites were exposed to artemisinin and mefloquine alone or with 10 µM SPP. The antimalarial effect of artemisinin and mefloquine was indeed abolished by SPP (fig. 5), strongly suggesting that ceramide is involved in the growth inhibition effect of these drugs.

Inhibitors of de novo synthesis of ceramide: lack of effect on the antimalarial action of artemisinin and mefloquine

The first potential source of ceramide in *P. falciparum* parasites we considered was de novo synthesis [34]. Two specific inhibitors of de novo synthesis of ceramide were used: L-cycloserine and fumonisin B1 [35, 36]. They were added 24 h before addition of the antimalarial drugs, to inhibit sphingolipid biosynthesis. L-cycloserine at 0.025 mM and fumonisin B1 at 10 µM, the maximal non-toxic concentrations for *P. falciparum*, had no effect on the inhibition of parasite growth by artemisinin and mefloquine (data not shown).

Suppression of the growth inhibitory effect of antimalarials by inhibitors of SMase activity

A second potential source of ceramide in *P. falciparum* parasites is hydrolysis of SM by SMases. To investigate whether the activity of acid SMase, neutral SMase, or both, is responsible for the killing effect of antimalarial drugs, two inhibitors of SMase activity were used. SR 33557 is a specific inhibitor of acid SMase with no effect on neutral SMase [37], while AD 2482, an analogue of sphingomyelin, is an inhibitor of both acid and neutral SMase [S. Gatt and A. Dagan, unpublished data]. SR 33557 at 2.5 µM and AD 2482 at 50 µM (concentrations devoid of any effects on parasite growth) nearly abolished the killing activity of artemisinin and mefloquine (fig. 6). These findings suggest that the antimalarial effect of both drugs is dependent on acid SMase activity.

Inhibition of *P. falciparum* growth by artemisinin and mefloquine: mediation by decrease in parasite glutathione levels

Since our results suggest that a decrease in the glutathione levels mediates ceramide-induced inhibition of *P. falciparum* growth (see above and fig. 3), parasites

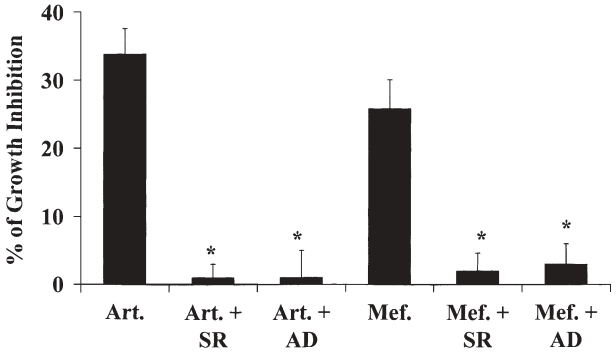

Figure 6. Effect of the SMase inhibitors, SR 33557 and AD 2482, on the antimalarial effect of artemisinin and mefloquine. Parasites were exposed to SR 33557 (2.5 µM, SR) and AD 2482 (50 µM, AD) for 30 min before the addition of the antimalarials. Artemisinin (Art.) and mefloquine (Mef.) were added at 100 nM and present in culture for 6 h. SR 33557 and AD 2482 abolished the killing effect of the antimalarials. * $p < 0.05$. Parasitaemia in control cultures was 25%.

were treated with the antimalarial drugs, and the level of glutathione was measured. Artemisinin and mefloquine at 100 nM decreased the level of glutathione to a similar extent up to 30 min. Artemisinin further decreased glutathione levels up to 180 min (fig. 7A). Both drugs decreased the level of glutathione dose dependently, the effect of artemisinin at 100 nM being significantly ($p < 0.01$) stronger than that of mefloquine at the same concentration (fig. 7B). Following this, artemisinin and mefloquine were added to the parasites together with the SMase inhibitors SR 33557 (2.5 µM) and AD 2482 (50 µM), and the glutathione level was measured. The SMase inhibitors prevented the decrease in glutathione levels caused by the antimalarial drugs (fig. 7C). The addition of SR 33557 and AD 2482 alone had no effect on the glutathione levels in the parasites. NAC, a precursor of glutathione, at 1 mM (a concentration devoid of any effect on parasite growth) abolished the cytotoxic effect of artemisinin and mefloquine (fig. 7D). For these experiments, antimalarial drugs were added with or without NAC for 6 h.

These results suggest that the decrease in glutathione levels caused by artemisinin and mefloquine is caused by SMase activity, and is essential for their inhibition of parasite growth.

Activation of acid and neutral SMases by artemisinin and mefloquine

To measure directly the activity of neutral and acid SMase, following the addition of artemisinin and mefloquine, *P. falciparum* parasites were exposed to the antimalarial drugs for 5–60 min (5 min was the minimal technically feasible time for exposure to the drug). Our results demonstrated that artemisinin is a stronger activator than mefloquine of acid SMase (fig. 8A). Activation of the enzyme by artemisinin peaked at 40 min, while activation of acid SMase by mefloquine already declined after 5 min. Figure 8B shows that mefloquine and artemisinin increased the activity of neutral SMase to a similar extent up to 40 min of exposure, but the effect of mefloquine further increased significantly ($p < 0.005$) at 60 min. Uninfected erythrocytes, either untreated or treated with the antimalarials, did not exhibit any SMase activity, acidic or neutral.

Ceramide does not induce apoptosis in *P. falciparum* parasites

Ceramide has been implicated as a second messenger in activating apoptotic cascades in cancer cells [5]. We hypothesized that artemisinin and mefloquine may induce killing of *P. falciparum* parasites through ceramide-induced apoptosis. Thus, we employed four methods to detect apoptosis: the TdT DNA fragmentation assay, DNA laddering, determination of apoptosis by electron microscopy and caspase-3 assay. We found that *P. falciparum* parasites, exposed to ceramide and antimalarial drugs, failed to exhibit characteristic apoptosis, as determined by these methods (data not shown). Our results suggest that ceramide and the antimalarial drugs used by us induce non-apoptotic death of *P. falciparum* parasites.

Discussion

In this study, we found that: (i) exogenously added ceramide and SMase exhibited a dose-dependent cytotoxic effect on *P. falciparum* parasites; (ii) ceramide decreased parasite glutathione levels; (iii) NAC, the precursor of glutathione, inhibited the antimalarial effect of ceramide; (iv) artemisinin and mefloquine increased the cellular levels of ceramide and enhanced the activity of SMase; (v) accordingly, inhibitors of SMase activity and of ceramide bioactivity inhibited the antimalarial effect of artemisinin and mefloquine; (vi) inhibitors of de novo synthesis of ceramide had no effect on the cytotoxic effect of the two antimalarial drugs; (vii) artemisinin and mefloquine induced a decrease in parasite glutathione levels; (viii) NAC inhibited the cytotoxicity of artemisinin and mefloquine and (ix) SMase inhibitors prevented the decrease in glutathione levels induced by the drugs.

Plasmodium, the malaria-causing parasite, generates large amounts of toxic ferriprotoporphyrin IX (FP) during digestion of acquired haemoglobin. About 30% of the toxic FP is detoxified by polymerisation to haemozoin [38], while the rest has to be degraded to ensure parasite survival. FP can exit the food vacuole and be degraded by cytosolic glutathione [39, 40]. FP degradation has also been suggested to occur inside the food vacuole, by reacting with hydrogen peroxide [41]. In the present study,

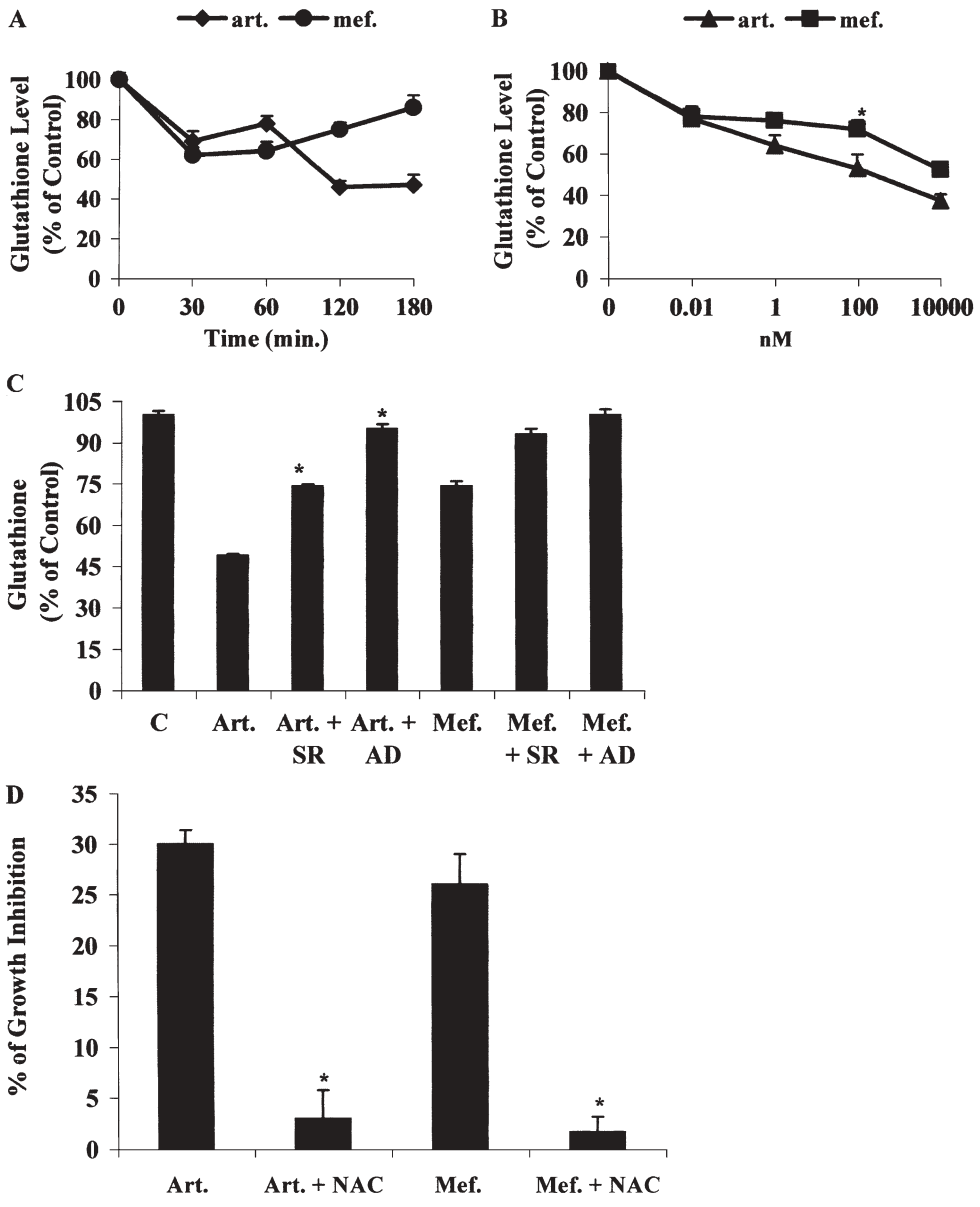

Figure 7. Depletion of parasite glutathione by artemisinin and mefloquine. (*A*) Artemisinin and mefloquine were added at 100 nM and induced a time-dependent decrease in glutathione levels. (*B*) Artemisinin and mefloquine produced dose-dependent inhibition of intracellular glutathione levels during a 2-h incubation period. The basal level of glutathione in control (untreated) parasites was 12.8 nmol/mg protein. (*C*) Parasites were exposed to SR 33557 (2.5 µM, SR) and AD 2482 (50 µM, AD) for 30 min before the addition of antimalarial drugs. Artemisinin (Art.) and mefloquine (Mef.) were added at 100 nM and incubation lasted for 2 h. Both SMase inhibitors markedly decreased the depletion of glutathione (* $p < 0.05$). (*D*) *P. falciparum* parasites were incubated with NAC (1 mM) for 48 h before addition of the drugs. Artemisinin (Art.) and mefloquine (Mef.) were present, at 100 nM, for 6 h. NAC abolished the cytotoxic effect of artemisinin and mefloquine (* $p < 0.05$). Parasitaemia in control (untreated) cultures was 22%.

we demonstrated that ceramide induces a dose- and time-dependent decrease in glutathione levels. NAC, a precursor of glutathione and an antioxidant, was employed to identify whether the decrease in glutathione level mediates the antimalarial effect of ceramide. NAC indeed abolished ceramide-induced cytotoxicity, suggesting that ceramide mediates death of *P. falciparum* parasites by decreasing glutathione levels. By this decrease, ceramide removes a physiological defence mechanism of the parasite towards toxic FP.

The involvement of FP degradation in the mechanism of action of various antimalarials has been investigated. Chloroquine was found to prevent FP degradation in the presence of glutathione [39] and hydrogen peroxide [41]. Thus, sufficiently high glutathione levels were suggested to protect plasmodia parasites against chloroquine and

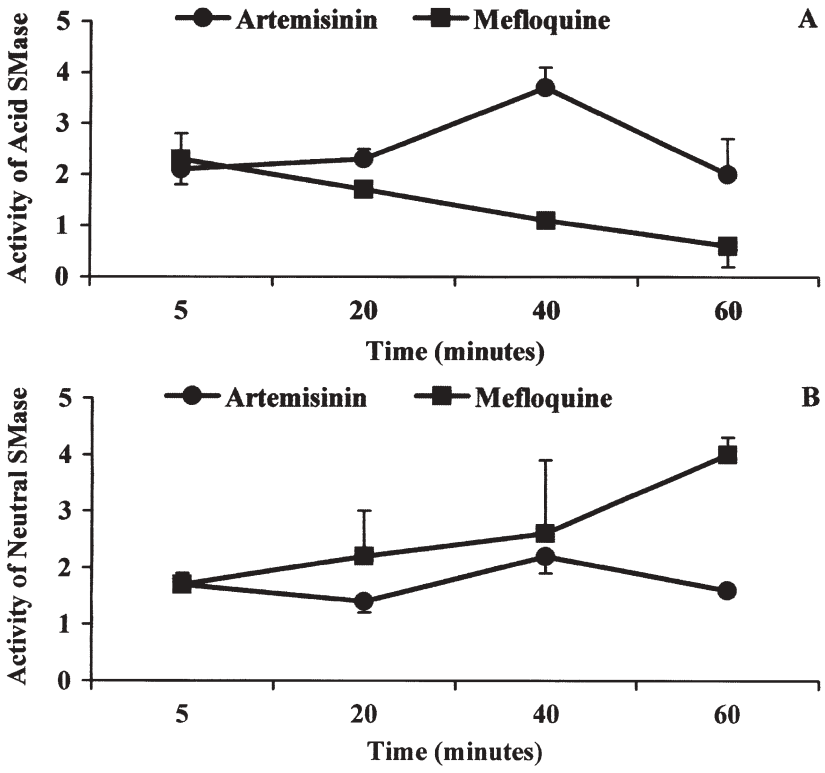

Figure 8. The activity of SMase in *P. falciparum* parasites treated with artemisinin and mefloquine. *P. falciparum* parasites were exposed to artemisinin and mefloquine, both at 100 nM, for 5–60 min. (*A*) Activity of acid SMase. The basal level of acid SMase (in untreated parasites) was 1.6 pmol/h per milligram protein and the results shown were calculated as the product of the activity divided by the basal level. Drug treatment enhanced the enzymatic activity significantly ($p < 0.05$), except for mefloquine at 60 min. (*B*) Activity of neutral SMase. The basal level of neutral SMase (in untreated parasites) was 10.3 pmol/h per milligram protein and the results shown were calculated as the product of the activity divided by the basal level. Drug treatment enhanced the enzymatic activity significantly ($p < 0.05$).

may thus be implicated in the development of chloroquine resistance [42]. On the other hand, artemisinin is incapable of inhibiting glutathione-mediated FP degradation [39], whereas mefloquine and quinine can prevent glutathione-mediated FP degradation in aqueous solution but not in association with membranes, where FP tends to dissolve easily and accumulate [40]. Furthermore, degradation of FP by glutathione occurs while it is still bound to the membrane, and not in aqueous medium following the dissociation of FP from the membrane. Thus, the ability of certain drugs to prevent glutathione-mediated FP degradation in aqueous solution is of no practical pharmacological relevance. Since our findings suggest that artemisisnin and mefloquine induce inhibition of *P. falciparum* growth via reduction in glutathione levels, and these drugs do not prevent glutathione-associated degradation of FP accumulated in membrane [39], we propose that the decrease in glutathione caused by these drugs mediates parasite death via a different mechanism, e. g. induction of oxidative stress.

Mechanisms of action have already been described for mefloquine and artemisinin. Mefloquine was suggested to accumulate in the acid vacuole of parasites and to prevent FP polymerization, resulting in the build-up of the toxic haem moiety, thereby inducing the death of the *P. falciparum* parasites [43]. Chloroquine accumulates in the food vacoule of the parasite, binds to FP, inhibits its polymerisation and causes a dose-dependent accumulation of FP in the membrane fraction of infected cells [44]. Artemisinin reacts with glutathione resulting in oxidised glutathione and the antimalarial action of artemisinin is antagonised by glutathione [45]. An additional mechanism of action has been proposed, i. e. artemisinin inhibits the haemoglobin degradation pathway and the haem detoxification system, and dose dependently destroys the malarial pigment [46]. Through another potential mechanism, artemisinin can alter membrane protein export from the vacuolar network and has an effect on the PVM and the tubolovesicular membrane (TVM) [47].

In this study, we demonstrated that inhibitors of SMase activity (SR 33557 and AD 2482) almost abolished the growth inhibitory effect of the antimalarial drugs used by us and inhibited the decrease in intracellular glutathione. Our findings suggest that ceramide generated by SMase action mediates the antimalarial effect of artemisinin and mefloquine by depletion of parasite glutathione. Nevertheless, our proposed mechanisms and those previously described are not necessarily mutually exclusive. SMase

activation may be a death-signalling event induced by different alterations in the cell functions that were mentioned above [43–47]. This signalling event, measurable within minutes following initiation of drug treatment, results in a decrease in glutathione levels, and eventually leads to a significant decrease in the number of parasites. Interestingly, in a different system (rat liver), mefloquine also decreases the GSH concentration significantly [48].

Mature uninfected erythrocytes have no intracellular organelles, lack nuclei and are therefore incapable of de novo synthesis of lipids or proteins [20, 49, 50]. One possible source of ceramide may be de novo synthesis by intraerythrocytic stages of *P. falciparum* parasites [34]. However, we showed that inhibitors of de novo synthesis of ceramide had no effect on the growth-inhibitory activity of artemisinin and mefloquine, suggesting that this pathway is not involved in their antimalarial effect. Another possible alternative source for ceramide is the hydrolysis of host and/or parasite SM by SMase. A recent study showed that *P. falciparum*-infected erythrocytes exhibit neutral SMase activity (EC 3.1.4.12; Mg^{2+} dependent and membrane bound), but not acid SMase activity [51]. However, in the present study, we established the existence of both acid and neutral SMase activities. To measure acid SMase, we treated the parasites with SLO, which releases the erythrocyte cytosol and leaves the PVM [21] and TVM [K. Lingelbach, personal communication] intact. *P. falciparum*-infected, but not uninfected, erythrocytes convert radiolabelled or fluorescent ceramide precursors into SM, providing evidence for the existence of parasite SM synthase [20, 48, 49, 52, 53]. *P. falciparum* parasites have at least two distinct forms of SM synthase, one of which is localised in the Golgi complex, and another, unique to *P. falciparum*, in the TVM [20]. Localisation of acid SMase in the TVM of the parasites (where the resynthesis of SM could also occur) [20] could explain the difference between our results and those of Hanada et al. [51], who isolated the parasites from the erythrocytes using a detergent which disintegrates biological membranes [21].

The rise in ceramide levels (fig. 4) may appear rather limited. Nevertheless, a similar rise of approximatly 25–50% in ceramide cellular concentrations mediates a death signal in U937 cells treated with daunorubicin [54] and in Molt-4 cells treated with PSC 833 [55]. Ceramide is a second messenger in cellular signalling. Accordingly, small quantities (possibly present in specific compartments) are sufficient to transmit the death signal.

Classically, apoptosis has been linked to the appearance of multicellular organisms. Recently, however, apoptosis has also been described in unicellular organisms, e.g. *Trypanosoma cruzi* [56], *T. brucei rhodesiense* [57] and some bacteria [58]. Since ceramide mediates the therapeutic effects of anticancer chemotherapy and radiation, and induces apoptosis [4, 5], we suspected that ceramide and antimalarial drugs kill *P. falciparum* parasites by an apoptotic mechanism. However, various assays employed by us did not reveal any biochemical or morphological apoptotic changes [59]. These results suggest that ceramide, artemisinin and mefloquine induce death of *P. falciparum* parasites by a non-apoptotic mechanism, similar to ceramide-induced killing of normal human T lymphocytes [60], malignant and normal B lymphocytes [61], JB6 tumour cells [8] and hepatocytes [62]. Nevertheless, since apoptosis in *P. falciparum* has not yet been really demonstrated, there is doubt concerning the drawn conclusions.

In summary, our study shows that production of intraparasitic ceramide can induce a decrease in parasite glutathione levels, resulting in inhibition of *P. falciparum* growth. Our results further suggest that this mechanism can mediate the antimalarial activity of artemisinin and mefloquine.

Acknowledgements. This work was supported by the Singer and Finghurt Foundations, Israel (E. F.), and by the Constantiner Institute, Israel (I. P.-K.). The authors are grateful to Prof. J. Golenser for providing the FCR-3 strain of *Plasmodium falciparum* and for his advice on its cultivation.

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