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Oxidative stress-mediated antimalarial activity of plakortin, a natural endoperoxide from the tropical sponge Plakortis simplex

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Keywords: Plakortin Endoperoxide 4-hydroxynonenal ROS Antimalarial drug Artemisinin resistance Plasmodium falciparum

Abstract
Plakortin, a polyketide endoperoxide from the sponge Plakortis simplex has antiparasitic activity against P. falciparum. Similar to artemisinin, its activity depends on the peroxide functionality. Plakortin induced stage-, dose- and time-dependent morphologic anomalies, early maturation delay, ROS generation and lipid peroxidation in the parasite. Ring damage by 1 and 10 mM plakortin led to parasite death before schizogony at 20 and 95%, respectively. Treatment of late schizonts with 1, 2, 5 and 10 mM plakortin resulted in decreased reinfection rates by 30, 50, 61 and 65%, respectively. In both rings and trophozoites, plakortin induced a dose- and time-dependent ROS production as well as a significant lipid peroxidation and up to 4-fold increase of the lipoperoxide breakdown product 4-hydroxynonenal (4-HNE). Antioxidants and the free radical scavengers trolox and N-acetylcysteine significantly attenuated the parasite damage. Plakortin generated 4-HNE conjugates with the P. falciparum proteins: heat shock protein Hsp70-1, endoplasmatic reticulum-standing Hsp70-2 (BiP analogue), V-type proton ATPase catalytic subunit A, enolase, the putative vacuolar protein sorting-associated protein 11, and the dynein heavy chain-like protein, whose specific binding sites were identified by mass spectrometry. These proteins are crucially involved in protein trafficking, transmembrane and vesicular transport and parasite survival. We hypothesize that binding of 4-HNE to functionally relevant parasite proteins may explain the observed plakortin-induced morphologic aberrations and parasite death. The identification of 4-HNE-protein conjugates may generate a novel paradigm to explain the mechanism of action of pro-oxidant, peroxidebased antimalarials such as plakortin, artemisinins and synthetic endoperoxides.

Introduction
Falciparum malaria still is one of the most widespread infectious diseases. The disease is caused by protozoan parasites of the genus Plasmodium that infect and destroy red blood cells (RBCs), leading to fever and in case of severe courses of disease, to severe anemia, respiratory distress, cerebral malaria and if untreated, death. According to WHO (Factsheet, December 2014) in 2013 there were an estimated 124–283 million cases of malaria worldwide, and an estimated 584,000 deaths. Approx. 90% of all Contents lists available at ScienceDirect journal homepage: www.elsevier.com/locate/freeradbiomed Free Radical Biology and Medicine
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Abbreviations: ARTEs, semisynthetic derivatives of artemisinin; DCF-DA, dichlorofluorescein-diacetate; DHE, dihydroethidium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EtBr, ethidium bromide; FACS, fluorescence-activated cell sorting; GM, growth medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hsp, heat shock protein; 4-HNE, 4-hydroxynonenal; MFI, mean fluorescence intensity; NAC, N-acetylcysteine; PBS, phosphate buffered saline; RBC, red blood cell; npRBC, non-parasitized RBC; pRBC, parasitized RBC; P. falciparum, malaria parasite Plasmodium falciparum; RPMI medium, Roswell Park Memorial Institute medium; SDSPAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; WM, wash medium. n Correspondence to: University of Torino, Department of Oncology, Torino, Italy, Via Santena 5 bis, 10126 Torino, Italy. Tel.: þ39 011 670 5846; fax: þ39 011 670 5845. E-mail addresses: olexii.skorokhod@unito.it (O.A. Skorokhod), denise.davalosschafler@unito.it (D. Davalos-Schafler), valentina.gallo@unito.it (V. Gallo), elena.valente@unito.it (E. Valente), daniela.ulliers@unito.it (D.

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Ulliers), agata.notarpietro@unito.it (A. Notarpietro), giorgia.mandili@unito.it (G. Mandili), franco.novelli@unito.it (F. Novelli), m.persico@unina.it (M. Persico), orazio.taglialatelascafati@unina.it (O. Taglialatela-Scafati), paolo.arese@unito.it (P. Arese), evelin.schwarzer@unito.it (E. Schwarzer). 1 These authors contributed equally to this work. Free Radical Biology and Medicine 89 (2015) 624–637 malaria deaths occur in Africa, where in 2013 an estimated 437,000 children died of malaria before their fifth birthday. The absence of an effective vaccine and expanding resistance against the nowadays almost exclusively used potent antimalarial artemisinin (Fig. 1) and its semisynthetic derivatives (ARTEs) [1–3], underline the urgent need for new drugs in order to achieve eradication [4]. Artemisinin is a sesquiterpene lactone with a peroxide bridge essential for antimalarial activity [5]. In the search for novel antimalarials beyond ARTEs, attention is focussed on other compounds containing the endoperoxide pharmacophore such as the 1,2,4-trioxolanes arterolane (OZ277) [6], and OZ439, a next generation trioxolane with improved in vivo properties [7], both in clinical trials. Peroxide-bridged compounds derived from marine organisms are of particular interest due to their peculiar chemical architecture and marked biological activities [8]. Among the several compounds of this class, plakortin (Fig. 1), isolated and purified from the marine sponge Plakortis simplex [9], is an endoperoxide with similarity to ARTEs as far as the crucial role of the peroxide ring conformation and functionality are concerned [10]. Plakortin can be considered a promising antimalarial lead compound characterized by fast-acting activity at medium-low concentrations in vitro particularly against chloroquine resistant strains [11]. General aim of this study was understanding the mechanism of action of plakortin at molecular level, an endeavour of key importance for the development of active synthetic, optimized derivatives containing the essential pharmacophoric scaffold. Specific aims were analysis of stage-dependent antimalarial activity of plakortin, its capacity to produce ROS and generate the lipoperoxidation product 4-hydroxynonenal (4-HNE) in parasitized red blood cells (pRBCs), and the identification of 4-HNE conjugates with parasite proteins with potential importance for the parasite survival and invasion fitness. 4-HNE-dependent modifications of specific parasite proteins may represent a novel mode of action of antimalarial drugs, adding potency and specificity to oxidative antimalarials. 2. Materials and methods Unless otherwise stated all materials were obtained from Sigma-Aldrich (St. Louis, MO). Plakortin, methyl 4,8-diethyl-6-methyl-3,6-peroxy-9-dodecenoate [12], was obtained from the organic extract of the Caribbean sponge Plakortis simplex and purified by combination of column chromatography and HPLC as described [13]. The final purity of the compound wasZ99%. 2.1. In vitro cultivation of Plasmodium falciparum and stage-specific enrichment of parasitized red blood cells Plasmodium falciparum (Palo Alto strain, Mycoplasma free) pRBCs were kept in permanent culture as described [14]. Heparin anti-coagulated blood from healthy adult donors of both sexes was obtained from the local blood bank (AVIS, Torino, Italy). RBCs were separated from plasma, platelets and white blood cells by 3 washes in wash medium (WM, RPMI 1640 medium with HEPES modification), and stored at 4 °C after resuspension in WM at 50% hematocrit and supplementation of 2% (v/v) SAG (150 mM NaCl/ 1.25 mM adenine /45 mM glucose). RBCs that were used for plakortin incubation experiments were from blood samples taken on the same day of the experiment. For synchronization of cultures by parasite stage, schizonts were collected from the 40% to 60% interface after passing a mixed stage culture through a discontinuous PercollTM (GE Healthcare, Uppsala, Sweden)-mannitol density gradient at 5000 g for 30 min. Schizonts were added to RBCs resuspended at 1% hematocrit in growth medium (GM; WM supplemented with 20 mM glucose, 2 mM glutamine, 0.025 mM adenine, 32 mg/ml gentamicin and 1% (w/v) Albumax I (Invitrogen, Monza, Italy)) and left to infect for 10-14 h at 37 °C and in a 90% N2, 5% CO2, 5% O2 atmosphere (standard culture conditions). Then free hemozoin and residual mature parasites were removed by passing the culture through a 80% Percoll-mannitol cushion at 5,000 g for 30 min and keeping the ring-stage parasitized bottom fraction under standard conditions for further synchronous culturing and stage-specific incubation with plakortin [15]. When high parasitemia was required, the ring fraction (early maturation stage) of a synchronous culture was further enriched in 90% Percoll-mannitol at 14 h after inoculation. Enriched trophozoites (advanced maturation stage) were harvested from the 70% to 80% interface and schizonts (late maturation stage) from the 40% to 60% interface after Percoll-mannitol separations of synchronous cultures performed at 30 and 48 h after inoculation, respectively. Parasite fractions collected from the Percoll gradient were washed once with WM and maintained under standard culture conditions in GM. Cells were allowed to recover for one h before addition of plakortin into the culture. 2.2. Plakortin treatment of pRBCs and non-parasitized (np) npRBCs and prior antioxidants supplementation Pure plakortin was dissolved at 50 mM in ethanol (stock solution) and diluted to 10 mM with DMSO prior to use. The stock solution was kept at -20 °C until use. Synchronous cultures were supplemented with a single dose of 0-10 mM plakortin (final concentration) at ring-stage (12-16 h post-invasion), at trophozoitestage (26-30 h post-invasion) or at schizont-stage (42-48 h postinvasion) and kept under standard culture conditions (see above) until analysis. Enriched parasite fractions and non-parasitized (np) RBCs were treated similarly and kept under the same standard culture conditions. For reinfection studies, fresh npRBC suspensions in GM (2% hematocrit) were inoculated with schizonts (42-48 h postinvasion, 495% parasitemia, see method paragraph 'In vitro cultivation of Plasmodium falciparum') and adjusted to 5% parasitemia. Plakortin was added at indicated concentrations for 3 h and removed thereafter by one washing step with WM. After resuspension in GM cultures were kept at 37 °C under 90% N2/ 5% CO2/ 5% O2 (vol/vol) atmosphere and the reinfection rate was followed by counting ring-stage pRBCs at indicated times after plakortin addition. To study the oxidative properties of plakortin, the antioxidant water-soluble vitamin E analog Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the thiol-reducing and radical- and 4-hydroxynonenal (4-HNE)-scavenging

N-acetylcysteine (NAC) were added 30 min prior to plakortin supplementation into the cultures at final concentrations of 100 mM. 2.3. Morphological analysis of plakortin-treated pRBCs by light microscopy Parasitemia, stage-specific parasite morphology and morphologic anomalies of parasite and host RBC were determined in thin Fig. 1. The chemical structures of artemisinin and plakortin. The crucial endoperoxide is marked in color. O.A. Skorokhod et al. / Free Radical Biology and Medicine 89 (2015) 624–637 625 blood smears made at indicated times from the plakortin-containing or control cultures. The light microscopic examination of the Diff-Quiks fix stained (Medion Diagnostics GmbH, Düdingen, Switzerland) smear was performed with a Leica DM IRB microscope equipped with a 100x oil planar apochromatic objective with 1.32 numeric aperture and a DFC420C camera and DFC software version 3.3.1 (Leica Microsystems, Wetzlar, Germany). Parasitemia and percentage of damaged intracellular parasites were assessed in triplicate counting 400 to 1000 cells per smear at a time. 2.4. Assessment of parasitemia and parasite life cycle progress by FACS after ethidium bromide staining The percentage and maturity of pRBCs were detected by FACS at indicated time points by labelling culture aliquots with ethidium bromide (EtBr; 5 mg/ml final concentration) [16]. The fluorescence of the labelled pRBCs was acquired on a FACSCalibur flow cytometer (BD Biosciences, Sunnyvale, CA, USA) and analyzed in the FL2 channel at 564–606 nm after argon laser excitation at 488 nm using the Cell Quest software (BD Biosciences). 2.5. Hemoglobin quantification The hemoglobin concentration in the culture supernatant was assayed by heme-dependent luminol-enhanced luminescence. Luminescence was measured in a double-injector luminometer (Sirius; Berthold, Pforzheim, Germany) as described [17]. All assays were performed in triplicate. 2.6. Assay of ROS in pRBCs and npRBCs with DCF-DA and DHE by FACS and fluorescence microscopy Plakortin-treated pRBCs and npRBCs and untreated controls were incubated during 3 h at 10% hematocrit in GM containing 100 mM of the ROS probe dichlorofluorescein-diacetate (DCF-DA) at 37 °C under orbital agitation. After labelling with DCF-DA, RBCs were washed three times with PBS-Glucose (PBS-G) and the samples were resuspended in EtBr at 10 mg/ml GM for 15 min at 37 °C under orbital agitation for DNA staining of pRBCs, which allows to distinguish pRBCs from npRBCs and the separate ROS analysis. After incubation, RBCs were washed three times and resuspended in PBS-G. The fluorescence of the labelled pRBCs and npRBCs was acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed for DCF in the FL1 channel at 515–545 nm and for EtBr in the FL2 channel at 564–606 nm after argon laser excitation at 488 nm using the Cell Quest software (BD Biosciences). In order to minimize emission overlap of the two fluorochromes, a minor electronic compensation was applied. The obtained data were analyzed with Flowing Software (developed by Perttu Terho, Turku Centre for Biotechnology, Turku, Finland). A second probe was applied to assess specifically the superoxide generation by plakortin in pRBCs [18,19]. Dihydroethidium (DHE) was added to the cell culture at final concentration of 25 mM for 30 minutes. Then the non-reacted DHE was washed out with PBS-G, and red emission from p- and npRBC was measured by flow cytometry in the red channel at 642 nm after laser excitation at 488 nm using a Guava flow cytometer (Merck Millipore, Billerica, MA, USA) and Guavasoft 3.1 software (Merck Millipore). Additional analysis of fluorescent probe-labelled, plakortintreated parasites was performed by fluorescence microscopy in wet smears following incubation with DCF-DA and EtBr (100 mM and 5 mg/ml final concentration, respectively). Fluorescent images were acquired with a Leica DR IRB fluorescence microscope (Leica Microsystems) equipped with a Leica DFC 420 C camera, a 100x oil planar apochromatic objective with 1.32 numerical aperture, a 450/490 nm excitation and LP 515 nm barrier filter and the version 3.3.1 of the Leica DFC image software (Leica Microsystems). Trophozoites were identified by the presence of hemozoin in bright field inspection and confirmed by EtBr staining. 2.7. Assay of 4-HNE conjugates in pRBC and npRBCs by FACS Cells were washed with PBS-G-bovine serum albumin 1% (PBSG-BSA), incubated with anti-4-HNE–conjugate antibody (Alpha Diagnostics International, San Antonio, TX, USA) at 1:50 in PBS-GBSA for 1 h and then washed twice with PBS-G-BSA. Bound anti-4- HNE-conjugate antibodies were revealed by FITC-conjugated F(ab) 2 goat anti-rabbit IgG, the percentage and maturity of pRBCs are detected by labelling with EtBr (5 mg/ml final). The fluorescence of the labelled pRBCs was acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed for FITC in the FL1 channel at 515– 545 nm and for EtBr in the FL2 channel at 564–606 nm after argon laser excitation at 488 nm using the Cell Quest software (BD Biosciences). In order to minimize emission overlap of the two fluorochromes, a minor electronic compensation was applied. The obtained data were analysed with the WinMDI software (The Scripps Research Institute, La Jolla, CA, USA) and presented as mean fluorescence intensity (MFI) [14,20]. 2.8. Preparation of protein extracts from pRBC and npRBC Washed and 1700 g sedimented npRBCs or pRBCs were hypoosmotically lysed in a 10–fold excess (v/v) of ice-cold lysis buffer (10 mM K_2HPO_4/KH_2PO_4 (pH 8.0), supplemented with a protease inhibitor cocktail containing: 1 mM EDTA, 250 mM phenyl-methylsulfonyl fluoride (PMSF), 1 nM leupeptine, 3 mM pepstatine, the phosphatase inhibitors sodium orthovanadate and sodium fluoride at 1 mM, each and 100 mM Trolox, during 5 min on ice. The ruptured membranes and organelles of parasite origin were sedimented at 16,200 g for 3 min at 4 °C and the supernatant was discarded. The pellet was washed 10 times by resuspension in fresh lysis buffer and subsequent sedimentation at 16,200 g for 1 min. npRBC-derived preparations contained pure RBC membranes, while pRBC preparations contained the host cell membrane as well as membranes and organelle debris from the parasite. The proteins were extracted with SDS-containing Laemmli buffer at 95 °C for 5 min. Solubilized proteins were kept at -20 °C prior to use and β-mercaptoethanol (5% v/v) was added to protein samples before loading to the SDS-PAGE. The proteins were quantified using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). 2.9. Analysis of proteins by western blotting The solubilized proteins (10-30 μg) were separated with an 8% acrylamide (w/v) SDS-PAGE and transferred onto a

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nitrocellulose membrane (Amersham Biosciences, Fairfield, CT) for identifying 4-HNE-protein-conjugates. The BSA-blocked membrane was incubated overnight at 4 °C with the monoclonal anti-4-HNE-conjugate antibody (Abcam, Cambridge UK; 1:1000 dilution). The membrane was washed and incubated with the secondary HRPconjugated anti-mouse antibody (Pierce, Rockford, IL) at a 1:10,000 dilution for 1 h. Antibody-positive bands were visualized by ECL and acquired with Chemidoc MP (Bio-Rad), using PDQuest software (Bio-Rad, version 7.2) according to the manufacturer's instructions. 2.10. Identification of 4-HNE modified proteins by mass spectrometric analysis and peptide mass fingerprinting For the identification of 4-HNE-conjugated proteins in plakortintreated parasites matrix assisted laser desorption ionization-time of 626 O.A. Skorokhod et al. / Free Radical Biology and Medicine 89 (2015) 624–637 flight (MALDI-TOF) mass spectrometric analysis was performed. Proteins extracted from enriched ring and trophozoite stage pRBCs and npRBCs cultured with or without 10 mM plakortin for 3 h were separated by SDS-PAGE. Gels were stained with colloidal Coomassie, protein bands of interest excised from the gel and in-gel trypsin digested as described in [21]. MS analysis was performed on a MALDI-TOF spectrometer (MALDI micro MX; Waters, Milford, MA) equipped with a delayed extraction unit, according to the tuning procedures suggested by the manufacturer, operating in reflectron mode. Peak lists were generated by Protein Lynx Global Server (Waters, Milford, MA). The 25 most intense masses were used for database searches against the SwissProt and NCBI databases using the free search program MASCOT (http://www.matrixscience.com). The parameters used for the searches were: taxa Plasmodium falciparum, trypsin digestion, 1 missed cleavage, methionine oxidation and 4-HNE as variable modifications with a maximum error allowed of 100 ppm. For Plasmodium falciparum, proteins taken on to consideration had a Mascot score higher than 37 for SWISSPROT searches and higher then 67 for NCBI searches, as suggested by Mascot to be "significant". 2.11. Statistical analysis Non-parametric Mann-Whitney U Test was used to determine the significance of difference between the groups´ means (PASW Statistics 18, SPSS IBM, Chicago, IL). If not otherwise indicated, p values o0.05 were considered to be statistically significant. 3. Results Plakortin has been shown to inhibit P. falciparum growth in vitro (IC50r1 mM) as determined in mixed stage cultures by the lactate dehydrogenase assay [11]. The yet unexplored stage-specific susceptibility of the parasite to plakortin and the mechanism of drug action are the main objectives of present study. 3.1. Plakortin delays parasite growth and damages ring-stage parasites The time-dependent effects of plakortin on parasite growth and morphology were studied in stage-separated pRBCs supplemented with 1, 2, 5 and 10 mM plakortin. At the beginning of plakortin treatment (time zero samples), synchronized ring-stages (12-16 h old) showed normal parasite morphology. Untreated parasites developed to morphologically normal older ring and trophozoite stages after 3, 6 and 24 h incubation (Fig. 2A). Plakortin-treated ring-stage parasites showed time- and dosedependent delay of intra-erythrocytic growth and intracellular damage at all tested plakortin concentrations. At 1 mM the majority of parasites were normally shaped but showed a strong maturation delay till 24 h when only little hemozoin was visible in the still young trophozoites which did not attain the typical morphology of 36-40 h old parasites (Fig. 2A, Table 1). The few normally shaped parasites observed in cultures exposed to Z2 mM plakortin showed a strong maturation delay till 24 h (Fig. 2A, Table 1). The delay of ring growth was confirmed by a significant and dose-dependent decrease of DNA content in ring-stage parasites treated for 6 h with plakortin (Fig. 3A). The delayed but otherwise inconspicuous parasites developed to fully mature schizonts 10- 20 h later than untreated controls (not shown). Parasite damage was characterized by anomalous shape of the stained parasite with distorted or broken ring forms up to the complete shrinkage of the parasite to a pyknotic clump. At 1 mM plakortin, few deformed parasites appeared after 6 h treatment while atZ2 mM plakortin, parasite damage was observed already Fig. 2. Morphological aberrations and growth delay of P. falciparum by plakortin. Representative microscopic images taken from Quick Diff stained smears show synchronized P. falciparum cultures which were treated at ring stage (12-16 h after reinfection) with 0-10 mM plakortin for indicated time (3-24 h; A, B), and of damaged pRBCs from synchronized cultures treated at trophozoite stage (26- 30 h after reinfection; C) and at schizont stage (42- 46 h after reinfection; D,E) with 2 mM plakortin for 6 and 9 h, respectively. Anomalous parasite shapes of ring-stage parasites with distorted, broken or shrunk, pyknotic forms are indicated by arrowheads (A). Stained dots in the host cell cytosol resembling Maurer's clefts are marked by arrows (A) and magnified in a selected pRBC after treatment with 1 mM plakortin for 24 h (B). O.A. Skorokhod et al. / Free Radical Biology and Medicine 89 (2015) 624–637 627 3 h after drug addition when deformed but also densely aggregated and pyknotic parasites appeared together with cytosolic dots in the RBCs hosting damaged parasites (Fig. 2A-B). The stained dots resembling Maurer's clefts were frequently observed in the cytosol of the plakortin-treated host RBCs but not in npRBCs and may suggest that plakortin targets cytoplasm reorganization and trafficking processes (Fig. 2A-B, Table 1). The percentage of ring-stage parasite damaged by plakortin increased dose- and time-dependently as judged by differential manual counting. Significantly higher percentages of damaged parasites were observed during 3 to 6 h of plakortin supplementation at Z2 mM compared to untreated control cultures (Fig. 4A). After 24 h, at 1, 2, 5 and 10 mM plakortin supplementation, the damaged forms of parasites contributed with, respectively, 18.8712.1, 24.479.7, 61.676.1 and 92.874.9% to the total parasitemia (assessed as percentage of pRBCs hosting both viable and damaged/dead parasites), all significantly higher compared to untreated cultures. Damaged parasites did not recover during further incubation and all parasites were dead 48 h after a single dose of 10 mM plakortin to ring-stage cultures (not shown). Note that the damage observed until 24 h occurred at ring or early trophozoite stage as plakortin treatment delayed the parasite cycle progress from rings to trophozoites (Fig. 2A). The parasitemia of undamaged viable parasites decreased significantly after plakortin treatment in a strictly dose- and time-

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dependent manner and reached 8.270.2, 6.870.4, 3.470.6 and 0.671.3% after 24 h of incubation with 1, 2, 5 and 10 mM plakortin, respectively, compared to 10.770.4% in control cultures (Fig. 4A). Notably, total parasitemia, that includes viable and non-viable parasites, did not significantly change under plakortin until 6 h and the damaged ring-stage parasites remained intracellular (Fig. 4A). The significant and concentration-dependent decrease of total parasitemia by plakortin supplemented for 24 h (8.3 vs. 11.0% at 10 mM and 0 mM, respectively) may be caused by the expulsion of parasites from the host cell or by degradation of microscopically visible structures. Loss of pRBCs by plakortin-induced cell lysis was excluded by sensitive hemoglobin measurement in the culture supernatants (not shown). Table 1 Concentration and time-dependent morphological changes of pRBCs caused by plakortin added to synchronized in vitro P. falciparum cultures at ring stage 12-16 h after reinfection. PLAKORTIN 0 lM 1 lM 2 lM 5 lM 10 lM 3 h damaged parasite þ þ þþþ 6 h damaged parasite þ þ þþ þþþ delayed maturationa þþþþ cytosolic dots in RBC þþþ 24 h damaged parasite þ þ þþ þþþ delayed maturationa þþþ ND cytosolic dots in RBC þþþþ hemozoin formation þþþ þþ þ þ ND a Delayed maturation was judged in apparently undamaged pRBC; ND: not detectable as to the high percentage of prematurely damaged parasites; -: no plakortin effect; þ, þ þ, þþþ: semi-quantitative description of parameter. Fig. 3. Inhibition of P. falciparum in vitro growth by plakortin. Synchronized P. falciparum cultures were treated at ring-stage (12-16 h after reinfection, A) and trophozoite-stage (26-30 h after reinfection, B) with 0-10 mM plakortin for 6 h. Parasite growth was assessed by flow cytometry after staining cells with ethidium bromide (EtBr). Means of mean fluorescence intensities (MFI)7SE are shown for N¼3-5 independent cultures. The significance of differences to untreated control cultures (0 mM) is indicated by n for po0.05 and nn for po0.1. Fig. 4. Damage of ring- and trophozoite-stage P. falciparum and inhibition of reinfection by plakortin. Synchronized P. falciparum cultures treated at ring-stage (12- 16 h after reinfection, A), trophozoite-stage (26-30 h after reinfection, B) and schizont stage (42-48 h after reinfection, C) with 0-10 mM plakortin were examined for parasitemia at indicated time points after plakortin addition by manual microscopic inspection of Quick stained smears. Parasites with irregular shapes (distorted, broken, pyknotic and fragmented forms) in the stained smears were counted as damaged pRBCs (gray columns) separately from pRBCs harboring viable parasites (white columns). The reinfection was determined as ring parasitemia at 3 and 9 h after inoculation of mature schizonts into a npRBC suspension and addition or not of plakortin (C). Means7SE of parasitemia are shown for independent cultures with RBCs from different donors (A: N¼4-7; B: N¼3; C: N¼5). The significance of differences to untreated control cultures (0 mM) is indicated by * for po0.05, ** for po0.1. Significance symbols above the grey portion of the column refer to damaged parasites, above the entire column to the total parasitemia (viable plus damaged parasites). 628 O.A. Skorokhod et al. / Free Radical Biology and Medicine 89 (2015) 624–637 3.2. Plakortin delays parasite growth and damages trophozoite-stage parasites The morphological effects of plakortin addition to synchronized trophozoites at about 26-30 h after invasion were consistent with those reported for the ring-stage fraction, i.e. delay and damage. At low (1-2 mM) plakortin concentrations a cycle progression delay was ascertained after 3-6 h of incubation by the smaller size of young trophozoites compared to the larger and more mature parasites in the untreated control culture and a delayed transition from trophozoites to schizonts after 12 h plakortin treatment (not shown). The DNA content in trophozoites exposed to plakortin from 30 to 36 h after invasion vs. plakortin-free cultures was significantly decreased at Z2 mM plakortin (Fig. 3B) and confirmed the microscopically observed life cycle delay. The main signs of damaged intracellular trophozoites were either an irregular shrunk and segmented shape or a completely aggregated pyknotic morphology, sometimes split in smaller fragments (Fig. 2C). Such morphologies were considered to indicate non-viable parasites. Plakortin addition to trophozoites resulted in a dose- and timedependent increase of damaged parasites (Fig. 4B). The treatment of synchronized vital trophozoites cultured at 11.1% parasitemia for 3 h in presence of 1, 2, 5 and 10 mM plakortin caused a drop of viable parasites to 9.3, 7.9, 4.8 and 3.0% and an increase of damaged trophozoites to 1.0, 2.2, 4.5 and 5.5%, respectively. The total parasitemia (including damaged pRBCs) decreased concentrationdependently and significantly at 6 and 12 h after plakortin supplementation, which suggests that up to 50% of parasites were expelled from their host RBCs (Fig. 4B). Since no hemoglobin was released into the culture supernatant (see Fig. 1 in [22]), hemolysis of pRBCs was not the cause of decreased parasitemia. While both rings and trophozoites suffered from very similar ‘long’-term damages comparing the share of damaged parasites, assayed after 24 h incubation of ring-stages (Fig. 4A) and 12 h incubation of trophozoite-stages with plakortin (Fig. 4B), ´shortterm’ damage at 3 and 6 h of plakortin supplementation was significantly more frequent in trophozoites than in rings, particularly at low plakortin concentrations. This may suggest an elevated sensitivity of trophozoites to plakortin compared to rings. 3.3. Plakortin impairs reinfection The effect of plakortin on schizonts, the last stage of the intraerythrocytic parasite cycle, and on reinfection was tested after inoculation of highly enriched, 42-48 h old schizonts into fresh RBC suspensions and supplementing plakortin during 3 h. Compared to control cultures run in parallel, the reinfection rate (determined as ring parasitemia during 3 and 9 h after inoculation) was inhibited dose-dependently by plakortin (Fig. 4C). At 3 h, 1-10 mM plakortin significantly inhibited reinfection and decreased the ring parasitemia by 40-60%. At this time strikingly more schizonts were counted in plakortin-treated cultures compared to controls (not shown) suggesting a delay in schizogony. As the ring parasitemia remained low under plakortin at 9 h compared to controls (Fig. 4C) and further on until 18 h, when all cultures were schizont-free (not shown), an impairment of reinfection occurs additionally to the delay. We suppose that one or both morphologic aberrations observed in several but not all schizonts after plakortin supplementation contribute to the decreased reinfection rate. Fig. 2D shows a relatively mature schizont with several sharply bounded holes very similar

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to those caused by lipid droplets and with few altered merozoites apparently without cytosol around the swollen nuclei. The second aberration (Fig. 2E) was loss of the characteristic schizont morphology and the appearance of several pyknotic fragments in hemozoin-rich pRBCs. No differences in the infection rate and morphology with respect to untreated controls were observed when the RBC suspension was pre-treated with plakortin before inoculating untreated schizonts (data not shown). 3.4. Plakortin generates free radicals and ROS in pRBCs The generation of free radicals and reactive oxygen species (ROS) by plakortin in vitro in presence of iron [23] or heme (unpublished observation) prompted us to test, first, the ability of plakortin to elicit the formation of ROS in p- and npRBCs and, second, the possibility to quench the plakortin effects by application of radical scavengers. The intracellular formation of ROS by plakortin in RBCs was detected with the fluorescein-based probe DCF-DA [24] and con- firmed by the superoxide-specific probe DHE [18] by comparing the fluorescence levels of plakortin-treated vs. untreated RBCs via flow cytometry. Plakortin treatment of ring-stage cultures for 3 h induced a dose-dependent increase in DCF fluorescence intensity in pRBCs (Fig. 5A). The ROS production in plakortin-treated pRBCs was significantly higher than in untreated pRBCs (by 92.079.2% at 2 μM and by 108.070.5% at 10 μM, respectively). The plakortin-elicited fluorescence achieved about 70% of the maximal fluorescence observed with the ROS-producing xanthine/xanthine oxidase system (Fig. 5A). The radical scavenger NAC decreased the fluorescence elicited by 10 mM plakortin by approximately 26%. The dose-dependent increase of the superoxide anion was detectable by DHE fluorescence as early as one h after plakortin supplementation in pRBCs and increased significantly at Z1 mM (Fig. 5C). Plakortin did not elicit detectable superoxide anion production in npRBCs. The modest but significant increase of DCF-fluorescence in co-cultured npRBCs after treatment with Z2 mM plakortin (pr 0.04; N¼ 3-5) remained clearly below the fluorescence in pRBCs at any concentration. Untreated pRBCs produced more ROS as npRBCs exposed to the highest plakortin concentration. Co-culturing with ring-pRBC without plakortin addition as well as treatment with 10 μM plakortin in absence of pRBCs did not increase the ROS level in npRBCs (Fig. 5A). The non-significantly (p¼0.07) increased ROS production in pRBCs vs. co-cultured npRBCs in untreated cultures implies parasite-specific ROS production. The flow cytometry results on ROS-dependent fluorescence in pRBCs were confirmed by fluorescence microscopy (Fig. 5B). In untreated control cultures the fade green fluorescence signal of DCF accumulated in the parasite and not in the host cell, indicated oxidative stress physiologically induced by the parasite itself. After plakortin treatment the fluorescence intensity co-localizing with the ring-stage parasites increased, indicating elevated oxidative stress level inside the parasitophorous vacuole. Under these conditions the RBC membrane also became marginally fluorescent (Fig. 5B). We then investigated the correlation between the plakortinelicited ROS generation and the alterations in parasite´ growth and morphology described before. Supplementing ring-stage cultures with the antioxidants and radical scavengers NAC and Trolox prevented the intracellular damaging effect of plakortin and the share of damaged parasites decreased significantly (Fig. 6A-B). Notably, NAC addition abrogated the plakortin effect and the percentages of vital parasites in the cultures pre-incubated with NAC before 2 and 10 mM plakortin addition, did not significantly differ from untreated control cultures (p40.05, N¼3). In contrast, significant differences were observed when plakortin was added alone (po0.05, N¼3; Figs. 4A, 6A). Fig. 6B clearly shows the protective effect against the morphologic damage induced in pRBCs by 10 mM plakortin during 3 h. While the untreated control displays synchronized rings with well-defined structures, the plakortin (10 μM) treated population shows aggregated and Fig. 5. ROS production by plakortin in pRBCs. Synchronized P. falciparum cultures were adjusted to approximately 10% parasitemia and treated at ring-stage (12-16 h after reinfection) with indicated final concentrations of plakortin. Where present, NAC was added 30 min prior to plakortin. In parallel, npRBCs were suspended in GM and treated with the pro-oxidant xanthine/xanthine oxidase as positive control for ROS production. Cells were stained with the ROS-sensitive fluorescent probe DCF-DA (A, B) at 3 h after pre-incubation with plakortin and superoxide-sensitive probe DHE (C) at 1 h after plakortin supplementation. Both probes were separately assessed in pRBCs and npRBCs as mean fluorescence intensity (MFI) of DCF or DHE by flow cytometry (A, C) and microscopy (B). A) pRBCs and npRBCs were distinguished by ethidium bromide. Means7SE of MFI obtained with independent cultures grown in RBCs from N¼ 3 -5 donors are plotted. The significance of differences is indicated by * for po0.05. B) Two representative pairs of phase contrast and fluorescence images for untreated (0 mM PLAKORTIN) and Plakortin-treated (10 mM PLAKORTIN) cultures are shown. Images were acquired with an inverted microscope Leica DM IRB (Leica Microsystems) equipped with a 100 X oil planar apochromatic objective with 1.32 numerical aperture and a Leica camera DFC 420 C. Leica DFC software (version 3.3.1) was applied. Imaging medium was PBS-G used for washing of cells after staining. C) pRBC and npRBC were distinguished by ethidium, the product of DHE. Means7SE of MFI obtained with independent cultures grown in RBCs from N¼3 donors are plotted. The significance of differences is indicated by * for po0.05. damaged forms, which were rarely seen with both anti-oxidants NAC and Trolox supplemented at 100 mM before plakortin addition to the cultures. 3.5. Plakortin induces lipoperoxidation and 4-HNE-protein conjugation in p- and npRBCs. Accumulation of 4-HNE-protein conjugates, the final products of ROS formation and lipoperoxidation was observed in the plakortin-treated RBCs. Untreated ring-pRBC showed a progressive, modest increase of 4-HNE conjugate formation during 24 h culture (Fig. 7A) consistent with previous results [25]. In plakortin-treated cultures the progressive time- and dose-dependent increase of 4-HNE conjugates in pRBCs was stronger and the conjugate level exceeded untreated cultures at plakortin concentrations ofZ2 mM added for

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at least 6 h (þ70% at 2 mM; Fig. 7A). The water soluble vitamin E-analog Trolox and the radicalscavenger NAC were highly efficient to impair the 4-HNE conjugate formation at any tested plakortin concentration during 24 h incubation of rings (Fig. 7E). The additional 4-HNE scavenging capacity of NAC may explain the highly efficient abrogation of plakortin-induced 4-HNE conjugation (Fig. 7E) and parasite damage (Fig. 6A-B) despite the minor attenuation of plakortindependent ROS production by NAC (Fig. 5A,C). In npRBCs from the same cultures, 4-HNE conjugation was low and plakortin-independent during 24 h with the exception of 10 mM plakortin that caused a significant increase in conjugate levels from 6 h on (Fig. 7B-D). These results and the lack of effect of 10 mM plakortin in npRBCs without co-incubation with pRBCs (not shown) can be explained by assuming that 4-HNE diffuses out of pRBCs and bind to the membrane of npRBCs. Consistently with previous observations [25], the 4-HNE conjugates in trophozoites exceeded those in ring forms (Fig. 7A,C). The high levels of 4-HNE conjugates in trophozoites were further increased by plakortin treatment, with peak levels reached after 6 h plakortin application. (Fig. 7C), and in correspondence with the damaging plakortin effect in mature parasites (Fig. 4B). 3.6. Molecular targets of 4-HNE and their potential functional importance In electrophoretically separated proteins extracted from plakortin-treated ring- and trophozoite-pRBCs significantly more 4-HNE conjugates were detectable compared to untreated pRBCs Fig. 6. Attenuation of the plakortin effects by antioxidants and free radical scavengers. Synchronized P. falciparum cultures were treated at ring-stage (12- 16 h after reinfection) with indicated final concentrations of plakortin for 3 h. Where present, Trolox or N-acetylcysteine (NAC) were added at 100 mM final concentration, each, 30 min prior to plakortin (A,B). A) Parasitemia was determined in Quick Diff-stained smears of the cultures by manual microscopic inspection. Parasites with irregular shapes (distorted, broken, pyknotic and fragmented forms) in the stained smears were counted as damaged pRBCs (gray columns) separately from pRBCs harboring viable parasites (white columns). Means7SE of parasitemias are shown for independent cultures from N¼3 RBC donors. The significance of differences of parasitemias is indicated by * for po0.05 (solid line for damaged parasites and dotted line for viable parasites). B) Representative microscopic images are shown for synchronized high parasitemia P. falciparum cultures (N¼5) after respective treatments. O.A. Skorokhod et al. / Free Radical Biology and Medicine 89 (2015) 624–637 631 (Fig. 8). The 4-HNE conjugated proteins were identified by MALDITOF mass spectrometry to see whether modification might contribute to the parasite damage elicited by plakortin. Table 2 lists parasite proteins which were specifically targeted by 4-HNE upon plakortin treatment, and indicates their modification sites and potential functional importance for the parasite survival. Fig. 7. Increase of 4-HNE conjugate formation in pRBCs by plakortin and its attenuation by scavengers of free radicals. Synchronized P. falciparum cultures were treated at ring-stage (12-16 h after reinfection, A,B;E) and trophozoite-stage (26-30 h after reinfection,C,D) with 0-10 mM plakortin for 24 h. 4-HNE conjugate formation in the culture was assessed in intact pRBCs (A,C) and npRBCs (B,D) at indicated time points after plakortin addition (at time 0) by flow cytometry and expressed as MFI (see methods). E) Where present, Trolox or NAC were added at 100 mM final concentration 30 min prior to plakortin. Means7SE are presented for independent cultures from 2-7 RBC donors (A, B), 3-6 RBC donors (C, D), 2-4 RBC donors (E). The significance of differences between plakortin treated versus non treated cultures (A-D) or between plakortin treated versus plakortin treated plus Trolox/NAC-supplemented RBCs (E) is indicated by * for po0.05. 632 O.A. Skorokhod et al. / Free Radical Biology and Medicine 89 (2015) 624–637 In ring-stage cultures 10 mM plakortin, added for 3 h generated 4-HNE conjugates with four P. falciparum proteins. Notably, these proteins are directly or indirectly involved in protein homeostasis and trafficking and transmembrane transport: HSP70-1, V-type proton ATPase catalytic subunit A, enolase, and the putative vacuolar protein sorting-associated protein 11 (formerly RING finger protein PFE0100w). In trophozoites the endoplasmatic reticulum–standing HSP 70-2 (BiP analogue) and the Dynein heavy chain-like protein became additionally modified by 4-HNE (Table 2). 4. Discussion Artemisinin-based Combination Therapies (ACTs) are the gold standard treatment for falciparum malaria recommended by WHO, due to their potent, rapid and broad stage specificity of action [49– 51]. Recently, artemisinin resistant strains were described to spread throughout several countries of the Great Mekong region [1,52,53]. In view of this resistance, there is an urgent need for novel antimalarials sharing the excellent properties of artemisinin, yet distinct from it to avoid cross-resistance. Present study was aimed at characterizing molecular mechanisms of the antiplasmodial activity of plakortin, lead compound of a class of 1,2-dioxane derivatives isolated from the Caribbean sponge Plakortis simplex. Plakortin and its 9,10-dihydro analogue were shown to possess in vitro antimalarial activity in mixed blood stage cultures in the submicromolar range (IC50 of 0.9 and 0.41 mM, respectively) and to be devoid of general cytoxicity [10,11,23,54]. More potent synthetic plakortin-based compounds are under development [54]. The antimalarial efficacy of plakortin as well as artemisinin has been reported to be dependent on the cleavage of their endoperoxide bond [10]. We will discuss the presented results with emphasis on i) stage-dependence and cellular localization of antimalarial activity; ii) ROS generation and pro-oxidant activity in pRBCs; iii) lipid peroxidation and formation of 4-HNE-protein conjugates in pRBCs. 4.1. Stage-dependence of plakortin activity Stage-sensitivity to plakortin was studied by analyzing parasite morphology and reinfection rates during parasite development. Our results indicate that rings (12-16 h after reinfection) were sensitive to plakortin after the first 3 h of drug exposure at low drug concentrations, while the effect was significant at higher drug concentrations (Figs. 2A, 4). In young trophozoites the effect was already significant at lower drug doses (Fig. 4) and treating highly mature schizonts with plakortin resulted in a significant decrease of reinfection at low concentrations (Fig. 4C). The different plakortin sensitivity in ring vs. trophozoite stages might be due to higher heme release during hemoglobin

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digestion and lower antioxidant (e.g. GSH) levels in the trophozoite compared to ring stages [55,56]. Morphological damages and dose-dependent decrease in DNA content indicate delay in parasite development, which was remarkable already in rings treated with the lowest plakortin concentration. In conclusion, the early morphological effects of plakortin in rings observed here are consistent with the early parasite damage elicited by artemisinin [57,58]. 4.2. ROS generation and pro-oxidant activity We show here for the first time the intracellular production of ROS by plakortin in pRBCs, but not in npRBCs. Dampening the ROS level by radical scavengers was accompanied by abrogation of the parasite damaging effect of plakortin in cultured P. falciparum suggesting a ROS-dependent anti-parasitic mechanism of plakortin. In analogy with artemisinin, two mechanisms may be suggested for plakortin-dependent ROS generation: a) An iron-dependent (e.g. via heme iron) scission of the endoperoxide bridge and the resultant radical formation may elicit ROS generation in the parasite. The identified final product of the in vitro reaction of plakortin with Fe2þ suggests the generation of transient free radicals that may represent the toxic intermediates [23] and starting point for the ROS generation observed here. b) The endoperoxide may rapidly oxidize reduced cofactors of the antioxidant GSH/thioredoxin system in the parasite, GSH loses the control on ROS generation and ROS rises in the parasite as suggested for artemisinin [59,60]. 4.3. Lipid peroxidation and generation of 4-HNE-protein conjugates in pRBCs Similary to artemisinin, plakortin may diffuse into the FV, Fig. 8. Detection of increased 4-HNE-protein conjugates in pRBCs incubated with plakortin. Ring (RINGS) and trophozoite (TROPHS) pRBCs at465 and495% parasitemia, respectively, were treated or not with 10 mM plakortin for 3 h. Extracted proteins were separated by SDS-PAGE, transferred to nitrocellulose and 4-HNE conjugated protein bands were recognized by a monoclonal anti-4-HNE conjugate antibody and visualized with a secondary peroxidase-conjugated antibody by ECL. ODs of ECL-positive bands were assessed by densitometry and the sum of ODs shown as mean7 SE of N¼3 independent experiments (RINGS) and N¼4 (TROPHS). Significant differences between plakortin-treated (10 mM) and untreated control cultures (0 mM) are indicated by * for p¼ 0.05 and ** for p¼0.08. 4-HNEbound albumin (BSA-HNE) was run as positive control. O.A. Skorokhod et al. / Free Radical Biology and Medicine 89 (2015) 624–637 633 Table 2 Plakortin induced protein modification at RING* stage and TROPHOZOITE§ stage, only. 4-HNE-MODIFIED PROTEIN (NAME, ALIAS; IDs) 4-HNE BINDING SITE (UniProt) EXPRESSION (BLOOD STAGE/ ASSESSMENT) INTRACELLULAR LOCALIZATION/ ASSESSMENT MOLECULAR FUNCTION (inferred by similarity (GO) or experimentally proven) POTENTIAL CONSEQUENCE OF CONJUGATION 1 n HSP70-1, HSP70_PLAFA K112, K122, K140, K149, H198, K199, C253, K258, K260, K335, K341, K342, K345, K370, K464, K471, H513, K520, C559, K563, K570 T [26,27],R,S,M [27] / MS (http://plas modb.org) and amino acid sequencing [28] cytosol [26,27,29,30]; nucleus [26,30] / immunohistology [26], MS (http://plasmodb.org) N-term. ATP-binding, constitutive and inducible chaperone [27]; clathrin-mediated vesicular transport predicted [34] (http://sites.huji.ac.il/malaria); cytosolic multi-chaperone complex [27]; heat shock response along with PfHsp90 [27]; FPIX binding after chloroquine treatment [35] cycle delay and death Uniprot: Q8IB24_PLAF7 NCBI: GI:124512406- PlasmoDB: PF3D7_0818900 FV / LC-MS/MS of subcellular structures [31] MC/ by LC-MS/MS of subcellular structures [32] essential for parasite growth and development by refolding nascent proteins [36]. PPV / MALDI-TOF-MS of subcellular structures [33] 2 n V-type proton ATPase catalytic subunit A (EC 3.6.3.14), vacuolar ATP synthase subunit a (vapA) K 579, K584 R,T,S / MS (http:// plasmodb.org) and [37,38] PM, FV, PVM, SCV/ IEM[39] proton pump, pH control in parasite cytosol and FV[40]; oxidation sensitive (H2O2) [40,41]: ATP drop due to GAPDH or other glycolytic enzyme response to low pH Parasite cytosol acidification, ATP decrease, impairment of vesicular transport, discoloration of the ring shape as deduced from [39– 41] FV/ functional study in isolated organelles [40] Uniprot: Q76NM6, VATA_PLAF7; cytosol and nuclear extract/ MS of parasite extracts [37] NCBI: GI:124512982 PlasmoDB: PF3D7_1311900 3 n Enolase (EC 4.2.1.11) K62, K66, K69, K79, K138, K146, K147, H198, K201, K205, K206, C412 R,T,S,M,G / IEM [42,43] cytosol, nucleus, FV, cytoskeleton, M membrane/ EM, LM, IFM [42,44], MC [45] glycolysis, plasminogen binding, antigenic [43], forms complexes with HSP70 and iron superoxide dismutase [36]. ATP decrease phosphopyruvate hydratase Uniprot:Q8IJN7, ENO_PLAF7; NCBI: GI:124802328 PlasmoDB: PF3D7_1015900 4 n Vacuolar protein sortingassociated protein 11, putative, former RING finger protein PFE0100w, K110, K389, K397, K399, K410, K445, K482, K672, K673, K748, K793, K799, K925 M (http://www.uni prot.org), R,T,S (http://plasmodb. org) membrane; single-pass membrane protein/ predicted; endosome (GO) (http://www.uniprot. org)]. apicoplast/ predicted (GO). intracellular protein transport (GO); vesicle-mediated transport, vesicle docking involved in exocytosis (GO); zinc ion binding (GO); contains clathrin heavy chain repeat (aa608-752) (http://www.uniprot.org/uniprot/Q8I480) impaired protein export, impaired vesicle fusion with plasma membrane, structural failure Uniprot:Q8I480- ZNRF2_PLAF7 NCBI: GI:124505963 PlasmoDB: PF3D7_0502000 5 §DYHC1_PLAF7; Dynein heavy chain-like protein, H967, H2165, H2475 S, M / immunochemistry and -histology [46,47] cytoplasm, microtubule/ EM [46] involved in invasion (dynein inhibitors reduced the number of R [46]; shuttle on cytoplasmic microtubules, intracellular transport processes [48]; motor for the intracellular retrograde motility of vesicles and organelles along microtubules. ATPase activity for power stroke (by similarity, http://www.uniprot. org/ uniprot/Q8IBG1) impaired reinfection of RBC by merozoites Uniprot:Q 8IBG1 NCBI: GI:296004907 PlasmoDB: PF3D7_0729900 6 §Heat shock protein 70 (HSP70-2); BiP; GRP78 K265, K268, K340, K341 high at late T, S/ MS of the proteome, amino acid sequencing [28] ER (GO) chaperone-assisted protein folding folding and export to the host cell (http://plasmodb.org) impaired protein trafficking with structural defects and cell death Uniprot: Q8I2 4_PLAF7 NCBI: GI:124506906 PlasmoDB: PF3D7_0917900

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SCV) small clear vesicles, argued to be involved in endo-/exocytosis; PM: parasite plasma membrane; PPV: parasitophorous vacuole; PVM: parasitophorous vacuole membrane; FV: food vacuole; IEM: Immune electron microscopy; R: ring stage; T: trophozoite stage; S: schizont stage; M: merozoite; MC: Maurer's clefts; GO: gene ontology term from (http://plasmodb.org); MS: mass spectrometry; LC: liquid chromatography; FPIX: ferriprotoporphyrin IX; UniProt refers to http://www.uniprot.org/. Protein identification, modified amino acids of known functional relevance and protein association with lipid rich structures and iron-containing proteins are in bold. O.A. Skorokhod et al. / Free Radical Biology and Medicine 89 (2015) 624 –637 634 become activated by heme-iron within the lipid environment of the membrane and oxidatively damage parasite membranes. In vitro, ROS-dependent luminol-enhanced luminescence revealed ROS production in an oxygenated cell-free system (PBS, pH 7.2) elicited by the sole addition of plakortin to purified npRBC membranes, while plakortin alone in absence of membranes did not generate ROS. Soluble hemin enhanced and substantially extended the lipid-induced ROS production (Schwarzer E, unpublished observations). These in vitro data point at the importance of lipid structures for plakortin action, in accordance with ROS enrichment in the parasite/parasitophorous vacuole and in the plasma membrane of plakortin-treated pRBCs (Fig. 5). The crucial role of lipids and heme may be underscored by the accumulation and activation of antimalarial trioxanes by neutral lipid-associated heme, resulting in enhanced ROS generation and oxidative membrane damage [6,61]. Further studies are needed to tackle the role of free heme or iron to activate plakortin in the intact pRBC and to identify the subcellular hotspot for ROS generation elicited by plakortin. The functional importance of lipids for plakortin action was recapitulated by the significant lipid peroxidation observed in pRBCs treated with plakortin and measured here by the appearance of the breakdown product of peroxidized fatty acids, the bioactive 4-HNE. Here, plakortin was shown to induce the covalent binding of 4-HNE to lysine, cysteine and histidine residues in specific proteins (see Table 2 and Tables 1 and 2 in [22]). Identification of the modified P. falciparum proteins and the aminoacid residues bound to 4-HNE was performed by a combined immunochemistry and mass spectrometry approach as discussed below. Binding of the hydrophobic 9-carbon chain of 4-HNE to multiple sites of proteins may considerably change their structure and function with substantial consequences [62–65]. and may clarify the potential impact of 4-HNE conjugates on parasite functionality and growth. 4.3.1. P. falciparum heat shock protein 70 (PfHsp 70-1 and PfHSP 70- 2) Heat shock proteins (Hsp) are considered essential to P. falciparum survival [27,33], as invasion and remodelling of host RBCs by the parasite require correct folding, transport and export of a large number of newly synthesized proteins under conditions of thermal and oxidative stress [66–68]; and elevated levels of PfHsp70-1 were found in an artemisinin-resistant falciparum strain [69]. PfHsp70-1, highly expressed in all parasite blood stages, was suggested to be involved in clathrin-mediated vesicular transport (http://sites.huji.ac.il/malaria), [34], and recombinant PfHsp70-1 suppresses protein aggregation and assists protein refolding [70]. For PfHsp70-1 functionality, the essential ATPase activity and nucleotide exchange are regulated by nucleotide exchange factors (NEFs) and co-chaperones that interact with Hsp70-1 and allow the refolding of peptide substrates in ATP-consuming cycles. Twenty amino acid residues in PfHsp70-1 are assumed to form the interface between Hsp70 and NEF and regulate the ATPase activity [71]. Linkage of 4-HNE to Lysine-260 (see Table 2), one crucial residue for the binding of NEFs, may hinder protein-protein interaction, and hence the release of correctly folded peptides. A disturbed interaction between the chaperone and the peptides under export may result in misfolding and dot-like precipitates as observed in plakortin-treated pRBCs. Therefore, we suggest that the modification of a functional relevant amino acid in Hsp70-1 may play a role in the anti-parasitic activity of plakortin. This view is underpinned by the antimalarial activity of small molecules that selectively modulate the interaction of PfHsp70-1 with co-chaperones [72]. The binding of ferriprotoporphyrin IX [35] and association with the food vacuole [31] of PfHsp70-1 may explain the modification of as many as 21 amino acids with 4-HNE upon plakortin treatment (Table2). PfHsp70-2, the corresponding endoplasmatic reticulum Hsp and homolog of the human protein exporter BiP/Grp78 [73] was suggested to be associated with several protein families such as the transcriptional and translational machinery and crucially involved in cell survival [74]. 4.3.2. V-type proton ATPase catalytic subunit A (EC 3.6.3.14) Plakortin treatment of pRBCs was accompanied by the binding of 4-HNE to two lysine residues in the C-terminal domain of Pf V-type proton ATPase catalytic subunit A. The V-type ATPase controls the pH of the parasite cytosol and food vacuole [38,39], pumping protons across the membrane out of the cytosol and into the parasitophorous vacuole and food vacuole. The sensitivity of the pump against oxidation was shown in intact parasites and isolated vacuoles [39–40]. The two 4-HNE molecules bound to the C-terminal domain may alter the conformational arrangement of the helices and lead to stability loss and functional impairment. The main consequences could be, first, the acidification of the parasite cytosol along with inhibition of glycolysis and ATP depletion; second, the alkalisation of the FV leading to impaired hemoglobin degradation and hemozoin production; and third, the alkalization of the parasitophorous intermembrane space with negative effects on protein traffic and export. Indeed, challenging the pump in situ with H2O2 induced acidification of the parasite cytosol [39] and alkalinization of the food and parasitophorous vacuole. Here, treatment of rings with plakortin produced accumulation of vesicles in the host cell, a staining defect of the parasitophorous vacuole and, at 10 mM, loss of the integrity of the parasite as expected after ATP depletion. 4.3.3. Enolase (EC 4.2.1.11) Plakortin-induced 4-HNE production modified enolase at several amino acid residues though not at the active site. Molecular interaction studies showed that the enzyme is complexed with PfHsp70-1, iron superoxide dismutase and the secretory multifunctional serine protease DegP and suggested to protect the parasite against thermal and oxidative stress [36]. The iron-containing superoxide dismutase in the complex and the

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association with the food vacuole [43] may explain the large number of modification sites of enolase when plakortin was added. As a glycolytic enzyme, enolase contributes to the main ATP-generating activity of the parasite, its structural modification may lead to activity loss, ATP decrease and functional and structural damage of the parasite [43]. 4.3.4. Vacuolar protein sorting-associated protein 11 4-HNE-conjugation sites are distributed along the whole protein and 3 of the 13 modifications reside in the clathrin heavy chain repeat domain, which assists in vacuole maintenance and protein sorting also in non-clathrin proteins. The repeats in the Pf putative protein are predicted (http://plasmodb.org) to mediate protein-protein interactions, or perform clathrin-like functions in the vesicle export. In absence of experimental data on the Pf protein consequences of its modification for vacuole trafficking remain uncertain. 4.3.5. Dynein heavy chain-like protein Dyneins are subcellular motor proteins implicated in the parasite replication, shuttle on cytoplasmic microtubules and transcellular transport in the pRBC [48]. Pf-dynein is expressed in late asexual stages [47] and suggested to play a role in invasion of RBCs by merozoites [46]. It is tempting to predict that the modifications described here for the first time may be related to the inhibition of reinvasion observed after plakortin (see Fig. 4C). Finally, it should be noted that, first, falciparum proteins that bound 4-HNE after plakortin-treatment were unaffected in absence of plakortin; second, all 4-HNE-modified proteins were localized in or at membrane-rich structures like PPV, FV, vesicles, and MCs and third, often associated with redox-active iron proteins, ferriprotoporphyrin or zink. The lipid-rich structures are a first line ‘scavenger’ for 4-HNE shed from peroxidized fatty acids by plakortin treatment. From in vitro studies we know that only lipid-rich membranes coupled to heme or Fe-containing proteins make plakortin a dangerous source of ROS, while plakortin alone in aqueous solution was a poor ROS producer. 5. Conclusions and outlook Focus of this study were pro-oxidative activities of plakortin that may offer a novel mechanistic explanation of its antimalarial activity, based on the plakortin-elicited lipoperoxidation and formation of a number of 4-HNE modified/inhibited critical parasite proteins. A large number of studies have shown that 4-HNE-protein conjugates may explain a multiplicity of diverse effects, such as inhibition cell cycle in differentiating erythroid cells; modulation of inflammation; breakage of DNA; inhibition of cell motility; and be responsible for a large number of other bioeffects [62–64]. The classical interpretation of antimalarial activity of dioxanes and trioxanes relies on the generation of C-centered radicals able to alkylate specific, functionally sensible targets leading to parasite damage and death, and on the production of ROS that may damage oxidation-sensitive targets and also lead to parasite destruction. Here we provide evidence that a third anti-parasite mechanism was operational after plakortin treatment, namely parasite damage elicited by 4-HNE, a secondary cytotoxic lipoperoxidation products. Cytotoxic secondary lipoperoxidation products may possibly help clarifying other pharmacologically interesting, malaria-unrelated activities of artemisinin [75] difficult to rationalize and harmonize with its antimalarial properties. Authorship Contribution: O.A.S., D.D., E.V., E.S., D.U., V.G., A.N., G.M., M.P., O. T.-S. performed the experiments; O.A.S, E.S., P.A. designed the experiments; all authors interpreted the results; O.A.S., D.D., P.A. and E.S. wrote and all authors revised the paper. Conflict-of-interest disclosure The authors declare no competing financial interests. Acknowledgments We wish to thank Dr. Giuseppina Chianese for assistance with isolation of plakortin and Dr. Emiliano Panieri (Department of Molecular Biotechnology and Health Sciences, University of Torino) for the gift of DHE. The study was supported by the Italian Ministry for Research (MIUR; PRIN2008, Leads ad Attività Antimalarica di Origine Naturale, Isolamento, Ottimizzazione e Valutazione Biologica), MIUR grant 2010C2LKKJ-007 (ES), the University of Torino, intramural funds (E.S.; O.A.S.) and EviMalaR (European Virtual Institute dedicated to Malaria Research) Project No.242095 (P.A.). D.D. was granted by CONACYT Mexico and the Italian Ministry of Foreign Affairs (MAE). References [1] A.M. Dondorp, F. Nosten, P. Yi, D. Das, A.P. Phyo, J. Tarning, et al., Artemisinin resistance in Plasmodium falciparum malaria, New Engl. J. Med 361 (2009) 455–467. [2] J.G. Breman, Resistance to artemisinin-based combination therapy, Lancet Infect. Dis. 12 (11) (2012) 820–822. [3] E.A. Ashley, M. Dhorda, R.M. Fairhurst, C. Amaratunga, P. Lim, S. Suon, et al., Tracking Resistance to Artemisinin Collaboration (TRAC). Spread of artemisinin resistance in Plasmodium falciparum malaria, N. Engl. J. Med. 371 (5) (2014) 411–423. [4] T.N.C. Wells, P. Alonso, W. Gutteridge, New medicines to improve control and contribute to the eradication of malaria, Nat. Rev. Drug Disc 8 (2009) 879–891. [5] S.R. Meshnick, T.E. Taylor, S. Kamchonwongpaisan, Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy, Microbiol. Rev. 60 (2) (1996) 301–315. [6] C.L. Hartwig, E.M. Lauterwasser, S.S. Mahajan, J.M. Hoke, R.A. Cooper, A. R. Renslo, Investigating the antimalarial action of 1,2,4-trioxolanes with fluorescent chemical probes, J Med Chem. 54 (23) (2011) 8207–8213. [7] S.A. Charman, S. Arbe-Barnes, I.C. Bathurst, R. Brun, M. Campbell, W. N. Charman, et al., Synthetic ozonide drug candidate OZ439 offers new hope for a single-dose cure of uncomplicated malaria, Proc. Natl. Acad. Sci. USA 108 (11) (2011) 4400–4405. [8] K. Gademann, J. Kobylinska, Antimalarial natural products of marine and freshwater origin, Chem. Rec. 9 (3) (2009) 187–198. [9] E. Fattorusso, O. Taglialatela-Scafati, Marine antimalarials, Mar. Drugs. 7 (2) (2009) 130–152. [10] C. Fattorusso, G. Campiani, B. Catalanotti, M. Persico, N. Basilico, S. Parapini, et al., Endoperoxide Derivatives from Marine Organisms: 1,2-Dioxanes of the Plakortin Family as Novel Antimalarial Agents, J. Med. Chem. 49 (2006) 7088–7094. [11] E. Fattorusso, S. Parapini, C. Campagnuolo, N. Basilico, O. Taglialatela-Scafati, D. Taramelli, Activity against Plasmodium falciparum of cycloperoxide compounds obtained from the sponge Plakortis simplex, J. Antimicrob. Chemother. 50 (2002) 883–888. [12] M.D. Higgs, D.J. Faulkner, Plakortin, an antibiotic from Plakortis halichondrioides, J. Org. Chem. 43 (18) (1978) 3454–3457. [13] F. Cafieri, E. Fattorusso, O. Taglialatela-Scafati, A. Ianaro, Metabolites from the sponge Plakortis simplex,

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Determination of absolute stereochemistry of plakortin. Isolation and stereostructure of three plakortin related compounds, Tetrahedron. 55 (1999) 7045–7056. [14] S. Uyoga, O.A. Skorokhod, M. Opiyo, E.N. Orori, T.N. Williams, P. Arese, et al., Transfer of 4-hydroxynonenal from parasitized to non-parasitized erythrocytes in rosettes. Proposed role in severe malaria anemia, Br. J. Haematol. 157 (1) (2012) 116–124. [15] E. Schwarzer, H. Kuhn, E. Valente, P. Arese, Malaria-parasitized erythrocytes and haemozoin nonenzymatically generate large amounts of hydroxy fatty acids that inhibit monocyte functions, Blood 101 (2003) 722–728. [16] V. Gallo, O.A. Skorokhod, E. Schwarzer, P. Arese, Simultaneous determination of phagocytosis of Plasmodium falciparum-parasitized and non-parasitized red blood cells by flow cytometry, Malar. J. 11 (2012) 428. [17] J.W. Lin, R. Spaccapelo, E. Schwarzer, M. Sajid, T. Annoura, K. Deroost, et al., Replication of Plasmodium in reticulocytes can occur without hemozoin formation, resulting in chloroquine resistance, J. Exp. Med. 212 (6) (2015) 893–903. [18] B. Kalyanaraman, V. Darley-Usmar, K.J.A. Davies, P.A. Dennery, H.J. Forman, M. B. Grisham, et al., Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations, Free Radical Biology & Medicine 52 (1) (2012) 1–6. [19] H.M. Peshavariya, G.J. Dusting, S. Selemidis, Analysis of dihydroethidium fluorescence for the detection of intracellular and extracellular superoxide produced by NADPH oxidase, Free Radic Res. 41 (6) (2007) 699–712. [20] O. Skorokhod, E. Schwarzer, T. Grune, P. Arese, Role of 4-hydroxynonenal in the hemozoin-mediated inhibition of differentiation of human monocytes to dendritic cells induced by GM-CSF/IL-4, Biofactors. 24 (2005) 283–289. [21] G. Mandili, A. Khadjavi, V. Gallo, V.G. Minero, L. Bessone, F. Carta, et al., Characterization of the protein ubiquitination response induced by Doxorubicin, FEBS J. 279 (12) (2012) 2182–2191. [22] Schwarzer, E.; Gallo, V.; Valente, E.; Ulliers, D.; Taglialatela-Scafati, O.; Arese, P.; Skorokhod, O.A. Preferential binding of 4-hydroxynonenal to lysine residues in specific parasite proteins in plakortin-treated Plasmodium falciparum-parasitized red blood cells Data in Brief (submitted). [23] O. Taglialatela-Scafati, E. Fattorusso, A. Romano, F. Scala, V. Barone, P. Cimino, et al., Insight into the mechanism of action of plakortins, simple 1,2-dioxane antimalarials, Org. Biomol. Chem. 8 (4) (2010) 846–856. [24] A. Gomes, E. Fernandes, J.L. Lima, Fluorescence probes used for detection of reactive oxygen species, J. Biochem. Biophys. Methods. 65 (2-3) (2005) 45–80. [25] A. Skorokhod, E. Schwarzer, G. Gremo, P. Arese, HNE produced by the malaria parasite Plasmodium falciparum generates HNE-protein adducts and decreases erythrocyte deformability, Redox Rep. 12 (1) (2007) 73–75. [26] E.R. Pesce, P. Acharya, U. Tatu, W.S. Nicoll, A. Shonhai, H.C. Hoppe, et al., The Plasmodium falciparum heat shock protein 40, Pfj4, associates with heat shock protein 70 and shows similar heat induction and localisation patterns, Int. J. Biochem. Cell Biol. 40 (12) (2008) 2914–2926. [27] P. Acharya, R. Kumar, U. Tatu, Molecular chaperoning a cellular upheaval in 636 O.A. Skorokhod et al. / Free Radical Biology and Medicine 89 (2015) 624–637 malaria: Heat shock proteins in Plasmodium falciparum, Mol. Biochem. Parasitol. 153 (2) (2007) 85–94. [28] B. Kappes, B.W. Suetterlin, R. Hofer-Warbinek, R. Humar, R.M. Franklin, Two major phosphoproteins of Plasmodium falciparum are heat shock proteins, Mol. Biochem. Parasitol. 59 (1) (1993) 83–94. [29] N. Issar, E. Roux, D. Mattei, A. Scherf, Identification of a novel post-translational modification in Plasmodium falciparum: protein sumoylation in different cellular compartments, Cell. Microbiol. 10 (10) (2008) 1999–2011. [30] N. Kumar, G. Koski, M. Harada, M. Aikawa, H. Zheng, Induction and localization of Plasmodium falciparum stress proteins related to the heat shock protein 70 family, Mol. Biochem. Parasitol. 48 (1991) 47–58. [31] M. Lamarque, C. Tastet, J. Poncet, E. Demettre, P. Jouin, H. Vial, et al., Food vacuole proteome of the malarial parasite Plasmodium falciparum, Proteomics Clin. Appl. 2 (2008) 1361–1374. [32] L. Vincensini, S. Richert, T. Blisnick, A. Van Dorsselaer, E. Leize-Wagner, T. Rabilloud, et al., Proteomic analysis identifies novel proteins of the Maurer's Clefts, a secretory compartment delivering Plasmodium falciparum proteins to the surface of its host cell, Mol. Cell. Prot 4 (2005) 582–593. [33] J. Nyalwidhe, K. Lingelbach, Proteases and chaperones are the most abundant proteins in the parasitophorous vacuole of Plasmodium falciparum-infected erythrocytes, Proteomics. 6 (2006) 1563–1573. [34] J.M. Bautista, P. Marín-García, A. Diez, I.G. Azcárate, A. Puyet, Malaria proteomics: insights into the parasite-host interactions in the pathogenic space, Proteomics J 97 (2014) 107–125. [35] O. Famin, H. Ginsburg, The treatment of Plasmodium falciparum-infected erythrocytes with chloroquine leads to accumulation of ferriprotopor phyrin IX bound to particular parasite proteins and to the inhibition of the parasite's 6-phosphogluconate dehydrogenase, Parasite. 10 (1) (2003) 39–50. [36] S. Sharma, M. Jadli, A. Singh, K. Arora, P. Malhotra, A secretory multifunctional serine protease, DegP of Plasmodium falciparum, plays an important role in thermo-oxidative stress, parasite growth and development, FEBS J. 281 (6) (2014) 1679–1699. [37] S.C. Oehring, B.J. Woodcroft, S. Moes, J. Wetzel, O. Dietz, A. Pulfer, et al., Organellar proteomics reveals hundreds of novel nuclear proteins in the malaria parasite Plasmodium falciparum, Genome Biol. 13 (11) (2012) R108. [38] B.N. Pease, E.L. Huttlin, M.P. Jedrychowski, E. Talevich, J. Harmon, T. Dillman, et al., Global analysis of protein expression and phosphorylation of three stages of Plasmodium falciparum intraerythrocytic development, J Proteome Res. 12 (9) (2013) 4028–4045. [39] M. Hayashi, H. Yamada, T. Mitamura, T. Horii, A. Yamamoto, Y. Moriyama, Vacuolar H(þ)-ATPase localized in plasma membranes of malaria parasite cells, Plasmodium falciparum, is involved in regional acidification of parasitized erythrocytes, J. Biol. Chem. 275 (2000) 34353–34358. [40] D.A. Van Schalkwyk, K.J. Saliba, G.A. Biagini, P.G. Bray, K. Kirk, Loss of pH control in Plasmodium falciparum parasites subjected to oxidative stress, PLoSOne 8 (3) (2013) e58933. [41] K.J. Saliba, R.J. Allen, S. Zissis, P.G. Bray, S.A. Ward, K. Kirk, Acidification of the malaria parasite's digestive vacuole by a Hþ-ATPase and a Hþ-pyrophosphatase, J. Biol. Chem. 278 (8) (2003) 5605–5612. [42] I.P. Bhowmick, N. Kumar, S. Sharma, I. Coppens, G.K. Jarori, Plasmodium falciparum enolase: stage-specific expressionand sub-cellular localization, Malar. J. 8 (2009) 179. [43] I. Pal-Bhowmick, M.

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Mehta, I. Coppens, S. Sharma, G.K. Jarori, Protective properties and surface localization of Plasmodium falciparum enolase, Infect. Immun. 75 (11) (2007) 5500–5508. [44] S. Das, S. Shevade, D.J. LaCount, G.K. Jarori, Plasmodium falciparum enolase complements yeast enolase functions and associates with the parasite food vacuole, Mol. Biochem. Parasitol. 179 (2011) 8–17. [45] M. Lanzer, H. Wickert, G. Krohne, L. Vincensini, C. Braun Breton, Maurer'- sclefts: A novel multi-functional organelle in thecytoplasm of Plasmodium falciparum-infected erythrocytes, Int. J. Par 36 (1) (2006) 23–36. [46] R.E. Fowler, A.M. Smith, J. Whitehorn, I.T. Williams, L.H. Bannister, G. H. Mitchell, Microtubule associated motor proteins of Plasmodium falciparum merozoites, Mol. Biochem. Parasitol. 117 (2) (2001) 187–200. [47] M.J. Gardner, N. Hall, E. Fung, O. White, M. Berriman, R.W. Hyman, et al., Genome sequenceofthe human malaria parasite Plasmodium falciparum, Nature. 419 (2002) 498–511. [48] J.C. Pinder, R.E. Fowler, L.H. Bannister, A.R. Dluzewski, G.H. Mitchell, Motile systems in malaria merozoites: How is the red blood cell invaded? Parasitol. Today. 16 (6) (2000) 240–245. [49] J. Golenser, J.H. Waknine, M. Krugliak, N.H. Hunt, G.E. Grau, Current perspectives on the mechanism of action of artemisinins, Int. J. Parasitol. 36 (14) (2006) 1427–1441. [50] C.W. Jefford, Why artemisinin and certain synthetic peroxides are potent antimalarials. Implications for the mode of action, Curr. Med. Chem. 15 (2001) 1803–1826. [51] S.R. Meshnick, Artemisinin: mechanisms of action, resistance and toxicity, Int. J. Parasitol. 32 (2002) 1655–1660. [52] H. Noedl, Y. Se, K. Schaecher, B.L. Smith, D. Socheat, M.M. Fukuda, Evidence of artemisinin-resistant malaria in western Cambodia, N. Engl. J. Med. 359 (24) (2008) 2619–2620. [53] World Health Organization (2013) World Malaria Report. The World Health Organization Website (online). ⟨http://www.who.int/malaria/publications/ world_malaria_report_2013/en/⟩. [54] M. Persico, S. Parapini, G. Chianese, C. Fattorusso, M. Lombardo, L. Petrizza, et al., Further optimization of plakortin pharmacophore: structurally simple 4-oxymethyl-1,2-dioxanes with promising antimalarial activity, Eur. J. Med. Chem. 70 (2013) 875–886. [55] K. Becker, L. Tilley, J.L. Vennerstrom, D. Roberts, S. Rogerson, H. Ginsburg, Oxidative stress in malaria parasite-infected erythrocytes: host-parasite interactions, Int J Parasitol. 34 (2) (2004) 163–189. [56] K. Luersen, R.D. Walter, S. Mueller, Plasmodium falciparum-infected red blood cells depend on a functional glutathione de novo synthesis attributable to an enhanced loss of glutathione, Biochem J. 346 (2000) 545–552. [57] Y. Maeno, T. Toyoshima, H. Fujioka, Y. Ito, S.R. Meshnick, A. Benakis, et al., Morphologic effects of artemisinin in Plasmodium falciparum, Am. J. Trop. Med. Hyg. 49 (4) (1993) 485–491. [58] D.W. Wilson, C. Langer, C.D. Goodman, G.I. McFadden, J.G. Beeson, Defining the timing of action of antimalarial drugs against Plasmodium falciparum, Antimicrob. Agents Chemother. 57 (3) (2013) 1455–1467. [59] R.K. Haynes, K.W. Cheu, D. N'Da, P. Coghi, D. Monti, Considerations on the mechanism of action of artemisinin antimalarials: Part 1-The' 'carbon radical' and' 'heme' hypotheses, Infec. Disord. Drug Targets 13 (2013) 217–277. [60] T. Antoine, N. Fisher, R. Amewu, P.M. O'Neill, S.A. Ward, G.A. Biagini, Rapid kill of malaria parasites by artemisinin and semi-synthetic endoperoxides involves ROS-dependent depolarization of the membrane potential, J. Antimicrob. Chemother. 69 (4) (2014) 1005–1016. [61] P.A. Berman, P.A. Adams, Artemisinin enhances heme-catalysed oxidation of lipid membranes, Free Radic. Biol. Med. 22 (7) (1997) 1283–1288. [62] G. Poli, R.J. Schaur, W.G. Siems, G. Leonarduzzi, 4-Hydroxynonenal: a membrane lipid oxidation product of medicinal interest, Med. Res. Rev. 28 (4) (2008) 569–631. [63] O.A. Skorokhod, L. Caione, T. Marrocco, G. Migliardi, V. Barrera, P. Arese, et al., Inhibition of erythropoiesis in malaria anemia: role of haemozoin and haemozoin generated 4-hydroxynonenal, Blood. 116 (2010) 4328–4337. [64] O.A. Skorokhod, V. Barrera, R. Heller, F. Carta, F. Turrini, P. Arese, et al., Malarial pigment hemozoin impairs chemotactic motility and transendothelial migration of monocytes via 4-hydroxynonenal, Free Radic. Biol. Med. 75 (2014) 210–221. [65] E. Schwarzer, P. Arese, O.A. Skorokhod, Role of the lipoperoxidation product 4-hydroxynonenal in the pathogenesis of severe malaria anemia and malaria immunodepression, Oxid. Med. Cell. Longev 2015 (2015) 638416. [66] A. Craig, A. Scherf, Molecules on the surface of the Plasmodium falciparum infected erythrocyte and their role in malaria pathogenesis and immune evasion, Mol. Biochem. Parasitol. 115 (2001) 129–143. [67] K. Haldar, N. Mohandas, Erythrocyte remodeling by malaria parasites, Curr. Opin. Hematol. 14 (2007) 203–209. [68] O.B. Akide-Ndunge, E. Tambini, G. Giribaldi, P.J. McMillan, S. Müller, P. Arese, et al., Co-ordinated stage-dependent enhancement of Plasmodium falciparum antioxidant enzymes and heat shock protein expression in parasites growing in oxidatively stressed or G6PD-deficient red blood cells, Malar. J. 8 (2009) 113. [69] B. Witkowski, J. Lelièvre, M.J.L. Barragán, V. Laurent, X. Su, A. Berry, et al., Increased tolerance to artemisinin in Plasmodium falciparum is mediated by a quiescence mechanism, Antimicrob. Agents Chemother. 54 (2010) 1872–1877. [70] G. Misra, R. Ramachandran, Hsp70-1 from Plasmodium falciparum: protein stability, domain analysis and chaperone activity, Biophys. Chem. 142 (1-3) (2009) 55–64. [71] A. Shonhai, A. Boshoff, G.L. Blatch, The structural and functional diversity of Hsp70 proteins from Plasmodium falciparum, Protein Sci. 16 (9) (2007) 1803–1818. [72] I.L. Cockburn, A. Boshoff, E.R. Pesce, G.L. Blatch, Selective modulation of plasmodial Hsp70s by small molecules with antimalarial activity, Biol. Chem. 395 (11) (2014) 1353–1362. [73] M.J. Gething, Role and regulation of the ER chaperone BiP, Semin. Cell. Dev. Biol. 10 (1999) 465–472. [74] D.J. LaCount, M. Vignali, R. Chettier, A. Phansalkar, R. Bell, J.R. Hesselberth, et al., A protein interaction network of the malaria parasite Plasmodium falciparum, Nature. 438 (2005) 103–107. [75] W.E. Ho, H.Y. Peh, T.K. Chan, W.S. Wong, Artemisinins: pharmacological actions beyond anti-malarial, Pharmacol. Ther. 142 (1) (2014) 126–139.

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