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Preferential binding of 4-hydroxynonenal to lysine residues in specific parasite proteins in plakortin-treated *Plasmodium falciparum*-parasitized red blood cells

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Abstract

The data show the frequencies by which the amino acid residues lysine, histidine and cysteine of six proteins of the malaria parasite *Plasmodium falciparum* are post-translationally modified by the lipoperoxydation endproduct 4-hydroxynonenal after challenging the parasitized red blood cell with plakortin. Plakortin is an antimalarial endoperoxide whose molecular anti-parasitic effect is described in [1]. The preference for lysine modifications is compared with other eukaryotic cells. Plakortin did not elicit hemoglobin leakage from host red blood cells and did not oxidize reduced glutathione.

Specifications Table

Subject area	Biology
More specific subject area	Molecular pharmacology, antimalarial drug action, lipoperoxidation in malaria pathophysiology, proteomic assessment of post-translational modifications
Type of data	Tables, graphs
How data was acquired	MALDI-TOF spectrometer (MALDI micro MX (Waters, Milford, MA, USA); Luminometer Sirius (Berthold, Pforzheim, Germany)
Data format	Processed data
Experimental factors	Parasitized and non-parasitized red blood cells were kept in in vitro

	cell culture up to 24 h. Chemical extraction for parameter assessment.
Experimental features	Heme-dependent luminol-enhanced luminescence assay for hemoglobin. Thiol-reaction with DTNB and OD at 412 nm. Mass spectrometric analysis of peptides after trypsin-digestion of proteins.
Data source location	University of Torino, Torino, Italy
Data accessibility	Data are provided with this article and are related to [1]

Value of the data

•The data show how endoperoxide-elicited oxidative stress might specifically target parasite proteins of functional importance by binding the lipoperoxidation endproduct 4-HNE inducing parasite damage at still modest free radical concentration.

•Data on endoperoxide-elicited modifications of proteins of a unicellular parasite as molecular cause for cell death may be of interest for similar studies with other microorganisms.

•Data for lethal modifications of specific microbial proteins with lipoperoxidation endproducts such as 4-HNE may suggest a novel strategy for high throughput drug research, e.g. antimalarials.

•The data on host red blood cells (RBC) intactness may be useful to monitor host cell damage by redox active substances.

Data

Frequencies of lysine residues modified by 4-HNE

In Table 1 we list the numbers of lysine, histidine and cysteine residues (K, H and C respectively) that were found conjugated with 4-HNE and compare it with the total number of each of these amino acids in the respective protein. The complete list of 4-HNE modified plasmodium falciparum proteins detected after plakortin treated and specific sites of modification are reported in [1].

There is no particular imbalance between the portion of 4-HNE-conjugated amino acids in the proteins extracted from plakortin treated parasites. We note that Plasmodium proteins contain elevated % of asparagine and lysine residues [2], even more then in vertebrates, where K frequency is 2-3 times higher than that of H and C [3].

Evidently, availability of the amino acid residue for 4-HNE is a crucial factor for binding. C residues engaged in disulfide bridges are no ligands for HNE, while the basic amino acid K might be more frequently exposed to the protein-solvent interface compared to H, and hence be more accessible to 4-HNE binding [4].

Table 1. Lysine (K), histidine (H) and cysteine (C) frequency in the primary structure of P. falciparum proteins. Uniprot: Universal Protein Resource, www.uniprot.org/; NCBI GI: The National Center for Biotechnology Information GenInfo Identifier, www.ncbi.nlm.nih.gov/; PlasmoDB: the Plasmodium genome resource, http://PlasmoDB.org.

	4-HNE-MODIFIED PROTEIN (NAME, ALIAS; IDs)	**4-HNE BINDING SITES (distribution between K, H, C)**	**K, H, C count in the protein sequence and the total number of amino acids**
1	***HSP70-1, HSP70_PLAFA** Uniprot: Q8IB24_PLAF7 NCBI GI:124512406 **PlasmoDB: PF3D7_0818900**	K:17 H:2 C:2	K:54 H:7 C:9 Tot 680 AA
2	***V-type proton ATPase catalytic subunit A (EC 3.6.3.14)**, vacuolar ATP synthase subunit a (vapA) Uniprot: Q76NM6, VATA_PLAF7; NCBI GI:124512982 **PlasmoDB: PF3D7_1311900**	K:2 H:0 C:0	K:42 H:6 C:11 Tot 611 AA
3	***Enolase (EC 4.2.1.11)** phosphopyruvate hydratase Uniprot: Q8IJN7, ENO_PLAF7; NCBI GI:124802328 **PlasmoDB: PF3D7_1015900**	K:10 H:1 C:1	K:38 H:4 C:6 Tot 446 AA
4	***Vacuolar protein sorting-associated protein 11, putative,** former RING finger protein PFE0100w, Uniprot: Q8I480-ZNRF2_PLAF7 NCBI GI:124505963 **PlasmoDB: PF3D7_0502000**	K:13 H:0 C:0	K:165 H:31 C:20 Tot 1272 AA

5	[§]**DYHC1_PLAF7; Dynein heavy chain-like protein,** Uniprot: Q 8IBG1 NCBI GI:296004907 **PlasmoDB:PF3D7_0729900**	K:0 H:3 C:0	K:477 H:79 C:69 Tot 4985 AA
6	[§]**Heat shock protein 70 (HSP70-2);** BiP;GRP78 Uniprot: Q8I2X4_PLAF7 NCBI GI:124506906 **PlasmoDB:PF3D7_0917900**	K:4 H:6 C:2	K:63 H:6 C:2 Tot 652 AA

Table 2. Examples from the literature for 4-HNE binding site distribution between K, H, C in selected proteins.

	4-HNE-MODIFIED PROTEIN (NAME, ALIAS; IDs)	4-HNE BINDING SITE distribution between K, H, C	K, H, C count in protein sequence and the number of all amino acids	Reference
1	**Liver fatty acid-binding protein, L-FABR, P12710**	K:4 H:1 C:1	K:17 H:1 C:1 Tot 127 AA	[5]
2	**Cytochrome c, P00004**	K:10 H:1 C:0	K:19 H:3 C:2 Tot 105 AA	[6]
	Alpha-synuclein, SNCA, P37840	K:2 H:1 C:0	K:15 H:1 C:1 Tot 140 AA	[7]

Data for hemoglobin release from RBC under plakortin treatment

Culture of RBC infected with trophozoite-stage *Plasmodium falciparum* were treated with 0-10 µM of plakortin and hemoglobin release was measured in culture supernatant (Figure 1). The concentration is indicated in nmol/l. The concentration in the whole RBC suspension was 1mM and assessed after complete lysis of RBCs in NaOH/Triton: this is the maximal achievable concentration of hemoglobin in the supernatant corresponding to 100% RBC-lysis.

As shown in Figure 1 the hemoglobin is not released from RBC even under maximum applied concentration of 10 µM. Very low lysis is detectable in non-parasitized RBC (npRBC) and a still very modest, although double as high value in RBCs parasitized with mature forms of *P.falciparum*- the trophozoite. This elevated value in trophozoites depends on the parasite biology and plakortin independent. Plakortin has no effect on the barrier function of the host RBC irrespective whether parasitized or not.

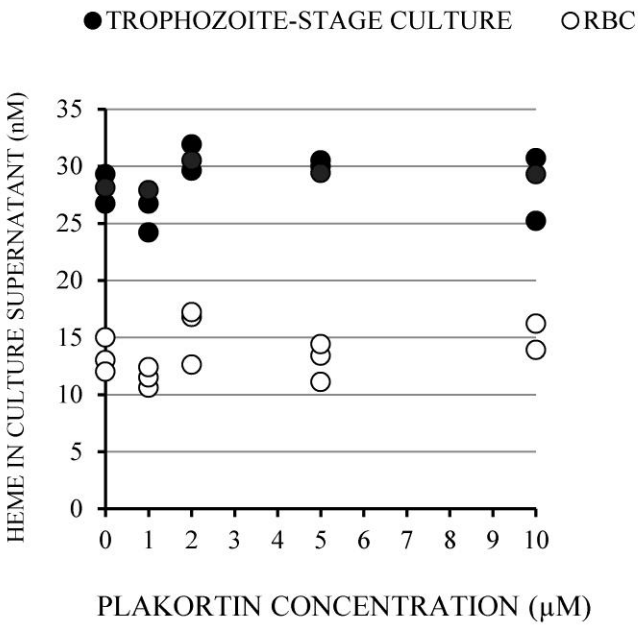

Figure 1. Hemoglobin release from incubated trophozoite-stage cultures and npRBC measured as heme concentration in culture supernatant. A single point represent the mean of 3 replicates of one experiment. N=3 independent experiments with 3 different blood donors.

Reduced glutathione concentrations in RBC after plakortin treatment

Reduced glutathione (GSH) was quantified in RBCs kept at 5% hematocrit in PBS supplemented with 20 mM glucose and plakortin for 24 h at 37°C. As presented in Figure 2 the GSH level in RBC remained unchanged after plakortin supplementation in a wide concentration range from 1 to 100 μM.

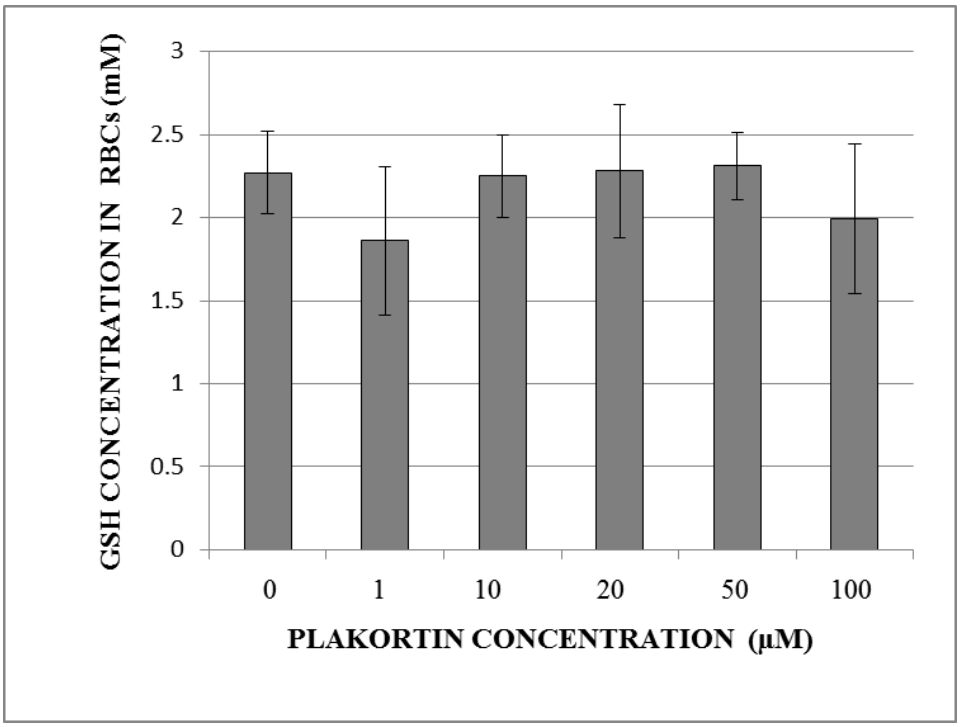

Fig. 2 Quantification of reduced glutathione (GSH) in RBCs treated or not with plakortin (1-100 µM, final concentration). Columns present mean GSH ± SD values from 2 replicates performed with RBCs from two donors.

Experimental Design, Materials and Methods

Unless otherwise stated all materials were obtained from Sigma-Aldrich (St. Louis, MO, USA). Plakortin, methyl 4,8-diethyl-6-methyl-3,6-peroxy-9-dodecenoate, was obtained from the organic extract of the Caribbean sponge *Plakortis simplex* and purified by combination of column chromatography and HPLC as described [8]. The final purity of the compound was $\geq$ 99%.

In vitro cultivation of Plasmodium falciparum and stage-specific enrichment of parasitized red blood cells

Plasmodium falciparum (Palo Alto strain, Mycoplasma free) parasites were kept in permanent culture as described [9-11]. RBCs that were used for plakortin incubation experiments were from blood samples taken on the same day of the experiment. Cultures were incubated in a humidified CO_2 cell incubator under a $N_2/CO_2/O_2$ atmosphere of 90vol%:5vol%:5vol%.

P**lakortin treatment of parasitized RBCs (pRBC) and npRBC.** 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 -20ºC until use. Synchronous cultures were supplemented with a single dose of 0-10 µM plakortin (final concentration) at trophozoite-stage (26-30 h post-invasion) and kept under standard culture conditions for 12-24 h until analysis. Enriched parasite fractions and npRBCs were treated similarly and kept under the same standard culture conditions.

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 [12-14]. All assays were performed in triplicate.

Preparation of protein extracts from pRBC

Washed and 1700g sedimented npRBCs or pRBCs were hypo-osmotically lysed in a 10–fold excess (v/v) of ice-cold lysis buffer (10mM K2HPO4/KH2PO4 (pH 8.0), supplemented with a protease inhibitor cocktail containing: 1 mM EDTA, 250 µM phenyl-methyl-sulfonyl fluoride (PMSF), 1nM leupeptine, 3 µM pepstatine, the phosphatase inhibitors sodium orthovanadate and sodium fluoride at 1 mM, each and 100 µM Trolox, during 5 min on ice. The ruptured membranes and organelles of parasite origin were sedimented at 16,200g 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 16,200g for 1 min. 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).

Identification of 4-HNE modified proteins by mass spectrometric analysis and peptide mass fingerprinting

For the identification of 4-HNE-conjugated proteins in plakortin-treated parasites matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometric analysis was performed. Proteins extracted from enriched ring and trophozoite stage pRBCs and npRBCs cultured with or without 10 µM plakortin for 3h were separated by SDS-PAGE. Gels were stained with colloidal Coomassie, protein bands of interest excised from the gel and in-gel trypsin digested. 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 [15, 16]. 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 (Uniprot) 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 than 67 for NCBI searches, as suggested by Mascot to be "significant".

GSH assay in RBCs

Assay of GSH in RBCs was performed according to [17]. GSH values were given as mM (μmol/ml packed RBCs).

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).

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