Experimental Parasitology 180 (2017) 64–70

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Full length article

Growth inhibitory effects of standard pro- and antioxidants on the human malaria parasite *Plasmodium falciparum*

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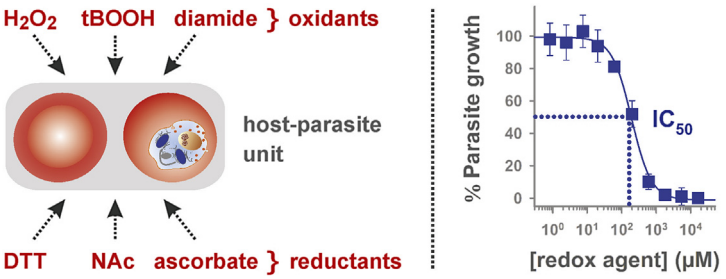
HIGHLIGHTS

- Oxidants and reductants both inhibit the growth of *Plasmodium falciparum*.
- IC_{50} values for comparable redox agents differ by up to three orders of magnitude.
- The host-parasite unit tolerates high levels of H_2O_2, acetylcysteine and ascorbate.
- The host-parasite unit is more susceptible to DTT, tBOOH and diamide.
- Reducing agents can affect SYBR green assays because of albumin gelation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 22 September 2016
Received in revised form
10 January 2017
Accepted 23 February 2017
Available online 24 February 2017

Keywords:
Plasmodium
Erythrocyte
Malaria
IC_{50} value
Redox
Albumin
Ascorbate
Diamide
DTT
H_2O_2
N-acetylcysteine

ABSTRACT

The redox metabolism of the malaria parasite *Plasmodium falciparum* and its human host has been suggested to play a central role for parasite survival and clearance. A common approach to test hypotheses in redox research is to challenge or rescue cells with pro- and antioxidants. However, quantitative data on the susceptibility of infected erythrocytes towards standard redox agents is surprisingly scarce. Here we determined the IC_{50} values of *P. falciparum* strains 3D7 and Dd2 for a set of redox agents using a SYBR green-based growth assay. Parasite killing in this assay required extremely high concentrations of hydrogen peroxide with a millimolar IC_{50} value, whereas IC_{50} values for *tert*-butyl hydroperoxide and diamide were between 67 and 121 µM. Thus, in contrast to *tert*-butyl hydroperoxide and the disulfide-inducing agent diamide, the host-parasite unit appears to be very robust against challenges with hydrogen peroxide with implications for host defense mechanisms. *N*-acetylcysteine, ascorbate, and dithiothreitol also had antiproliferative instead of growth-promoting effects with IC_{50} values around 12, 3 and 0.4 mM, respectively. So-called antioxidants can therefore also inhibit parasite growth with implications for clinical trials and studies on 'oxidative stress'. Furthermore, the addition of reductants to parasite cultures resulted in the gelation of albumin, the formation of methemoglobin and hemolysis. These effects can alter the fluorescence in SYBR green assays and have to be taken into account for the determination of IC_{50} values. In summary, standard oxidants and reductants both inhibit the growth of *P. falciparum* with IC_{50} values differing by three orders of magnitude.

1. Introduction

Malaria parasites of the genus *Plasmodium* multiply in hepatocytes and erythrocytes of their vertebrate hosts. The

Abbreviations: DTT, dithiothreitol; H_2O_2, hydrogen peroxide; NAc, *N*-acetyl-L-cysteine; tBOOH, *tert*-butyl hydroperoxide.

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http://dx.doi.org/10.1016/j.exppara.2017.02.017

symptoms of malaria are affected by the parasite strain, the parasitemia in the blood stream as well as the host immune response (Schofield and Grau, 2005; Gazzinelli et al., 2014). Parasite survival and parasite clearance by the immune system have both been hypothesized to depend on the redox metabolism of the host-parasite unit (Becker et al., 2004; Jortzik and Becker, 2012; Müller, 2015; Sorci and Faivre, 2009). For example, the degradation of hemoglobin in the digestive vacuole of malaria parasites was proposed to be a major source of endogenous 'oxidative stress' that affects parasite survival (Becker et al., 2004; Jortzik and Becker, 2012). Oxidative stress of exogenous origin was suggested to be exerted on infected erythrocytes by the host immune system (Jortzik and Becker, 2012; Sorci and Faivre, 2009). In addition, sickle-cell hemoglobin S and glucose-6-phosphate dehydrogenase deficiency – which are both selected genetic traits in malaria-endemic areas (Cappellini and Fiorelli, 2008; Kwiatkowski, 2005) – might also promote oxidative stress in parasitized human erythrocytes. These genetic variations were speculated to trigger a signal that results in the phagocytic clearance of infected erythrocytes similar to senescent erythrocytes (Becker et al., 2004; Luzzatto et al., 1969; 1970; Arese et al., 2005) and to affect the remodeling of the erythrocyte actin cytoskeleton resulting in a reduced cytoadherence (Cyrklaff et al., 2011; 2016; Rug et al., 2014).

Does so-called oxidative stress really play a physiological role for parasite survival or clearance? While several studies point towards a physiological relevance of the redox state of the host-parasite unit for parasite survival and immune responses, mechanistic insights are scarce to support the diverse hypotheses on oxidative stress (which is defined as a prolonged redox imbalance that results in the accumulation of oxidized and damaged molecules (Sies, 1986)). A common approach to test hypotheses in redox research *in vitro* is to challenge or rescue cells using bolus treatments with pro- and antioxidants. Much to our surprise there is rather limited data available regarding the effects of standard redox agents on parasite survival, in particular, there seems to be a lack of IC_{50} values. This lack is also remarkable taking into account that some redox agents have been already used as adjuvants in clinical trials (Isah and Ibrahim, 2014; Charunwatthana et al., 2009; Treeprasertsuk et al., 2003; Watt et al., 2002). In the present study, we determined the IC_{50} values of the oxidizing agents diamide, hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (tBOOH) as well as the reducing agents ascorbate, *N*-acetyl-L-cysteine (NAc) and dithiothreitol (DTT) in a standardized fluorometric growth assay for the blood stages of the human malaria parasite *Plasmodium falciparum*. The IC_{50} values might serve as a guideline to manipulate the redox state of the host-parasite unit and to study its relevance for the pathology of malaria.

2. Material and methods

2.1. Standard cell culture

P. falciparum blood stage parasites of strains Dd2 and 3D7 were cultured according to standard protocols (Trager and Jensen, 1976) at 37 °C, 5% CO_2, 5% O_2, 90% N_2 and 80% humidity in complete RPMI medium with 0.45% (w/v) albumax II, 0.2 mM hypoxanthine, 25 μg/mL gentamicin and human A erythrocytes as previously described (Wezena et al., 2016). The volume of standard cultures was 14 mL and the hematocrit was 3% unless otherwise stated. Synchronized 3D7 parasites were obtained after treatment with 5% sorbitol (Lambros and Vanderberg, 1979). Strain Dd2 was cultured for several weeks to reach a maximum asynchrony.

2.2. IC_{50} value determination and growth inhibition

IC_{50} values were determined using a SYBR green 1 assay according to established protocols (Smilkstein et al., 2004; Bacon et al., 2007). The parasitemia of a standard culture was evaluated by light microscopy using Giemsa-stained blood smears. The culture was subsequently transferred to a 15 mL falcon tube and centrifuged at 300 *g* for 5 min at room temperature. The supernatant was discarded and cells were adjusted with fresh erythrocytes in complete RPMI medium containing 2x albumax II (0.9% w/v) to a hematocrit of 3% and a parasitemia of 0.5%. Likewise, a suspension containing only uninfected erythrocytes with a hematocrit of 3% was prepared in complete RPMI medium containing 2x albumax II as a control. Albumax-free medium (50 μL) was dispensed in each of the 96 wells of sterile black fluotrac microplates (Greiner). The outer wells (including the first column) of each plate were supplemented with 50 μL of the suspension that contained only uninfected erythrocytes. These wells served as parasite- and drug-free controls to determine the background fluorescence at a final volume of 100 μL complete RPMI medium and a hematocrit of 1.5%. Stock solutions of H_2O_2, tBOOH, diamide, ascorbate, NAc and DTT were freshly prepared in albumax-free RPMI medium and filter-sterilized. Aliquots (25 μL) of these stock solutions were transferred to the second column of the microplates. After mixing, 25 μL of the 75 μL solution in column 2 were transferred to column 3 yielding a 1:3 dilution. This step was repeated for columns 3 to 10 (the last 25 μL from column 10 were discarded). Column 11 served as a control without redox agent. Columns 2 to 11 were supplemented with 50 μL of parasitized erythrocytes yielding a final volume of 100 μL complete RPMI medium, a hematocrit of 1.5% and a parasitemia of 0.5%. The reductants ascorbic acid, *N*-acetylcysteine and DTT were found to produce a background fluorescence. Thus, microplates with unparasitized erythrocytes and the above mentioned dilutions of these redox agents were set up in parallel as controls. All microplates were incubated for 72 h and sealed with parafilm before storage at −80 °C.

Prior to analysis, the microplates were thawed at room temperature for at least 1 h. For each plate 1.2 μL of a 10,000x SYBR green 1 solution was freshly diluted in 10 mL lysis buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.08% Triton X-100 and 0.008% saponin at room temperature. The microplates were supplemented with 100 μL of complete lysis buffer per well, covered with aluminium foil, mixed thoroughly on a horizontal shaker for 2 min and incubated in the dark for 1 h at room temperature. The fluorescence intensity was determined using a microplate reader (BMG Latech, Germany) with a gain of 60, an excitation wavelength of 485 nm and an emission wavelength of 535 nm. All data were corrected for the background fluorescence of uninfected erythrocytes, normalized to the growth of control parasites in column 11, and fitted to a sigmoidal dose-response curve using the four parameter Hill function in SigmaPlot 12.5.

A time-dependent growth inhibition by ascorbate was monitored for strain 3D7. Stock solutions of 200 mM ascorbate were freshly prepared in complete medium, filter-sterilized and directly diluted with complete medium that was added daily to standard parasite cultures. The parasitemia was determined by light microcopy after every 24 h by counting 750–1500 erythrocytes per Giemsa-stained blood smear.

3. Results

3.1. Antiproliferative effects of H_2O_2, tBOOH and diamide

First, we determined the IC_{50} values of asynchronous cultures of the chloroquine-resistant *P. falciparum* strain Dd2 for H_2O_2, tBOOH