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

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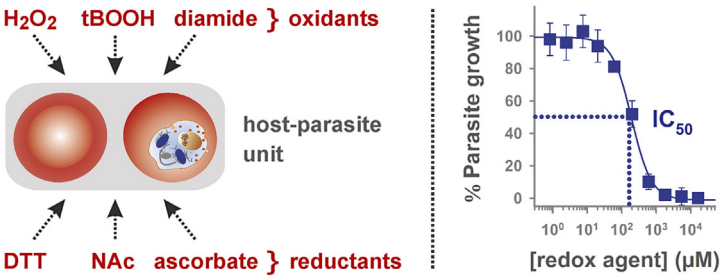
HIGHLIGHTS

- 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



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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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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

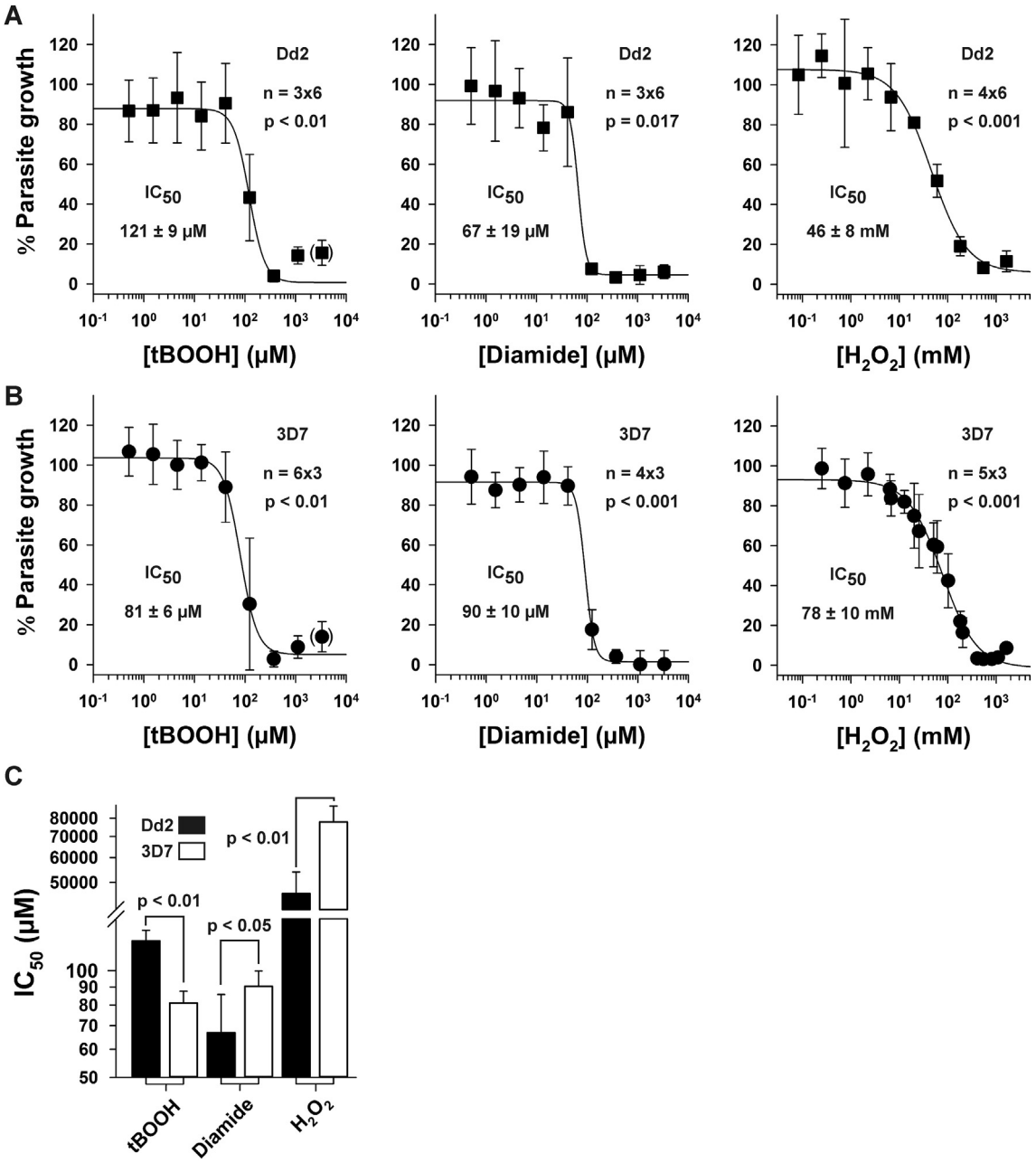

Fig. 1. Growth inhibitory effects of oxidizing agents on *P. falciparum* cell cultures. IC_{50} values were determined in 96-well plates using a SYBR green assay for bolus treatments with tBOOH, diamide or H_2O_2. The initial hematocrit and parasitemia were 1.5% and 0.5%, respectively. The number of replicates is indicated in each panel (e.g., n = 3 × 6 for three independent sextuplicate measurements). (A) IC_{50} values for asynchronous cultures of strain Dd2. (B) IC_{50} values for synchronous ring-stage parasites of strain 3D7. (C) Comparison of the IC_{50} values. P values were calculated using the one way ANOVA on ranks method in SigmaPlot 12.5.

and diamide. Asynchronous cultures were used in order to monitor an averaged effect of the redox agents on different developmental stages. The alkyl hydroperoxide tBOOH and the disulfide-inducing agent diamide were quite toxic with steep slopes in the IC_{50} plots and low IC_{50} values of 121 and 67 µM, respectively (Fig. 1A). Similar IC_{50} values for tBOOH and diamide were also determined for synchronous cultures of the chloroquine-sensitive strain 3D7 (Fig. 1B). However, synchronous 3D7 ring-stage parasites appeared to be more sensitive towards tBOOH and less sensitive towards diamide suggesting strain- or stage-specific differences of the redox metabolism. The chloroquine resistance of asynchronous Dd2 parasites was analyzed in a single sextuplicate measurement as a control, revealing a more than ten times higher IC_{50} value than for synchronous 3D7 parasites (data not shown). IC_{50} values of asynchronous Dd2 and synchronous 3D7 parasites for H_2O_2 around 46 mM and 78 mM were unexpectedly high and complete parasite killing necessitated H_2O_2 concentrations that caused an intense catalase-dependent oxygen production and foaming. In order to validate that the detected fluorescence in the SYBR green assay resulted (predominantly) from viable parasites, we confirmed the presence of intact parasites in Giemsa-stained blood smears from standard Dd2 cultures that were grown for 72 h after a bolus treatment with either 0.75 mM or the IC_{50} concentration of H_2O_2. The IC_{50} values for the oxidants are summarized in Fig. 1C and Table 1 for comparison.

Table 1
Summary of the IC_{50} values determined in this study.

Strain	Oxidants			Reductants		
	H_2O_2 (mM)	tBOOH (μM)	Diamide (μM)	Ascorbate (mM)	NAc (mM)	DTT (μM)
Dd2[a]	46.1 ± 7.8	121 ± 9	66.8 ± 18.9	3.0 ± 0.3	12.4 ± 3.0	389 ± 26
3D7[b]	77.8 ± 9.6	81.1 ± 6.4	90.3 ± 9.5	4.3 ± 0.3	16.6 ± 2.1	245 ± 18

[a] Bolus treatment of asynchronous parasite cultures.
[b] Bolus treatment of synchronous ring-stage parasite cultures.

3.2. Reducing agents promote albumin gelation and hemolysis

Next, we tested the effect of reducing agents on *P. falciparum* cell cultures. Pre-trials to determine the order of magnitude of the IC_{50} values revealed that high concentrations of reducing agents as well as prolonged incubation periods promote gelling of the medium, the formation of methemoglobin and hemolysis (Fig. 2A). While the gelation was also detected at DTT concentrations lower than 1 mM, methemoglobin formation and hemolysis appeared to require higher DTT concentrations. The gelation effect was time-dependent, also occurred in the absence of (infected) erythrocytes, and was only observed in the presence of albumax (Fig. 2B). Albumin is the most likely component of albumax to explain the gelation effect. This interpretation is supported by the literature on albumin aggregates and hydrogels, which can be generated with the help of reducing agents (Yang et al., 2015; Raja et al., 2015).

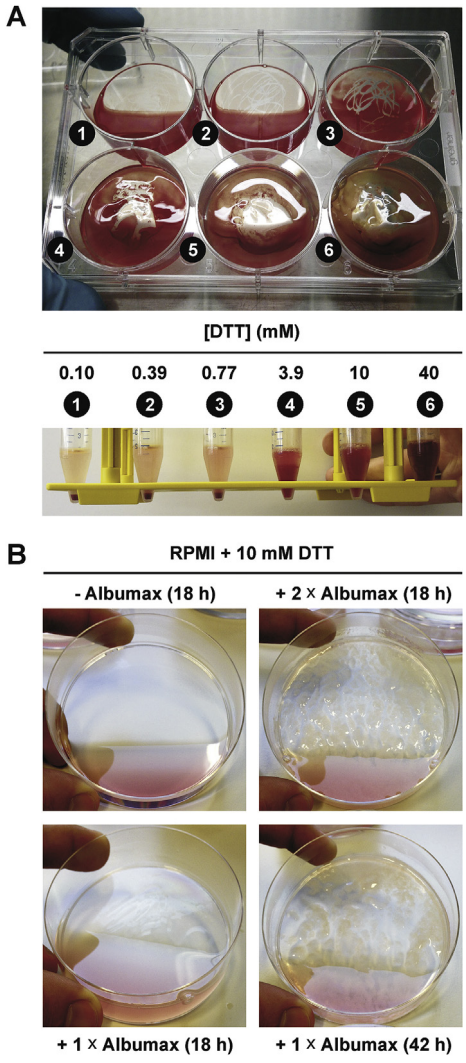

Fig. 2. Reducing agents cause the gelation of albumin, the formation of methemoglobin and hemolysis. (A) Concentration-dependent effects of DTT on infected erythrocytes in standard albumax-containing RPMI medium 48 h after a single bolus treatment. The initial hematocrit and parasitemia were 1.5% and 0.5%, respectively. Intense gelling of the medium and the formation of brown methemoglobin were observed at DTT concentrations >1 mM (upper panel). Sedimentation analyses also revealed a concentration-dependent hemolysis (lower panel). (B) Time- and albumax-dependent gelling of RPMI medium in the presence of 10 mM DTT. Incomplete medium without albumax served as a control.

3.3. Antiproliferative effects of ascorbate, NAc and DTT

In subsequent experiments we determined the IC_{50} values for ascorbate, NAc and DTT. To take into account a gelation-dependent increase of the background fluorescence, measurements were corrected for each concentration using reference values for uninfected erythrocytes that were incubated in parallel in the presence of the same amount of the reducing agent. Without subtraction of the reference values a plateau fluorescence was obtained that would have been equivalent to a residual parasitemia around 10–20%. Giemsa-stained blood smears confirmed, however, that the samples of the plateau fluorescence did not contain viable parasites. Thus, the subtraction of the reference values was validated by the blood smears.

All three reducing agents had antiproliferative instead of growth-promoting effects. Measurements for NAc and ascorbate revealed rather high IC_{50} values for strain Dd2 around 12 and 3 mM, respectively (Fig. 3A). DTT was far more toxic with a steeper slope in the IC_{50} plot and an IC_{50} value of 0.4 mM. Similar IC_{50} values for NAc, ascorbate and DTT were also determined for synchronous cultures of strain 3D7 (Fig. 3B). However, the latter parasites appeared to be slightly less sensitive towards NAc and ascorbate and slightly more sensitive towards DTT suggesting strain- or stage-specific differences of the redox metabolism. The IC_{50} values for the reducing agents are summarized in Fig. 3C and Table 1 for comparison.

3.4. The antiproliferative effect of ascorbate has slow kinetics

In order to analyze the antiproliferative effect of ascorbate in further detail, we treated standard cultures of synchronous 3D7 parasites with different concentrations of ascorbate and monitored the parasitemia over six days. A daily treatment with 2.5 and 5 mM ascorbate in fresh medium resulted in methemoglobin formation and hemolysis over time. These effects appeared to coincide with the growth inhibition, which became only detectable after prolonged incubation and required millimolar ascorbate concentrations in accordance with the IC_{50} value from the SYBR green assay (Fig. 4). In summary, the growth inhibition of standard *P. falciparum* cultures by ascorbate has slow kinetics with an IC_{50} value > 1 mM.

4. Discussion

One of several hypotheses on oxidative stress and malaria is that an impaired production of external reactive oxygen species by leukocytes might exacerbate malaria infections (Becker et al., 2004; Sorci and Faivre, 2009; Clark and Hunt, 1983; Descamps-Latscha et al., 1987; Greve et al., 1999). One problematic aspect of this hypothesis is that the host-parasite unit is packed with numerous concentrated hydroperoxidases and that some redox agents such as the hydroxyl radical are too reactive to diffuse across several membranes. The very high IC_{50} value for H_2O_2 indeed suggests that little H_2O_2 reaches the parasite (or that the effective concentration

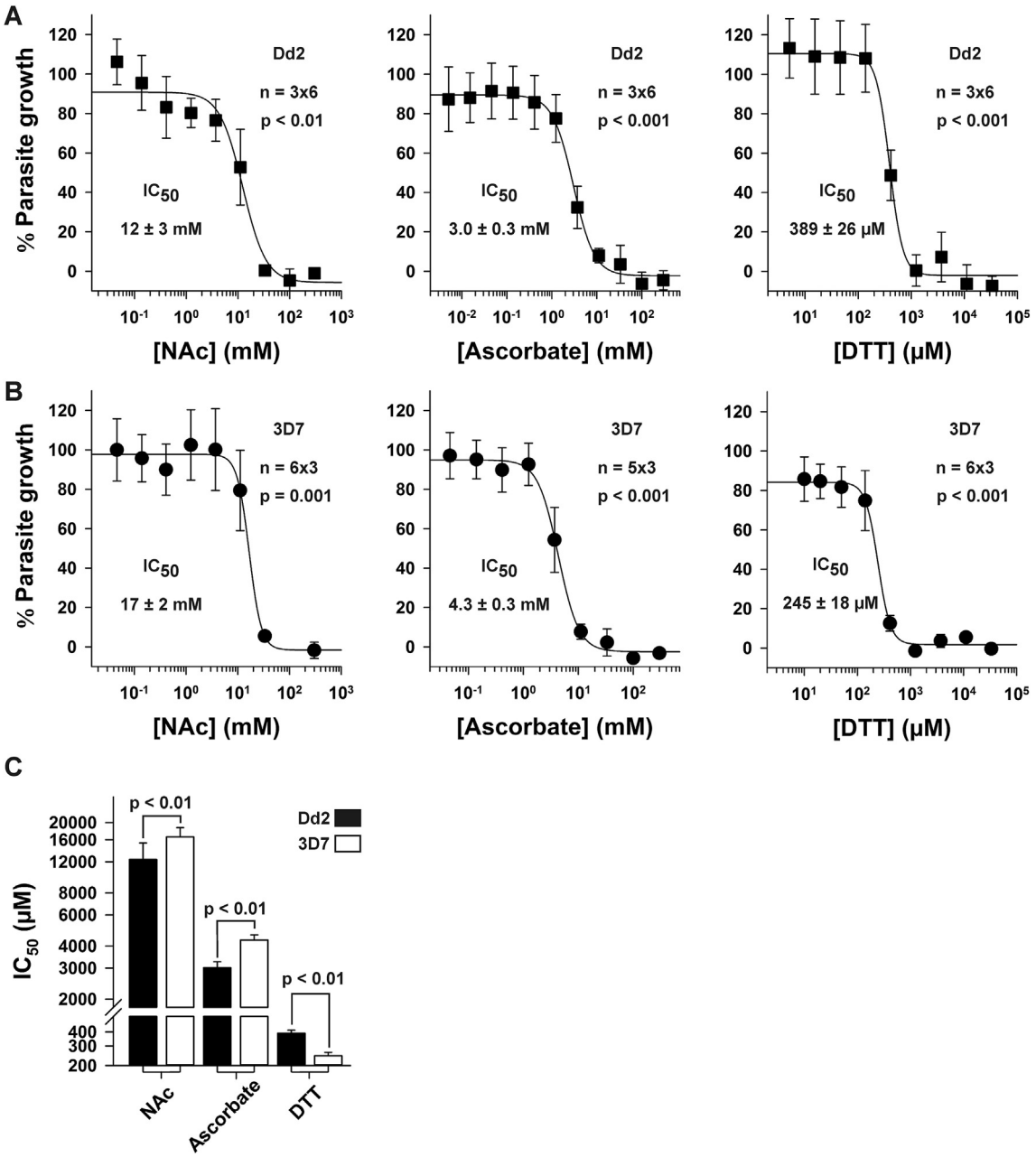

Fig. 3. Growth inhibitory effects of reducing agents on *P. falciparum* cell cultures. IC_{50} values were determined in 96-well plates using a SYBR green assay for bolus treatments with NAc, ascorbate or DTT. The initial hematocrit and parasitemia were 1.5% and 0.5%, respectively. The number of replicates is indicated in each panel (e.g., n = 3 × 6 for three independent sextuplicate measurements). (A) IC_{50} values for asynchronous cultures of strain Dd2. (B) IC_{50} values for synchronous ring-stage parasites of strain 3D7. (C) Comparison of the IC_{50} values. P values were calculated using the one way ANOVA on ranks method in SigmaPlot 12.5.

is too low to generate more detrimental redox species such as the hydroxyl radical *in situ*). A similar finding was made using supraphysiological doses of the strong oxidant peroxynitrite in a mouse model (Sobolewski et al., 2005), whereas treatment of *Plasmodium vinckei* with tBOOH was shown to result in iron-dependent hemolysis and parasite death *in vivo* (Clark et al., 1984). One factor that is worth taking into account is that our IC_{50} values were determined 72 h after a single bolus treatment. A continuous challenge with H_2O_2 might significantly alter the outcome as demonstrated previously for human embryonic kidney 293 cells (Sobotta et al., 2013). Furthermore, prolonged growth assays might reveal detrimental effects for subsequent cell cycles, even at lower H_2O_2 concentrations, and different growth conditions *in vitro* and *in vivo* (such as oscillating CO_2 and O_2 partial pressures or the presence of human serum instead of albumax) might also play an important role regarding the IC_{50} value. Nevertheless, our experiments show that the hydroperoxidase system of the host-parasite unit is extremely robust against oxidative challenges with H_2O_2, whereas it is far more sensitive towards tBOOH. The inability of erythrocyte catalase to reduce alkyl hydroperoxides might explain this finding. However, highly abundant human peroxiredoxin 2 is able to convert tBOOH (Koncarevic et al., 2009) and the parasite also harbors several peroxiredoxins, including a dual-localized peroxiredoxin V-type enzyme that efficiently reduces tBOOH (Deponte et al., 2007; Kawazu et al., 2008; Djuika et al., 2013, 2015; Staudacher et al., 2015). Treatment of

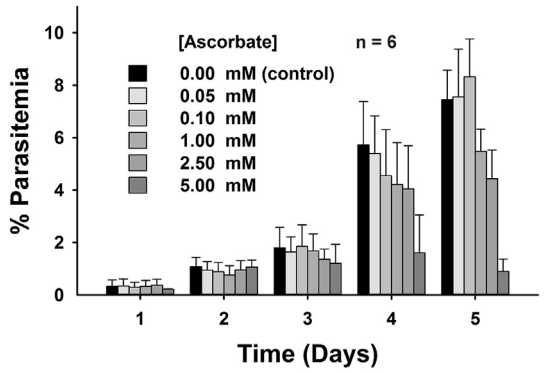

Fig. 4. Time-dependent growth inhibition of *P. falciparum* by ascorbate. The parasitemia of standard cell cultures of synchronized 3D7 parasites was monitored over six days in the presence of different concentrations of ascorbate. The initial parasitemia of ring-stage parasites at day 0 was 0.1% at a hematocrit of 3%. The ascorbate-containing medium was replaced daily and the parasitemia was determined for six independent measurements by counting infected erythrocytes from Giemsa-stained blood smears.

erythrocytes with tBOOH has previously been shown to result in lipid peroxidation and hemoglobin degradation (Trotta et al., 1982; 1983) and these effects are presumably far less pronounced at similar H_2O_2 concentrations. Another potential explanation for the higher susceptibility of *P. falciparum* towards tBOOH might be that tBOOH is transported more rapidly across the three membranes surrounding the parasite cytosol.

Diamide treatment results in an unspecific oxidation of thiols (Kosower and Kosower, 1995). Since glutathione is expected to be the most abundant thiol in the parasite, diamide treatment should predominantly yield glutathione disulfide, which is usually reduced by glutathione reductase at the expense of NADPH (Jortzik and Becker, 2012; Müller, 2015; Deponte, 2013). A temporary increase of the glutathione disulfide concentration after parasite treatment with 0.2 mM diamide has indeed been demonstrated previously (Barrand et al., 2012). In this respect it is interesting to note that our IC_{50} value for diamide correlates well with recent glutathione measurements using a redox-sensitive fluorescent sensor. This sensor was most oxidized at diamide concentrations $\geq$0.1 mM (Mohring et al., 2016). A plausible interpretation of our data is, therefore, that high diamide concentrations resulted in an increased glutathione disulfide concentration which might have depleted the NADPH pool or led to a detrimental protein glutathionylation.

Taking into account that malaria parasites infect vertebrates and harbor an apicoplast of algal origin (Janouskovec et al., 2010; van Dooren and Striepen, 2013), we were also interested in the effects of ascorbate on the growth of *P. falciparum*. Stage- and host-dependent growth-promoting as well as antiproliferative effects of ascorbate were previously detected at extremely high concentrations (Marva et al., 1989). Furthermore, inverse correlations between serum ascorbate concentrations and malaria have been reported for infected and uninfected adults and children (Raza et al., 2010; Hassan and Maryam, 2004; Onyesom, 2010). Our ascorbate IC_{50} values of 4.3 and 3.0 mM for strains 3D7 and Dd2 are in good agreement with a reported IC_{50} value around 5.0 ± 0.7 mM for strain D6 using a hypoxanthine incorporation assay (Winter et al., 1997). These values, as well as the shape of the diagrams in Fig. 3, indicate that physiological ascorbate concentrations around 50 μM (Moeslinger et al., 1995) do not play a role for the growth of *P. falciparum*. Other factors support this conclusion. First, RPMI medium does not contain ascorbate (although we cannot exclude trace amounts within the washed erythrocytes). Second, searches in PlasmoDB (Aurrecoechea et al., 2009) revealed no gene candidates that encode ascorbate-metabolizing enzymes. Third, even a continuous treatment with physiological ascorbate concentrations of 50 μM had no effect on standard *P. falciparum* cell cultures (Fig. 4). Thus, if ascorbate really has an influence on malaria, the mode of action either relies on non-erythrocyte host cells or requires additional factors that are absent in the standard medium.

One reason for the non-physiological antiproliferative effects of ascorbate, NAc and DTT might be that these so-called antioxidants provide electrons for Fenton reactions within the host-parasite unit. Thus, such electron donors might actually serve as pro-oxidants as suggested previously for ascorbate (Marva et al., 1989; Winter et al., 1997; Marva et al., 1992). A pro-oxidative effect of NAc has been demonstrated previously in Drosophila (Albrecht et al., 2011), whereas studies in *P. falciparum* revealed no effect for NAc alone at concentrations of 0.9 μM and 0.9 mM (Arreesrisom et al., 2007; Njomnang Soh et al., 2012). Our IC_{50} values for NAc around 17 and 12 mM for strain 3D7 and Dd2 explain the absent effects in these previous studies. Beside detrimental Fenton reactions, ascorbate, NAc and DTT might also reduce crucial disulfide bonds. While the reduction of albumin disulfide bonds might have an indirect antiproliferative effect, the reduction of erythrocyte or merozoite surface proteins might directly alter the invasion efficiency. The latter effects might be better detectable using asynchronous parasite cultures or prolonged growth assays (because SYBR green does not discriminate between the DNA of growth-arrested schizonts, extracellular merozoites or successfully invaded merozoites).

In conclusion, the host parasite unit in our assay system was extremely robust towards redox challenges by H_2O_2, NAc and ascorbate, whereas micromolar IC_{50} values were determined for diamide, tBOOH and DTT. The underlying mechanisms remain to be unraveled. In addition, an increased fluorescence owing to albumin gelation and hemolysis has to be taken into account for the determination of IC_{50} values of redox agents using SYBR green assays.

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