Molecular & Biochemical Parasitology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Comparison of methods probing the intracellular redox milieu in *Plasmodium falciparum*

Franziska Mohring, Esther Jortzik, Katja Becker*

Biochemistry and Molecular Biology, Interdisciplinary Research Center, Justus Liebig University Giessen, 35392 Giessen, Germany

ARTICLE INFO

Article history:
Received 13 May 2015
Received in revised form 28 October 2015
Accepted 10 November 2015
Available online xxx

Keywords:
Glutathione redox potential
Malaria
Oxidative stress
Plasmodium falciparum
roGFP
Thiols

ABSTRACT

Glutathione plays a crucial role in the redox regulation of the malaria parasite *Plasmodium falciparum* and is linked to drug resistance mechanisms, especially in resistance against the antimalarial drug chloroquine (CQ). The determination of the glutathione-dependent redox potential was recently established in living parasites using a cytosolically expressed biosensor comprising redox-sensitive green fluorescent protein coupled to human glutaredoxin 1 (hGrx1-roGFP2). In order to further elucidate redox changes induced by antimalarial drugs and to consolidate the application spectrum of the ratiometric biosensor we systematically compared it to other methods probing thiol and redox metabolism. Among these methods were cell disruptive and non-disruptive approaches including spectrophotometric assays with Ellman's reagent and naphthalene dicarboxyaldehyde as well as molecular probes such as ThiolTracker™ Violet and the dichlorofluorescein-based probe CM-H_2DCFDA. To directly compare the methods, blood stages of the CQ-sensitive *P. falciparum* 3D7 strain were challenged with the oxidative agent diamide and the antimalarial drugs artemisinin and CQ for 1 h, 4 h, and 24 h. For all conditions, dose-dependent changes in the different redox parameters could be monitored which are compared and discussed. We furthermore detected slight differences in thiol status of parasites transiently transfected with hGrx1-roGFP2 in comparison with control 3D7 cells. In conclusion, ThiolTracker™ Violet and, even more so, the hGrx1-roGFP2 probe reacted reliably and sensitively to drug induced changes in intracellular redox metabolism. These results were substantiated by classical cell disruptive methods.

1. Introduction

Despite all efforts to eradicate malaria, an estimated 3.3 billion people are still at risk of getting infected. In 2013 there were about 600,000 malaria deaths worldwide, with 90% in Sub-Saharan Africa, mostly due to *Plasmodium falciparum* [1]. The spread of resistance against antimalarial drugs and insecticides demands the search for new drug targets and compounds [2]. Cellular redox reactions play important roles in antioxidant defense and redox regulation but also in the mechanisms of drug action and drug resistance in malaria parasites [3,4]. To ensure redox balance in its various hosts and developmental stages, *P. falciparum* has developed an elaborate redox network mainly depending on glutathione and thioredoxin as electron donors [5–7]. Glutathione (γ-L-glutamyl-L-cysteinylglycine) represents the most abundant low-molecular-weight thiol in malaria parasites and the GSH:GSSG thiol-switch operates as a central redox regulator [8–11]. The biological functions of thiol-based redox switches in parasites and detection methods applied in parasite research were recently reviewed [12]. In spite of a variety of methods that can be applied to investigate the parasite thiol and oxidative stress status, data reproducibility and comparability remain a challenge. Factors contributing to this are the usage of cell-disruptive versus non-disruptive methods, susceptibility of parasite components to oxidative modification during sample preparation, diverse sensitivity, specificity, and speed of action of different probes and reactants used, but also substantial parasite stage and strain-specific differences and subtle variations in cell cultivation and sample preparation protocols. Systematic comparison indicating strengths and weaknesses of the different methods when used in *Plasmodium* has not yet been carried out.

ThiolTracker™ Violet is a fluorescent dye used as a marker for the thiol status in intact cells [13]. In comparison with monochlorobimane, ThiolTracker™ Violet is much brighter and allows for efficient measurement of glutathione in various cell types both by traditional microscopy and high-content imaging [14]. To our knowledge the probe has not yet been applied to quantifications of thiols in *Plasmodium*.

* Corresponding author at: Biochemistry and Molecular Biology, Interdisciplinary Research Center, Justus Liebig University, Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany. Fax: +49 641 9939129.
E-mail address: katja.becker@uni-giessen.de (K. Becker).

http://dx.doi.org/10.1016/j.molbiopara.2015.11.002
0166-6851/

CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) is a marker for oxidative stress and reacts with organic peroxides, nitric oxide, and hydrogen peroxide as well as peroxinitrite in the presence of iron or hematin, which leads to oxidation of H_2DCF to the fluorescent DCF [15]. So far the probe has been used in flow cytometry approaches to analyze *P. falciparum* stage-specific oxidative stress and the contribution of hemoglobin digestion in artemisinin (ART) activity [16,17].

A milestone in research of the glutathione redox state was the development of redox-sensitive green fluorescent protein (roGFP) [18,19]. Fused to human glutaredoxin 1 (hGrx1) it directly and sensitively indicates the intracellular glutathione redox potential in living cells [20,21]. Recently, the genetically encoded probe hGrx1-roGFP2 was established for the real-time imaging of the glutathione redox potential in *P. falciparum* [22] and has been used to analyze redox changes induced by oxidative or pharmacological stress. The ratiometric fluorescence microscopy readout after excitation at 405 and 488 nm indicates that with a glutathione redox potential of about −314 mV, the redox milieu of the parasite cytoplasm is highly reducing. First correlations between the glutathione-dependent redox potential determined with the hGrx1-roGFP2 sensor and intracellular glutathione and thiol concentrations determined by cell disruptive biochemical methods have been described [22].

In the present work we aimed to systematically compare different methods probing the redox status in *P. falciparum*. For this comparison we used the cytosolic glutathione biosensor hGrx1-roGFP2 as well as the fluorescent probes ThiolTracker™ Violet for thiol quantification [13] and CM-H_2DCFDA for investigation of general oxidative stress generation in living cells. In parallel, biochemical cell-disruptive methods were applied including the reaction with 5,5′dithiobis-(2-nitrobenzoic acid) (DTNB, also known as Ellman's reagent) to quantify total and free non-protein-bound thiols [23] as well as total glutathione when coupled to a glutathione reductase system [10,22], and finally naphthalenedicarboxaldehyde (NDA) derivatization for GSH quantification [24]. To compare the methods under conditions of redox and pharmacological stress, the parasites were challenged with the oxidative agent diamide (DIA) and the redox-active antimalarial drugs artemisinin (ART) and chloroquine (CQ). Furthermore, we studied if the episomal expression of the genetically encoded hGrx1-roGFP2 biosensor in *P. falciparum* has influences on the glutathione redox milieu of the cells.

2. Materials and methods

2.1. P. falciparumcell *culture*

The chloroquine (CQ)-sensitive *P. falciparum* 3D7 strain was cultured as described [25]. The strain was propagated in RBCs (A+) in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 0.5% Albumax, 9 mM glucose, 0.2 mM hypoxanthine, 2.1 mM L-glutamine, and 22 μg/ml gentamicin at 3.3% hematocrit and 37 °C in a gaseous mixture consisting of 3% O_2, 3% CO_2 and 94% N_2. Synchronization of *P. falciparum* parasites was achieved by repetitive treatment with 5% (w/v) sorbitol [26]. *P. falciparum* trophozoites were enriched with magnet separation [27]. EC_{50} values on the *P. falciparum* 3D7 strain were determined after 72 h incubation with the drugs using [^{3}H]-hypoxanthine incorporation and were 8.6 ± 0.4 nM for CQ and 17.3 ± 6.1 nM for ART [22].

2.2. Determination of thiol and glutathione concentrations in parasite lysate

Stock solutions of DIA and CQ (Sigma–Aldrich, Steinheim, Germany) were dissolved in distilled H_2O and ART (Aldrich Chemical Co. (Milwaukee, Wis.)) in DMSO. *P. falciparum 3D7*-infected erythrocytes (ring stage 6–10 h post invasion) were incubated with 4×EC_{50} of ART, 4×EC_{50} of CQ or 100 μM DIA for 24 h. For 4 h drug treatment, trophozoites (26–34 h post invasion) were incubated with 50×EC_{50} ART, 50×EC_{50} CQ. For 1 and 4 h challenge with different concentrations of DIA trophozoites (26–34 h post invasion) were incubated. Cell lysate was obtained via saponin lysis [28]. Total thiols in fresh parasite lysate were measured spectrophotometrically on the basis of their reaction with DTNB [22,23]. Briefly, the lysate was mixed with 150 mM potassium phosphate buffer, pH 8.0 and 400 μM DTNB, and directly measured at 412 nm at room temperature taking control absorption values into account. Free non-protein thiols were measured analogously in the supernatant of a parasite lysate previously precipitated with 3 volumes of 5% sulfosalicylic acid (SSA). Total glutathione was measured with the glutathione reductase-coupled 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)-GSH-recycling assay [10]. Briefly, 10 μl of SSA supernatant was mixed with 490 μl buffer containing 142 mM NaH_2PO_4, 6 mM EDTA, and 0.3 mg/ml NADPH, pH 7.5. After adding 0.6 mM DTNB, the mixture was incubated for 10 min at room temperature. Human glutathione reductase (0.5 U/ml) was added and DTNB increase detected immediately for 30 s at 25 °C.

GSH was quantified with the naphthalene dicarboxaldehyde (NDA) assay, according to [24]. One volume of SSA supernatant was added to ten volumes of freshly prepared NDA derivatisation mix (1 ml of 10 mM NDA in DMSO, 7 ml of 50 mM Tris, pH 10, and 1 ml of 0.5 M NaOH), and the fluorescence was measured at ex 485 nm/em 520 nm together with a glutathione standard curve in 96-well plates in a Tecan M200 microplate reader.

The two parasite lines 3D7 and 3D7$_{[hGrx1\text{-}roGFP2]}$ were grown, treated and studied independently from each other (including all biological replicates). For biochemical assays, four biological replicates were produced for each condition and stored. All samples were then tested on the same day to exclude systematic errors. Two replicates were pooled and measured in two technical replicates each resulting in four data values for statistical analysis. The graphs were plotted using the GraphPad Prism 4 software (San Diego, CA, USA). Also for data analysis GraphPad Prism 4.0 was used. In order to calculate the significance of changes in thiol levels after drug treatment, all groups were compared with the control by the Dunnett's Multiple Comparison Test. For comparison of 3D7 and 3D7$_{[hGrx1\text{-}roGFP2]}$ a two-tailed, unpaired *t*-test was used.

2.3. Determination of total thiols in living parasites

The cell-permeable fluorescent dye ThiolTracker™ Violet (life technologies) was used to measure total thiol concentrations in *P. falciparum* living cells according to the manufacturers' guidelines. *P. falciparum 3D7*-infected erythrocytes (trophozoite stage 24–28 h post invasion) were enriched with a magnet and returned to standard cell culture conditions for 2 h to recover. Drug treatment of 20 × 10^6 cells was carried out in LoBind tubes (Eppendorf) for 1 h or 4 h. For 24 h treatment, ring stage infected red blood cells (0–10 h post invasion, 10% parasitemia) were incubated. Cells were then washed in pre-warmed PBS, resuspended in 20 μM ThiolTracker™ Violet solution in PBS, and incubated for 30 min at 37 °C. Dye was removed by washing in PBS. ThiolTracker™ Violet fluorescence of 2 × 10^6 cells per treatment in PBS was measured at ex 404 nm/em 525 nm in the plate reader (M200, Tecan) or with a Leica TCS SP5 inverted microscope equipped with the objective HCX PL APO 63.0 × 1.30 GLYC 37 °C UV and a 37 °C temperature chamber. The probe was excited by the 405 nm laser line and the emission was detected through a 499–568 nm bandpass filter. Scanning was performed at 400 Hz frequency. The smart gain and smart offset were 950 V and −0.9%, respectively. Parasites were seeded on poly-L-lysine-coated μ-slides VI (Ibidi, Martinsried, Germany). All experiments were carried out three times in independent biological

Please cite this article in press as: F. Mohring, et al., Comparison of methods probing the intracellular redox milieu in *Plasmodium falciparum*, Mol Biochem Parasitol (2015), http://dx.doi.org/10.1016/j.molbiopara.2015.11.002

replicates. For analysis of microscope images the ImageJ software was used. At least 10 microscopy images of 100–300 cells were processed each time and the number of cells was calculated. The relative fluorescence units (RFU) of the respective image were divided by the number of cells per image to calculate the mean fluorescence per cell. Single cell images were taken at a zoom factor of 16.

2.4. Determination of oxidative stress with dichlorofluorescein in living parasites

The general oxidative stress indicator CM-H_2DCFDA (life technologies) was used to quantify reactive oxygen species in the cell according to the manufacturers' guidelines. After passive diffusion the acetate groups of the probe are cleaved by esterases, and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent adduct that is trapped inside the cell, thus facilitating long-term studies. Fluorescence can be detected at ex 492 nm/em 527 nm [13]. *P. falciparum 3D7*-infected erythrocytes (trophozoite stage 24–28 h post invasion) were enriched with a magnet and returned to cell culture conditions for 2 h to recover. Cells were washed with prewarmed PBS and incubated with 5 μM CM-H_2DCFDA solution in PBS for 30 min at 37 °C. Dye was removed by washing in PBS. Drug treatment of 20×10^6 cells was carried out in LoBind tubes (Eppendorf) for 1 h or 4 h in cell culture medium. For 24 h drug incubations, ring stage parasites (4–10 h post invasion) were washed with prewarmed PBS and incubated with 5 μM CM-H_2DCFDA solution in PBS for 30 min at 37 °C. Dye was removed by washing with PBS and the following 24 h drug treatment was carried out in culture medium under standard cell culture conditions. Further procedures were as described in Section 2.3. The probe was excited by the 496 nm laser line and the emission was detected through a 510–537 nm bandpass filter.

2.5. hGrx1-roGFP2 transfection and live cell imaging

The transfection and CLSM imaging of *P. falciparum* 3D7 was carried out as described [22]. Leica and Batch Ratio Analysis software [29] for data analysis were used. The graphs were plotted using GraphPad Prism 4 software (San Diego, CA, USA). Before image analysis, LAS AF Lite software was used to exclude the DIC channel by using the crop tool, and images were exported in .tif format. LSM Image Browser was used to split RGB images and to convert to .lsm format. The RRA Batch menu program was used to measure

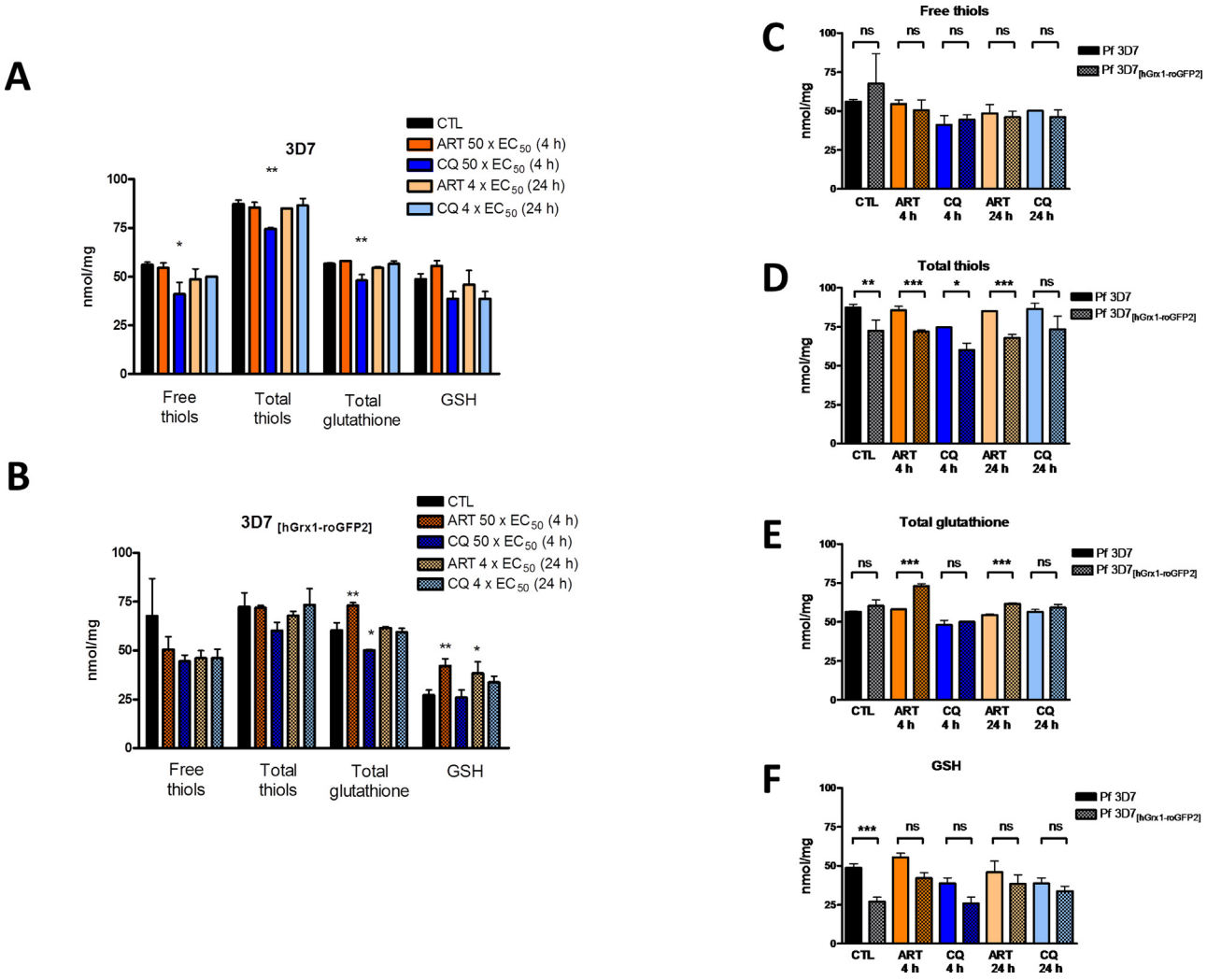

Fig. 1. Effects of diamide (DIA), artemisinin (ART) and chloroquine (CQ) on the thiol and glutathione status of *P. falciparum* 3D7 and 3D7$_{[hGrx1\text{-}roGFP2]}$. (A) Drug effects on 3D7 and (B) drug effects on 3D7$_{[hGrx1\text{-}roGFP2]}$. Direct comparison of (C) free thiols, (D) total thiols, (E) total glutathione, and (F) reduced glutathione (GSH) in drug treated *P. falciparum* 3D7 and 3D7$_{[hGrx1\text{-}roGFP2]}$. Asterisks above the columns in (A) indicate significant differences compared to the control (black) of every thiol groups, asterisks in (B) indicate significant changes of the thiol group compared to the thiol group in (A), asterisks in (C–F) indicate significant differences of 3D7 compared to 3D7$_{[hGrx1\text{-}roGFP2]}$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant.

Please cite this article in press as: F. Mohring, et al., Comparison of methods probing the intracellular redox milieu in *Plasmodium falciparum*, Mol Biochem Parasitol (2015), http://dx.doi.org/10.1016/j.molbiopara.2015.11.002

the 405/488 nm ratios. The settings were as follows: *x–y* average: 3, *t* average: 1, SD thresh: 2, saturation: 0.9, ratio order: normal, background: white. Autofluorescence images were simultaneously taken at ex 430–450 nm/em 510 nm and defined together with the background individually for every image.

3. Results and discussion

3.1. The glutathione biosensor influences intracellular thiol levels in P. falciparum 3D7

In order to assess potential effects of episomal hGrx1-roGFP2 expression on the intracellular redox milieu of *P. falciparum* we directly compared non-transfected CQ-sensitive 3D7 parasites with transiently hGrx1-roGFP2 expressing parasites ($3D7_{[hGrx1\text{-}roGFP2]}$). Cells were treated with ART or CQ for 4 h or 24 h. Total and free thiol concentrations as well as total glutathione and GSH levels were then determined in the freshly prepared cell lysates.

Fig. 1 summarizes the results of drug effects on the four assessed redox parameters in *P. falciparum* 3D7 parasites (Fig. 1A) and in $3D7_{[hGrx1\text{-}roGFP2]}$ parasites (Fig. 1B). In Fig. 1C–F the results for the four redox parameters are shown separately, which allows to directly compare the two parasite populations.

As illustrated, biosensor-expressing parasites showed under most treatments lower levels of total thiols (as determined using Ellman's reagent; Fig. 1D) and GSH (as determined with NDA; Fig. 1F) although statistical significance was not reached in all cases. Free, non-protein bound thiols (as determined using Ellman's reagent; Fig. 1A) and total glutathione concentrations (determined in the DTNB recycling assay) were less affected although a slight increase in total glutathione became significant in some cases (Fig. 1E). Based on the present data set, a shift in the GSH:GSSG ratio might occur in the population containing transfected cells. Since GSH and GSSG levels were determined by different assays and since the cell population studied consists of cells with and without biosensor expression we decided to refrain from calculating intracellular GSH:GSSG ratios and believe that this phenomenon needs to be carefully discussed. For many years it had been presumed that intracellular GSH:GSSG ratios range between 20:1 and 100:1 until redox probes led to suggested ratios of up to 10,000:1. Recently, it was observed that in yeast the GSH is mainly stored in lysosome-like vacuoles [30], which might – together with differences in sample preparation protocols – explain the tremendous disparity of data. In isolated *P. falciparum* trophozoites a GSH:GSSG ratio of 285:1 was estimated by using HPLC detection of *O*-phthalaldehyde-derivatized GSH and GSSG [11]. The presence of a GSH storage

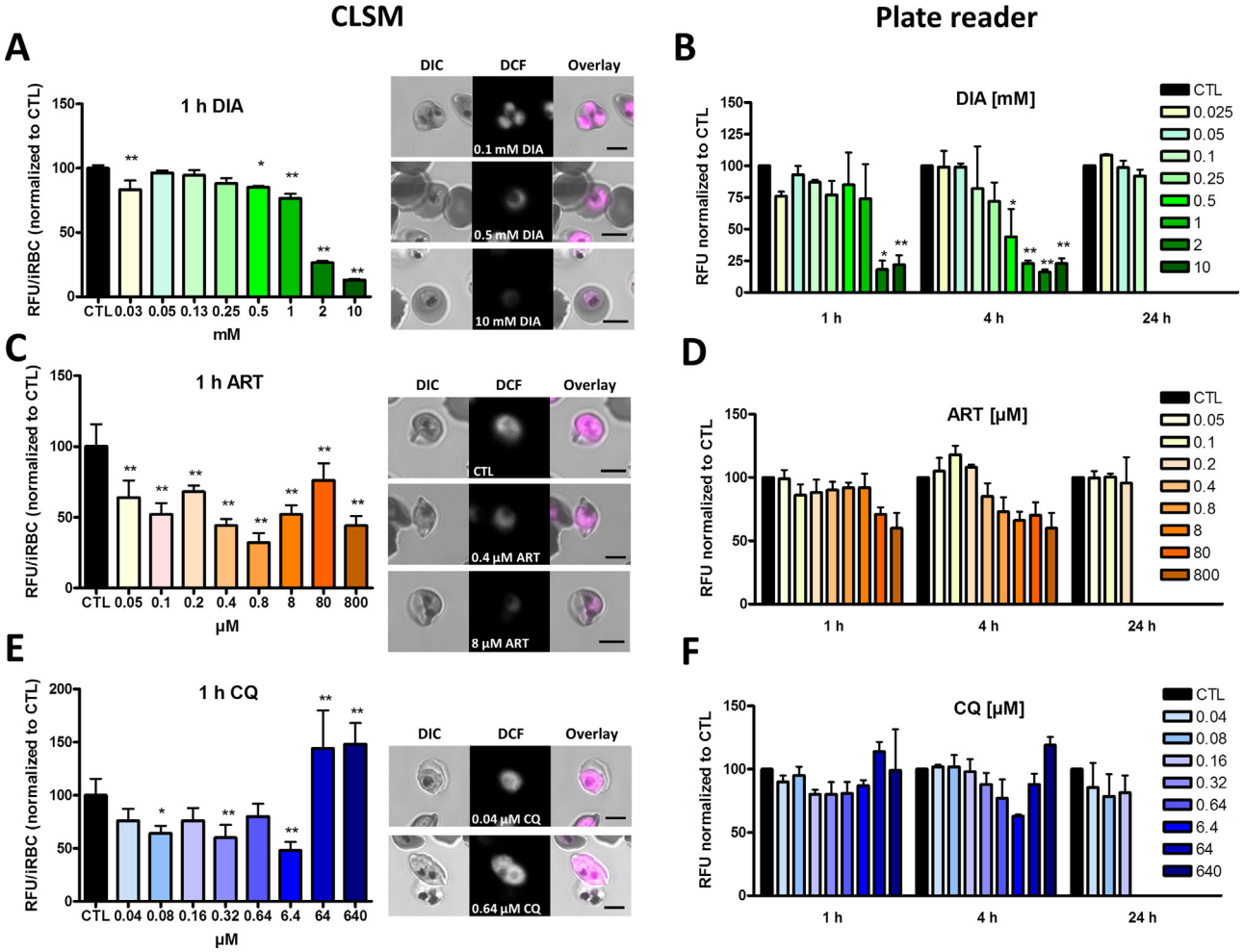

Fig. 2. Total thiols of *P. falciparum* 3D7 quantified with ThiolTracker™ Violet after diamide (DIA), artemisinin (ART) and chloroquine (CQ) treatment detected by confocal laser scanning microscopy (CLSM) or plate reader. (A) relative fluorescence units (RFU)/cell after 1 h DIA treatment detected by CLSM and example images of microscopy. (B) RFU after 1 h, 4 h, and 24 h DIA treatment detected by plate reader. (C) RFU/cell after 1 h ART treatment detected by CLSM and example images of microscopy. (D) RFU after 1 h, 4 h, and 24 h ART treatment detected by plate reader. (E) RFU/cell after 1 h CQ treatment detected by CLSM and example images of microscopy. (F) RFU after 1 h, 4 h, and 24 h CQ treatment detected by plate reader. Asterisks indicate significant differences $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Every drug and every concentration was investigated in 2–6 independent experiments via plate reader detection and 2 independent experiments via CLSM. Scale bars indicate a size of 5 µm.

Please cite this article in press as: F. Mohring, et al., Comparison of methods probing the intracellular redox milieu in *Plasmodium falciparum*, Mol Biochem Parasitol (2015), http://dx.doi.org/10.1016/j.molbiopara.2015.11.002

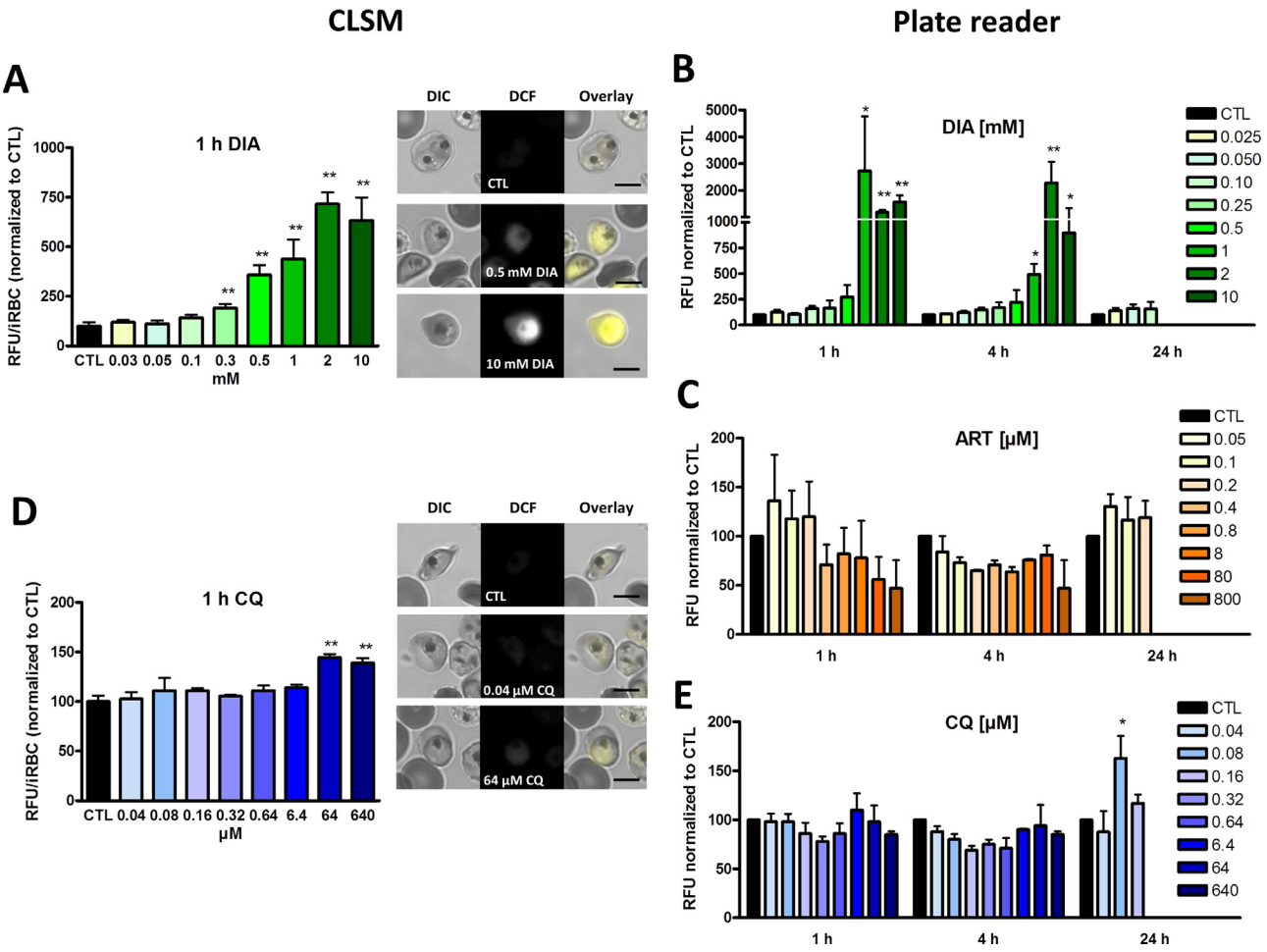

Fig. 3. Oxidative stress in *P. falciparum* 3D7 quantified with CM-H_2DCFDA after diamide (DIA), artemisinin (ART) and chloroquine (CQ) treatment and detected by confocal laser scanning microscopy (CLSM) or plate reader. (A) RFU/cell after 1 h DIA treatment detected by CLSM and example images of microscopy. (B) RFU after 1 h, 4 h, and 24 h DIA treatment detected by plate reader. (C) RFU after 1 h, 4 h, and 24 h ART treatment detected by plate reader. (D) RFU/cell after 1 h CQ treatment detected by CLSM and example images of microscopy. (E) RFU after 1 h, 4 h, and 24 h CQ treatment detected by plate reader. Asterisks indicate significant differences $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Every drug and every concentration was investigated in 2–6 independent experiments via plate reader detection and 2 independent experiments via CLSM. Scale bars indicate a size of 5 µm.

compartment has not been described so far, and glutathione import and export processes between subcellular compartments have not been studied in detail in malaria parasites [8,9,31]. Notably, however, an enhanced transport of GSH into the parasite's digestive vacuole has been shown for parasites carrying a mutant CQ resistance transporter gene (PfCRT) [32].

In our approach, reasons for the observed shift in the GSH:GSSG status of the cells studied could be exposure of the cells to the antifolate WR99210 (required for selecting the parasites) or a direct influence of the probe on the cellular redox milieu including reactions with low molecular weight thiols and redox active proteins but also influences on gene expression. Further potential reasons for the observed changes could be the transfection process itself (although this took place about 10 parasite generations before the experiments), plasmid expression, but also interaction of the probe with assay reagents used including DTNB and NDA. Whether or not similar effects can be observed in other organisms or cell types needs to be studied in further detail. In contrary to our results, Lukyanov et al. [33] (and references therein) suggest that the more in Grx1 activity in cell compartments leads to a more reducing redox environment, observed by comparing E_{GSH} levels determined by hGrx1-roGFP2 and rxYFP in yeast mitochondrial intermembrane space. In Gutscher et al., 405/488 nm ratio changes of hGrx1-roGFP2 and roGFP2 were examined in Hela cells after H_2O_2 challenge. Both biosensors had the same basal ratio with a higher response to oxidation in hGrx1-roGFP2. These studies contradict the hGrx1 theory [20].

The cell population in our study (containing parasites with and without transient expression of the biosensor) was determined to have the same life cycle time (44 h) as non-transfected parasites. However, the transfected population had a lower multiplication rate (3 ± 0.5) compared to (7 ± 0.5) non-transfected parasites. This is likely to be due to the presence of the selection marker WR99210 that eliminates parasites without plasmid expression. This selection marker might indeed also affect the intracellular redox milieu. A stable integration of the redox sensor (which we plan in the near future) will be likely to have advantages over the presently used episomal expression. Stably biosensor-expressing parasites need not be exposed to a selection drug such as WR99210 and are likely to have a much higher percentage of biosensor expressing cells.

When assessing drug effects on thiol status of transfected and non-transfected *Pf* 3D7 cell lines slight differences but also commonalities were observed (Fig. 1A and B). In *Pf* 3D7, 4 h ART exposure slightly increased GSH levels in *Pf* 3D7, whereas 4 h and 24 h ART exposure enhanced GSH levels but reduced total thiols in $3D7_{[hGrx1\text{-}roGFP2]}$. Notably, in both parasite populations 4 h CQ consistently diminished all four assessed thiol markers (Fig. 1A and B). DIA concentration-dependently decreased free thiols, total thiols,

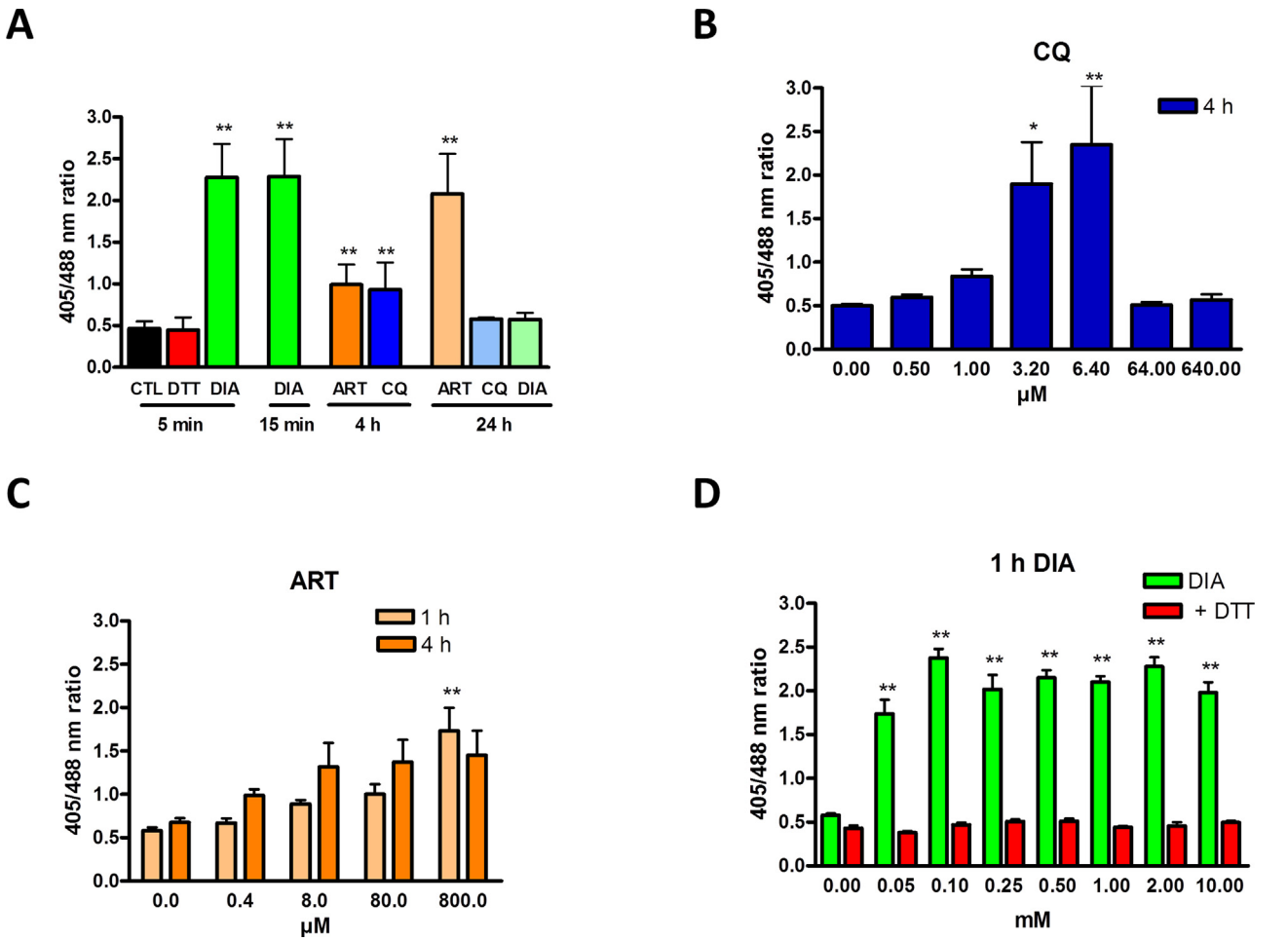

Fig. 4. Detection of the 405/488 nm ratio of *P. falciparum* 3D7$_{[hGrx1\text{-}roGFP2]}$ after diamide (DIA), artemisinin (ART), and chloroquine (CQ) treatment. (A) 405/488 nm ratio of genetically encoded hGrx1-roGFP2 after treatment with 10 mM DTT and 1 mM DIA for 5 min, 1 mM DIA for 15 min, 50xEC_{50} ART or CQ for 4 h, and 4xEC_{50} of ART or CQ or 100 µM DIA for 24 h detected by CLSM. (B) 1 h treatment with different concentrations of DIA (green) or 1 h DIA treatment and subsequent 5 min treatment with the reducing agent DTT (red). (C) 1 h and 4 h (orange) treatment with different concentrations of ART. (D) 4 h treatment with different concentrations of CQ (blue) (0.32, 6.4, 64, and 640 µM showed no effect on the ratio after 1 h treatment). Asterisks indicate significant differences $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Every drug and every concentration was investigated in 3 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

total glutathione and GSH levels after 1 h and 4 h treatment (Supplementary Fig. S1A and B). The results are in partial agreement with previous results on total thiol and total glutathione levels of non-transfected *P. falciparum* 3D7 trophozoites [22].

3.2. ThiolTracker™ Violet detects drug concentration-dependent shifts in fluorescence

In order to compare the hGrx1-roGFP2 signal systematically with other parameters probing the intracellular redox milieu we determined the signals obtained with ThiolTracker™ Violet after short, medium and long-term incubations with redox active agents and drugs. Prior to the fluorescence measurements using confocal microscopy (Fig. 2, left column) and a plate reader (Fig. 2, right column), we optimized the loading time, and cell number for *P. falciparum* blood stage parasites (Supplementary Fig. S2A and B). Uninfected RBCs as well as the cytosol of intact infected red blood cells (iRBC) did hardly show any ThiolTracker™ Violet fluorescence signals (Fig. 2A). Parasites treated with ART, CQ or DIA without ThiolTracker™ staining did not exhibit fluorescence (data not shown).

Fluorescence microscopy revealed at high DIA concentrations a dose-dependent decrease of thiols (Fig. 2A), whereby the highest concentrations of 2–10 mM led to disruption of the infected red blood cell (iRBC) unit and low fluorescence signals. The results were fully reproducible on a plate reader (Fig. 2B). The antimalarial drugs ART and CQ reduced the ThiolTracker™ Violet signal after 1 and 4 h treatment in a concentration-dependent manner. However, at artificially high drug concentrations, the signals increased again (Fig. 2C–F). Also these results were in principle reproducible on the plate reader although some drug effects were found to be less pronounced. This discrepancy is likely to be due to the fact that in CLSM experiments single, viable cells with good fluorescence are studied whereas in the plate reader the cell population is assessed as an entity. After 24 h no significant change in reduced thiols was observed, but plate reader detection had limitations because for technical reasons parasites could not be magnetically enriched prior to ThiolTracker™ Violet staining (see Section 2) and fluorescence signals were therefore weak.

In summary, ThiolTracker™ Violet seems to be a suitable reagent to assess the thiol status of living intraerythrocytic *P. falciparum* parasites. Assessment at the microplate reader is easier to handle, is not influenced by variations in the focal plane, allows for the detection of a great amount of cells in parallel, and yields good and reproducible signals.

Please cite this article in press as: F. Mohring, et al., Comparison of methods probing the intracellular redox milieu in Plasmodium falciparum, Mol Biochem Parasitol (2015), http://dx.doi.org/10.1016/j.molbiopara.2015.11.002

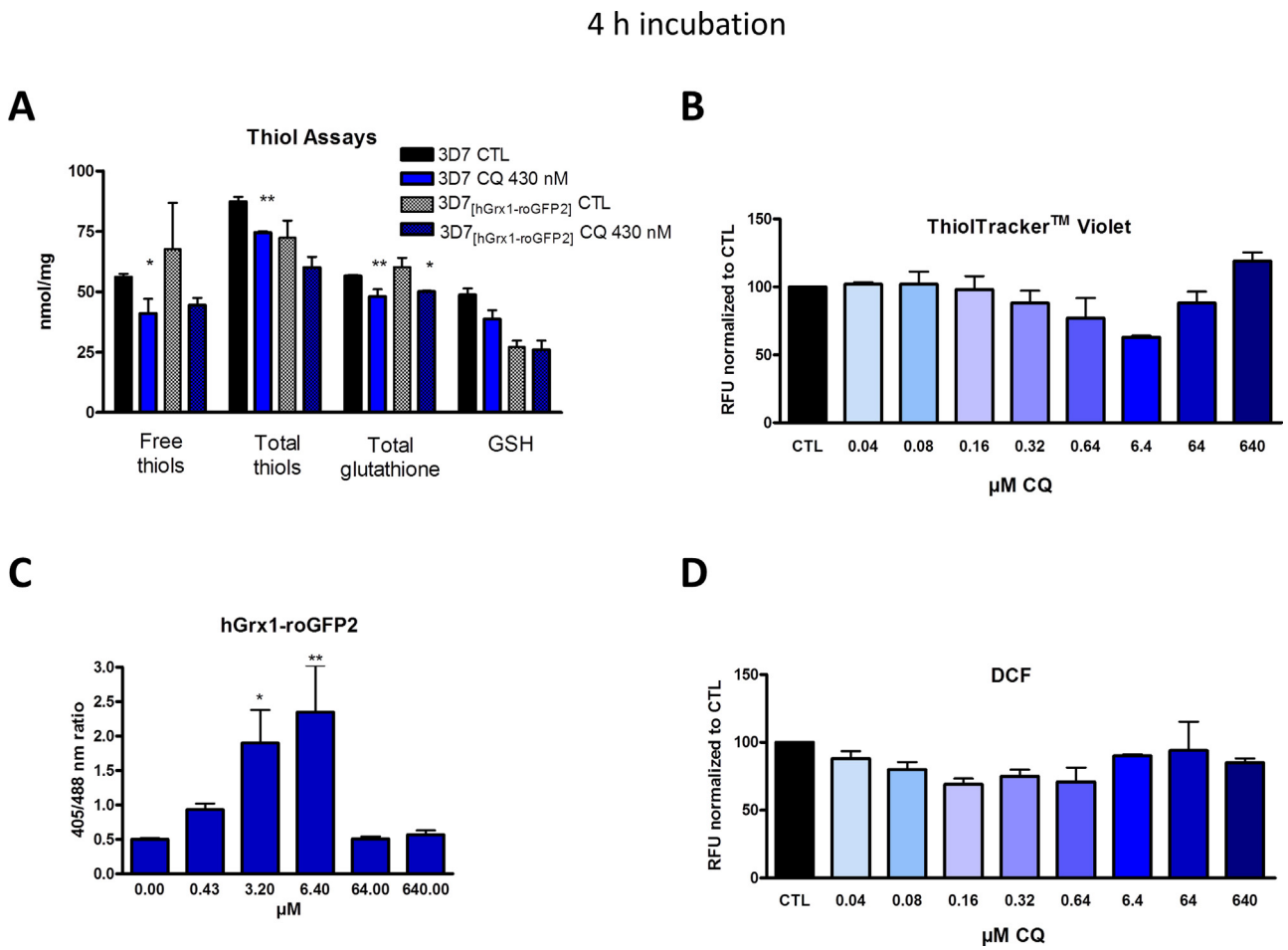

Fig. 5. Direct comparison of parameters probing the redox status of *P. falciparum* 3D7 after 4 h chloroquine treatment. (A) Concentrations of total and non-protein thiols, total glutathione determined with Ellman's reagent and GSH determined with naphthalenedicarboxaldehyde, determined in non-transfected and transfected parasites after 4 h treatment with 430 nM CQ. (B) Cytosolic thiols determined with ThiolTracker™ Violet. (C) Glutathione dependent redox potential probed with the hGrx1-roGFP2 biosensor. (D) General oxidative stress determined with CM-H_2DCFDA.

3.3. The fluorescein based probe CM-H_2DCFDA detects shifts in oxidative challenge

As a marker for general oxidative and nitrosative stress, the oxidation of H_2DCF to the fluorescent DCF was employed in *P. falciparum* in direct comparison with the other methods. Prior to the fluorescence measurements we optimized the loading time, and cell number for *P. falciparum* blood stage parasites (Supplementary Fig. S2C and D).

DIA increased DCF fluorescence after 1 h and 4 h incubation in a concentration-dependent manner, as detected by CLSM (Fig. 3A) and plate reader detection (Fig. 3B). After incubation with 10 mM DIA for 1 h also the RBC compartment revealed DCF fluorescence signals. Barrand et al. [34] showed with DTNB-based assays that the parasite exports glutathione in the form of GSH into the RBC compartment while the RBC itself exports GSH under physiological conditions and GSSG under oxidative challenge. Under strong DIA stress the antioxidant defense mechanisms of the parasite-host cell unit were obviously not sufficient anymore to maintain the redox balance in the RBC thus resulting in DCF signals.

ART or CQ exposure for 1 h and 4 h hardly changed the DCF response (Fig. 3C–E; please compare *y*-axis in Fig. 3A and B with those in Fig. 3C–E). Klonis et al. [17] also analyzed the effect of ART on CM-H_2DCFDA-loaded *P. falciparum* 3D7 trophozoites and observed only a 1.6-fold increase in fluorescence signals after 2 h treatment with 1 µM. As indicated by our data and discussed below, DCF seems to be more suited for measuring direct and strong oxidative stress rather than the more subtle changes in thiol status induced by antimalarial drugs. Furthermore, it should be pointed out that DCF is quite sensitive to light [35,36] and multiple factors influencing DCF measurements should be taken into account (as reviewed in Tetz et al. [37]).

3.4. Drug effects on the glutathione redox status obtained with hGrx1-roGFP2

Finally we used CLSM detection of the hGrx1-roGFP2 405/488 nm ratio in *P. falciparum* 3D7[hGrx1-roGFP2] to assess the effects of oxidative and pharmacologic stressors and compare them between different methods. As illustrated in Fig. 4A, hGrx1-roGFP2 real-time imaging revealed changes in the glutathione dependent redox potential after 4 h treatment with ART or CQ ($50\times EC_{50}$), which is in agreement with previously reported results [22]. Long-term incubation (24 h, $4\times EC_{50}$) with ART also increased the ratio, while CQ and DIA showed only small effects. In our previous study [22] we had also observed a strong ratio increase after 24 h treatment with ART and even stronger CQ effects (405/488 nm ratio of about 1.4).

Short-term treatment with DTT and DIA served as controls for the fully reduced and fully oxidized state of the glutathione redox probe. As shown in Fig. 4B, CLSM imaging of 3D7[hGrx1-roGFP2] revealed full oxidation already after 1 h treatment with 50 µM DIA,

Please cite this article in press as: F. Mohring, et al., Comparison of methods probing the intracellular redox milieu in *Plasmodium falciparum*, Mol Biochem Parasitol (2015), http://dx.doi.org/10.1016/j.molbiopara.2015.11.002

suggesting the direct oxidation of the probe to be fast and long lasting. In all cases complete reversion of the signal increase could be achieved by reduction with DTT (even after treatment with 10 mM DIA for 1 h). As shown in Fig. 4C and D, ART (1 h or 4 h) and CQ (4 h) led to a dose-dependent increase of the 405/488 nm ratio (again with reduced signals at the highest pharmacologically not relevant CQ concentrations).

3.5. Comparison of the different methods probing the thiol/glutathione status in P. falciparum

Using the experimental data on CQ as an example, we directly compared the results of the different methods applied in this study in order to allow for a cross validation and facilitate the planning of experimental setups for future studies in the field. The data are summarized in Fig. 5. All data obtained resulted after 4 h incubation of the parasites with various concentrations of CQ. All four biochemical assays in parasite extracts indicated a clear decrease in thiol and glutathione concentrations after 4 h incubation with 430 nM CQ (free thiols 26–35%; total thiols 14–17%; total glutathione 14–17%, GSH 4–21%). These effects were detectable in both 3D7 parasites transfected and not transfected with the hGrx1-roGFP2 probe (Fig. 5A). ThiolTracker™ Violet demonstrated a nicely comparable dose-dependent decrease in intracellular thiols at concentrations of 160 nM (2%), 320 nM (12%), 640 nM (23%), and 6.4 μM (37%) CQ (Fig. 5B), which, however, did not quite reach levels of significance. Also the hGrx1-roGFP2 probe started to increase its 405/488 ratio at 430 nM CQ (19% increase at 430 nM, 86% at 3.2 μM, 370% at 6.4 μM) indicating an impairment of the glutathione dependent redox potential (Fig. 5C). Therefore, in direct comparison with ThiolTracker™ Violet, the hGrx1-roGFP2 probe reacted even more sensitively to the redox changes indicating its particular suitability in this concentration range. In contrast to all other parameters, the DCF signal (Fig. 5D, see also Fig. 3E) hardly changed in this CQ concentration range (please compare to Fig. 3B which indicates pronounced changes observed under diamide stress and take into account the different numbering on the y-axis). This result can be explained by the fact that massive and direct oxidative stress is not imposed on the cells. To conclude, ThiolTracker™ Violet and, even more so, the hGrx1-roGFP2 probe reacted sensitively to CQ induced changes in intracellular redox metabolism. These results were well substantiated by classical cell disruptive methods. DCF can rather be employed as a chemical probe measuring direct and unspecific oxidative challenge.

Glutathione metabolism has been linked to CQ resistance [38,39]. GSH forms a complex with heme in the parasite digestive vacuole to prevent oxidative damage, whereas the CQ–heme complex is toxic [40]. Remarkably, GSH levels were shown to be lower in CQ resistant parasite lines [41]. Conserved in CQ resistant parasite lines is the K76T mutation in the chloroquine-resistance transporter (PfCRT), a protein in the digestive vacuole membrane, as reviewed in [42]. It was demonstrated that independent of intracellular GSH levels, glutathione transport processes are important for CQ resistance. Mutant PfCRTs revealed glutathione transport activity, indicating an increased transport of GSH into the digestive vacuole in CQ resistant parasite lines [41].

The involvement of glutathione in the mechanism of action of ART has, however, been more difficult to demonstrate although the conversion of GSH to GSSG under ART treatment was already proposed in 2001 [43] and there is consent that ART generates free radicals upon interaction with iron [44]. Later hypotheses include Fe-ART adducts that bind the PfATP6, a SERCA homolog, preventing calcium binding and as a consequence resulting in a loss of function of the Ca^{2+} pump as a mode of drug action [45]. Furthermore, the ART resistance marker K13 is linked to oxidative stress [46]. It was also observed that ART activity depends on hemoglobin uptake and hydrolysis in the digestive vacuole, while at the same time ART inhibits hemoglobin uptake [17]. Not long ago, exported protein 1 (EXP1), which is located mainly to the parasitophorous vacuolar membrane and to cytosolic compartments in iRBCs, was shown to have glutathione *S*-transferase activity, conjugating glutathione onto toxic hematin, and on top of that it was evidenced to be inhibited by the ART-derivative artesunate [47].

In conclusion, we believe that it is of great interest to further study the changes in intracellular thiol and glutathione levels of *P. falciparum* under drug treatment. Particularly the hGrx1-roGFP2 probe offers great potential for sensitive monitoring of the glutathione redox potential in living cells and a combination with the other biochemical and fluorescence-based methods presented here can greatly help interpreting and cross-validating the obtained results. To study detailed mechanisms of drug action and enhance reproducibility of the data, ideally highly synchronized cell lines with isogenic background should be tested in at least 3–4 independent biological replicates with standardized protocols taking into account the specific demands of the different methods. Furthermore, employing the hGrx1-roGFP2 probe in *Plasmodium* would greatly benefit from stable transfection of parasites and targeting of the redox probe to subcellular compartments, which is presently followed up in our laboratory in collaboration with other expert groups.

Acknowledgements

The authors wish to thank Marina Fischer and Siegrid Franke for their excellent technical assistance, Markus Schwarzländer for introduction to the Batch Ratio Analysis software [29] and Andreas Meyer for providing the hGrx1-roGFP2 probe. The study was supported by the Deutsche Forschungsgemeinschaft (grant BE1540/23-1 to K.B. within SPP 1710).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2015.11.002.

References

[1] WHO, World Malaria Report, World Health Organization, Geneva, Switzerland, 20142014.
[2] S. Takala-Harrison, M.K. Laufer, Antimalarial drug resistance in Africa: key lessons for the future, Malar. Adv. Pathophysiol. Biol. Drug Dev. 1342 (2015) 62–67.
[3] R.L. Krauth-Siegel, H. Bauer, R.H. Schirmer, Dithiol proteins as guardians of the intracellular redox milieu in parasites: old and new drug targets in trypanosomes and malaria-causing plasmodia, Angew. Chem. Int. Ed. Engl. 44 (2005) 690–715.
[4] S. Rahlfs, K. Becker, Interference with redox-active enzymes as a basis for the design of antimalarial drugs, Mini Rev. Med. Chem. 6 (2006) 163–176.
[5] S.M. Kanzok, The thioredoxin system of the malaria parasite *Plasmodium falciparum*: glutathione reduction revisited, J. Biol. Chem. 275 (2000) 40180–40186.
[6] E. Jortzik, K. Becker, Thioredoxin and glutathione systems in *Plasmodium falciparum*, Int. J. Med. Microbiol. 302 (2012) 187–194.
[7] S. Muller, Thioredoxin reductase and glutathione synthesis in *Plasmodium falciparum*, Redox Rep. 8 (2003) 251–255.
[8] K. Becker, S. Rahlfs, C. Nickel, R.H. Schirmer, Glutathione—functions and metabolism in the malarial parasite *Plasmodium falciparum*, Biol. Chem. 384 (2003) 551–566.
[9] R.L. Krauth-Siegel, A.E. Leroux, Low-molecular-mass antioxidants in parasites, Antioxid. Redox Signal. 17 (2012) 583–607.
[10] K. Becker, M. Gui, A. Traxler, C. Kirsten, R.H. Schirmer, Redox processes in malaria and other parasitic diseases. Determination of intracellular glutathione, Histochemistry 102 (1994) 389–395.
[11] H. Atamna, H. Ginsburg, The malaria parasite supplies glutathione to its host cell—investigation of glutathione transport and metabolism in human erythrocytes infected with *Plasmodium falciparum*, Eur. J. Biochem. 250 (1997) 670–679.

[12] M. Rahbari, K. Diederich, K. Becker, R.L. Krauth-Siegel, E. Jortzik, Detection of thiol-based redox switch processes in parasites—facts and future, Biol. Chem. 396 (2015) 445–463.
[13] B.S. Mandavilli, M.S. Janes, Detection of intracellular glutathione using ThiolTracker violet stain and fluorescence microscopy, Curr. Protoc. Cytom. (2010), Chapter 9: Unit 9.
[14] A.J. Neuwelt, Y.J. Wu, N. Knap, M. Losin, E.A. Neuwelt, M.A. Pagel, et al., Using acetaminophen's toxicity mechanism to enhance cisplatin efficacy in hepatocarcinoma and hepatoblastoma cell lines, Neoplasia 11 (2009) 1003–1011.
[15] H. Wang, J.A. Joseph, Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader, Free Radic. Biol. Med. 27 (1999) 612–616.
[16] Y. Fu, L. Tilley, S. Kenny, N. Klonis, Dual labeling with a far red probe permits analysis of growth and oxidative stress in *P. falciparum*-infected erythrocytes, Cytometry A 77 (2010) 253–263.
[17] N. Klonis, M.P. Crespo-Ortiz, I. Bottova, N. Abu-Bakar, S. Kenny, P.J. Rosenthal, et al., Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 11405–11410.
[18] G.T. Hanson, R. Aggeler, D. Oglesbee, M. Cannon, R.A. Capaldi, R.Y. Tsien, et al., Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators, J. Biol. Chem. 279 (2004) 13044–13053.
[19] C.T. Dooley, T.M. Dore, G.T. Hanson, W.C. Jackson, S.J. Remington, R.Y. Tsien, Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators, J. Biol. Chem. 279 (2004) 22284–22293.
[20] M. Gutscher, A.L. Pauleau, L. Marty, T. Brach, G.H. Wabnitz, Y. Samstag, et al., Real-time imaging of the intracellular glutathione redox potential, Nat. Methods 5 (2008) 553–559.
[21] B. Morgan, M.C. Sobotta, T.P. Dick, Measuring E(GSH) and H2O2 with roGFP2-based redox probes, Free Radic. Biol. Med. 51 (2011) 1943–1951.
[22] D. Kasozi, F. Mohring, S. Rahlfs, A.J. Meyer, K. Becker, Real-time imaging of the intracellular glutathione redox potential in the malaria parasite *Plasmodium falciparum*, PLoS Pathog. 9 (2013) e1003782.
[23] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77.
[24] D. Menon, P.G. Board, A fluorometric method to quantify protein glutathionylation using glutathione derivatization with 2,3-naphthalenedicarboxaldehyde, Anal. Biochem. 433 (2013) 132–136.
[25] W. Trager, J.B. Jensen, Human malaria parasites in continuous culture, Science 193 (1976) 673–675.
[26] C. Lambros, J.P. Vanderberg, Synchronization of *Plasmodium falciparum* erythrocytic stages in culture, J. Parasitol. 65 (1979) 418–420.
[27] F. Paul, S. Roath, D. Melville, D.C. Warhurst, J.O. Osisanya, Separation of malaria-infected erythrocytes from whole blood: use of a selective high-gradient magnetic separation technique, Lancet 2 (1981) 70–71.
[28] N. Sturm, E. Jortzik, B.M. Mailu, S. Koncarevic, M. Deponte, K. Forchhammer, et al., Identification of proteins targeted by the thioredoxin superfamily in *Plasmodium falciparum*, PLoS Pathog. 5 (2009) e1000383.
[29] M.D. Fricker (2015). Quantitative redox imaging software. Antioxid. Redox Signal. (Epub ahead of print).
[30] B. Morgan, D. Ezerina, T.N. Amoako, J. Riemer, M. Seedorf, T.P. Dick, Multiple glutathione disulfide removal pathways mediate cytosolic redox homeostasis, Nat. Chem. Biol. 9 (2013) 119–125.
[31] S. Muller, Redox and antioxidant systems of the malaria parasite *Plasmodium falciparum*, Mol. Microbiol. 53 (2004) 1291–1305.
[32] E.M. Patzewitz, E.H. Wong, S. Muller, Dissecting the role of glutathione biosynthesis in *Plasmodium falciparum*, Mol. Microbiol. 83 (2012) 304–318.
[33] K.A. Lukyanov, V.V. Belousov, Genetically encoded fluorescent redox sensors, Biochim. Biophys. Acta 1840 (2014) 745–756.
[34] M.A. Barrand, M. Winterberg, F. Ng, M. Nguyen, K. Kirk, S.B. Hladky, Glutathione export from human erythrocytes and *Plasmodium falciparum* malaria parasites, Biochem. J. 448 (2012) 389–400.
[35] E. Marchesi, C. Rota, Y.C. Fann, C.F. Chignell, R.P. Mason, Photoreduction of the fluorescent dye 2′-7′-dichlorofluorescein: a spin trapping and direct electron spin resonance study with implications for oxidative stress measurements, Free Radic. Biol. Med. 26 (1999) 148–161.
[36] C. Rota, Y.C. Fann, R.P. Mason, Phenoxyl free radical formation during the oxidation of the fluorescent dye 2′,7′-dichlorofluorescein by horseradish peroxidase. Possible consequences for oxidative stress measurements, J. Biol. Chem. 274 (1999) 28161–28168.
[37] L.M. Tetz, P.W. Kamau, A.A. Cheng, J.D. Meeker, R. Loch-Caruso, Troubleshooting the dichlorofluorescein assay to avoid artifacts in measurement of toxicant-stimulated cellular production of reactive oxidant species, J. Pharmacol. Toxicol. Methods 67 (2013) 56–60.
[38] H. Ginsburg, O. Famin, J. Zhang, M. Krugliak, Inhibition of glutathione-dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action, Biochem. Pharmacol. 56 (1998) 1305–1313.
[39] 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 (2004) 163–189.
[40] G.M. Nayyar, J.G. Breman, P.N. Newton, J. Herrington, Poor-quality antimalarial drugs in southeast Asia and sub-Saharan Africa, Lancet Infect. Dis. 12 (2012) 488–496.
[41] E.M. Patzewitz, J.E. Salcedo-Sora, E.H. Wong, S. Sethia, P.A. Stocks, S.C. Maughan, et al., Glutathione transport: a new role for PfCRT in chloroquine resistance, Antioxid. Redox Signal. 19 (2013) 683–695.
[42] P.G. Bray, R.E. Martin, L. Tilley, S.A. Ward, K. Kirk, D.A. Fidock, Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance, Mol. Microbiol. 56 (2005) 323–333.
[43] S. Mukanganyama, Y.S. Naik, M. Widersten, B. Mannervik, J.A. Hasler, Proposed reductive metabolism of artemisinin by glutathione transferases in vitro, Free Radic. Res. 35 (2001) 427–434.
[44] P.M. O'Neill, V.E. Barton, S.A. Ward, The molecular mechanism of action of artemisinin—the debate continues, Molecules 15 (2010) 1705–1721.
[45] A. Shandilya, S. Chacko, B. Jayaram, I. Ghosh, A plausible mechanism for the antimalarial activity of artemisinin: a computational approach, Sci. Rep. 3 (2513) (2013).
[46] F. Ariey, B. Witkowski, C. Amaratunga, J. Beghain, A.C. Langlois, N. Khim, et al., A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria, Nature 505 (2014) 50–55.
[47] A.M. Lisewski, J.P. Quiros, C.L. Ng, A.K. Adikesavan, K. Miura, N. Putluri, et al., Supergenomic network compression and the discovery of EXP1 as a glutathione transferase inhibited by artesunate, Cell 158 (2014) 916–928.

Please cite this article in press as: F. Mohring, et al., Comparison of methods probing the intracellular redox milieu in *Plasmodium falciparum*, Mol Biochem Parasitol (2015), http://dx.doi.org/10.1016/j.molbiopara.2015.11.002