

Supporting Information

Peroxide antimalarial drugs target redox homeostasis in *Plasmodium falciparum* infected red blood cells

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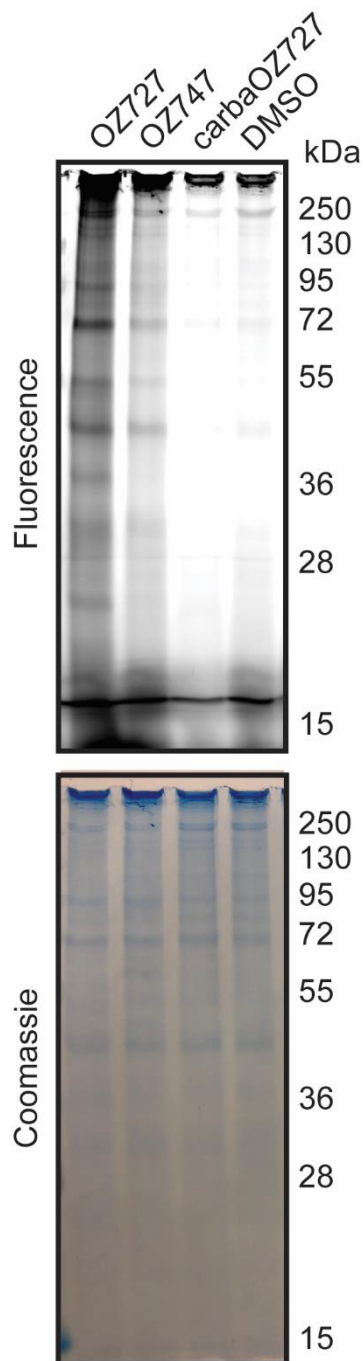
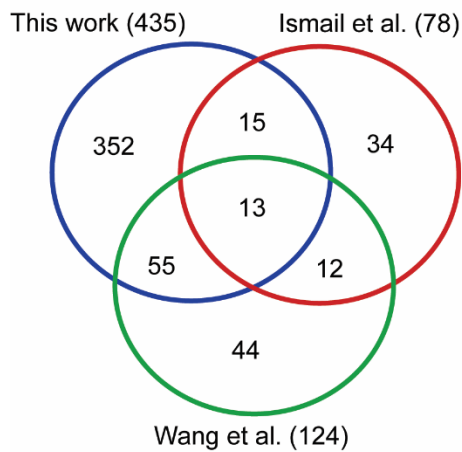


Figure S1. In-gel fluorescence analysis of ozonide alkylated proteins in *P. falciparum*. SDS-PAGE gel showing protein alkylation in samples treated with the active ozonide probes, OZ727 and OZ747. No labelling was observed in samples treated with the inactive ozonide clickable probe, carbaOZ727 or the untreated (DMSO) control. Synchronised trophozoite stage cultures (10% parasitaemia and 2% Hct) were exposed to OZ727, OZ747, carbaOZ727 (all 300 nM) or an equivalent volume of DMSO for 3 h. Protein extracts were prepared from the treated live parasites. Alkylated proteins containing an alkyne tag were clicked with a fluorescent dye (Alexa Fluor 647) using copper catalysed click chemistry, resolved by SDS-PAGE and visualised with fluorescence scanning.

A

Enriched proteins that are common between this work (Siddiqui et al.), Wang et al., and Ismail., et al.	
Gene ID	Protein description
PF3D7_1015900	Enolase (ENO)
PF3D7_1246200	Actin I (ACT1)
PF3D7_0608800	Ornithine aminotransferase (OAT)
PF3D7_0523000	Multidrug resistance protein (MDR1)
PF3D7_1324900	L-lactate dehydrogenase (LDH)
PF3D7_0322900	40S ribosomal protein S3A, putative (S3A)
PF3D7_1408000	Plasmepsin II (PM II)
PF3D7_0903700	α tubulin 1
PF3D7_0709000	Chloroquine resistance transporter (CRT)
PF3D7_0102200	Ring-infected erythrocyte surface antigen (RESA)
PF3D7_0207600	Serine repeat antigen 5 (SERA5)
PF3D7_0930300	Merozoite surface protein 1 (MSP1)
PF3D7_0824600	Fe-S cluster assembly protein DRE2, putative (DRE2)

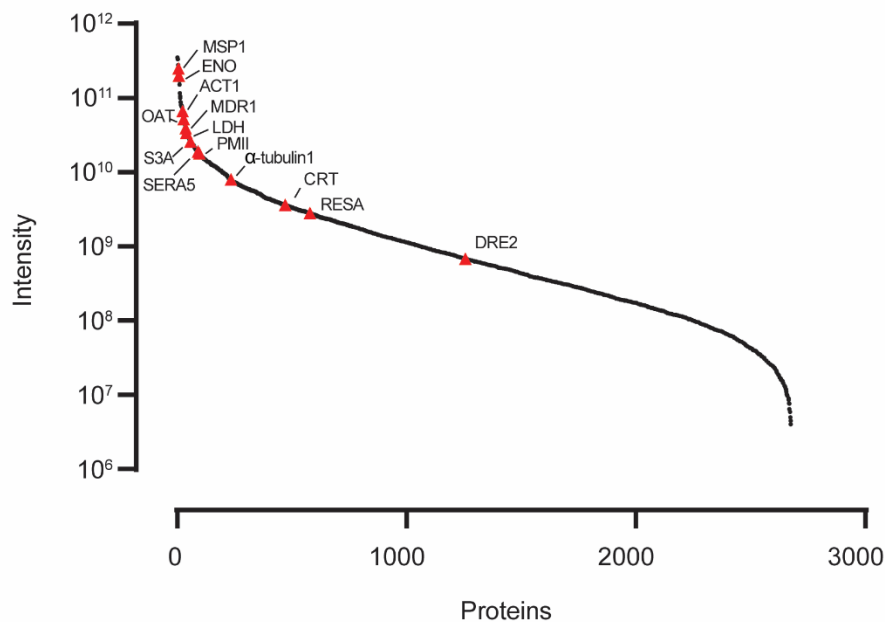
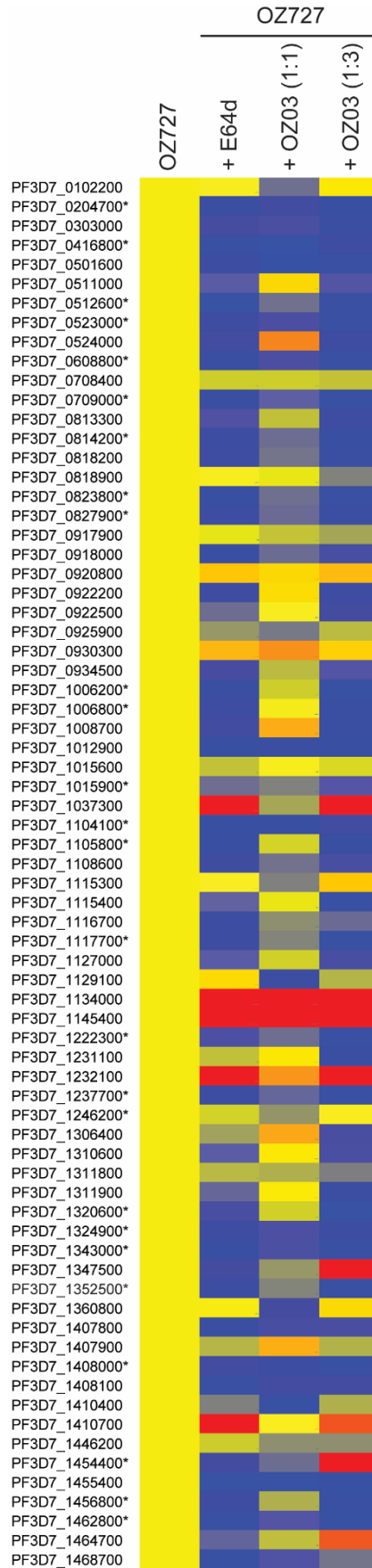
B

Figure S2. Comparison of pulldown results with previously published datasets. (A) Venn diagram showing the overlap between proteins enriched with peroxide clickable probes in the current study (Siddiqui *et al.*), Ismail *et al.*¹ and Wang *et al.*² and table with the 13 commonly identified proteins. (B) Scatterplot showing that the 13 commonly identified proteins (red triangles) from each of the three studies are generally highly expressed proteins within the *P. falciparum* blood stage proteome, except DRE2. The relative abundance of proteins in the *P. falciparum* blood stage proteome (Siddiqui *et al.*, unpublished data) are shown as black circles.



Normalised abundance (%)

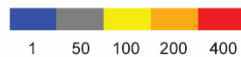


Figure S3. Heatmap representation of the effect of OZ03 co-incubation or E64d pre-treatment on OZ727 labelling of food vacuole proteins (GO:0020020). The extent of food vacuole protein (GO:0020020) alkylation is expressed relative to levels obtained after treatment with 300 nM of OZ727 alone for 1 h (column 1). OZ727 activation was blocked by pre-treatment (30 min) of live parasite cultures with 10 μ M of the cysteine protease inhibitor, E64d, followed by incubation with OZ727 for an additional 1 h (column 2). Competition of OZ727 binding to alkylated target proteins, was achieved by co-incubation with 300 nM of OZ727 and either an equivalent (300 nM, column 3) or excessive (900 nM, column 4) concentration of OZ03 for 1 h. The average protein abundance from at least three biological replicates is presented. Yellow features represent no change compared to OZ727 alone. Increased and decreased levels are represented by red and blue, respectively. Significantly enriched food vacuole proteins identified as OZ727 targets are indicated (*).

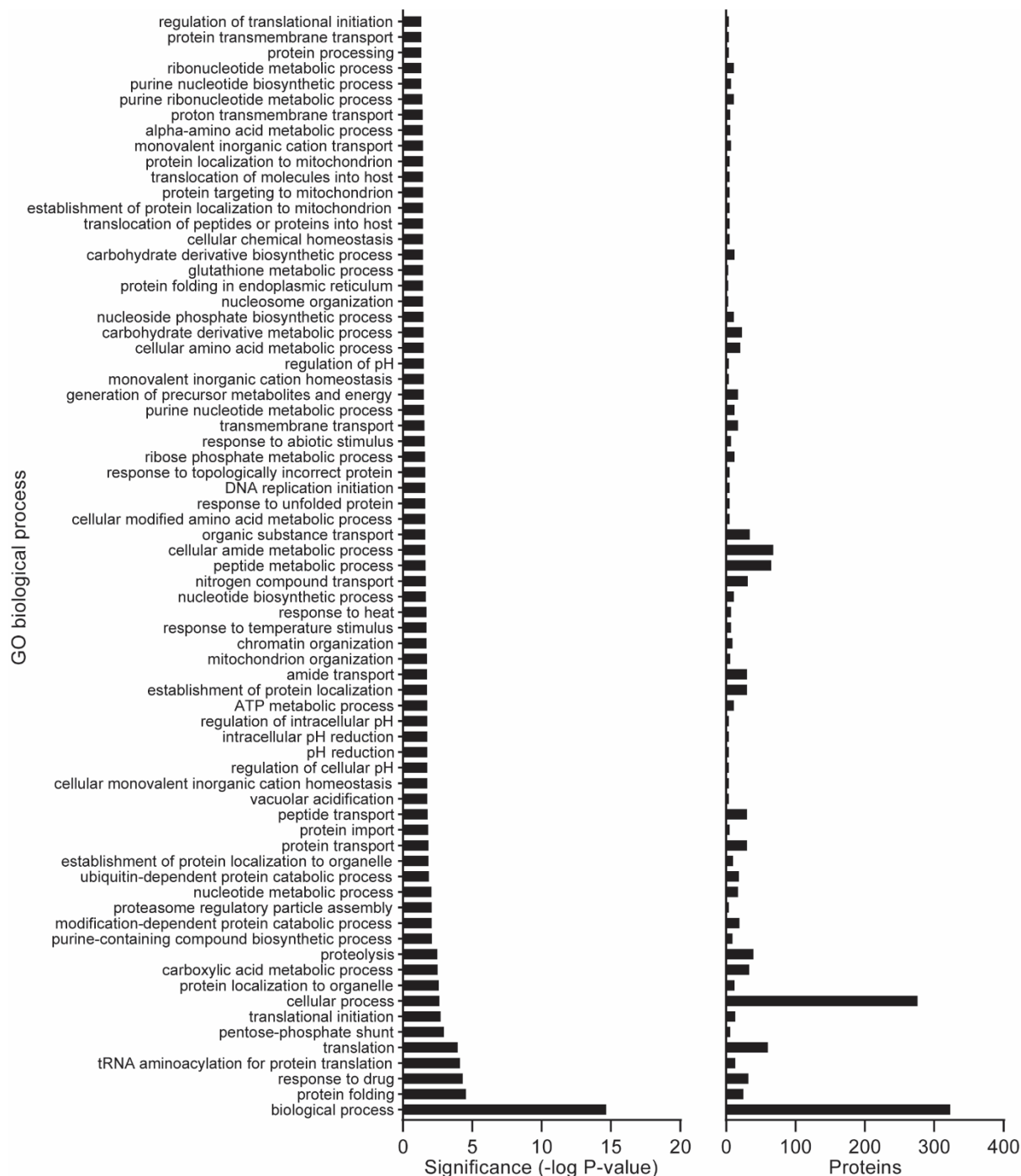


Figure S4. GO biological process enrichment analysis of control dataset. Proteins removed by our filtering strategy were subjected to GO analysis for biological process using topGO. Black bars represent the $-\log P\text{-value}$ for significantly enriched GO biological process terms (left) and the number of proteins identified within each term (right).

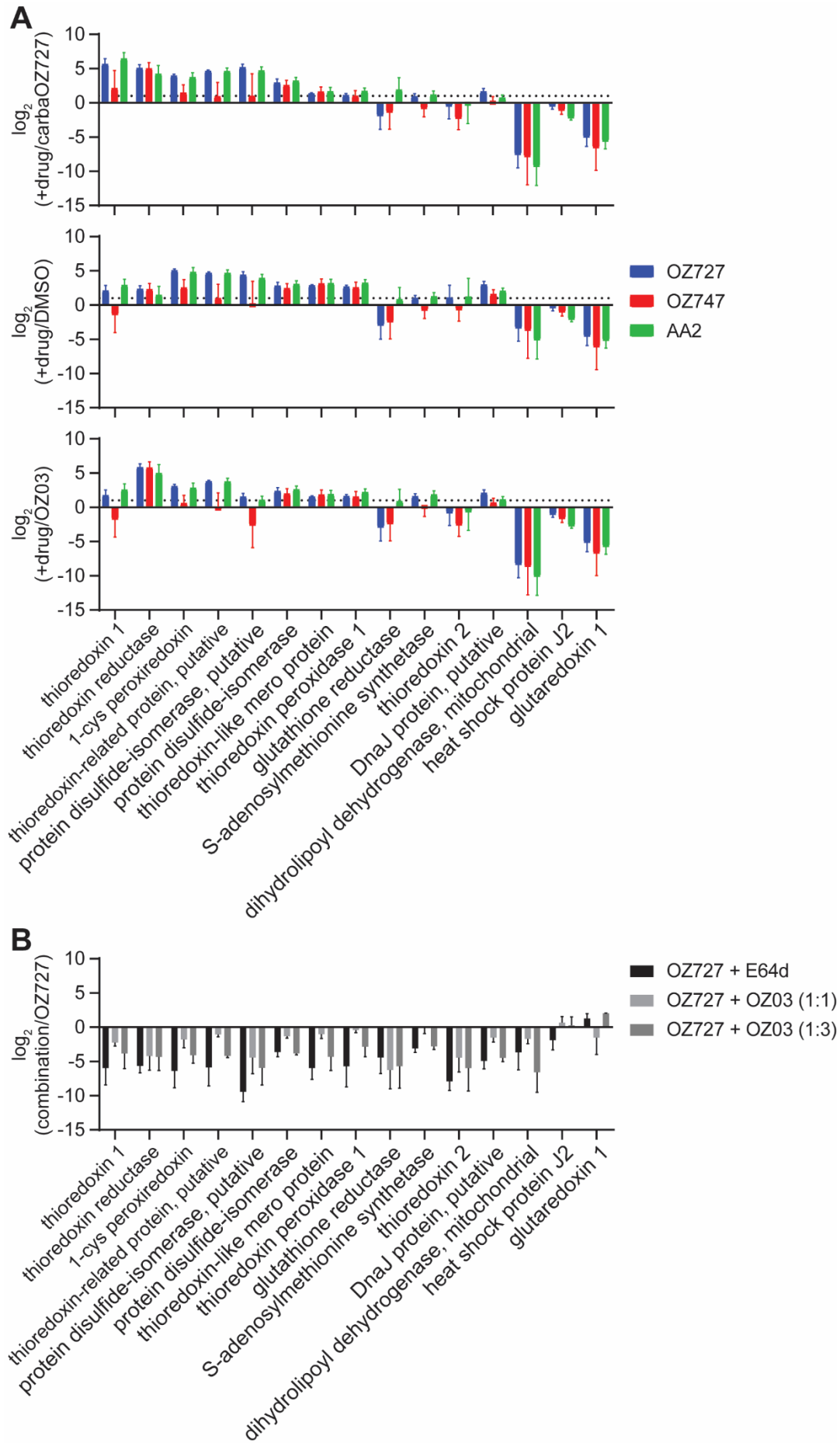


Figure S5. Redox homeostasis proteins (GO:0045454) are alkylation targets of peroxide antimalarials. (A) Enrichment of redox homeostasis proteins (GO:0045454) with OZ727 (blue), OZ747 (red) and AA2 (green) compared to the carbaOZ727 (inactive clickable peroxide), DMSO and OZ03 (active non-clickable parent peroxide) controls. All treatments were for 1 h with 300 nM of the probe. The dashed line represents the two-fold threshold for a peroxide target. (B) Effect of OZ03 co-incubation or E64d pre-treatment on OZ727 labelling of redox homeostasis proteins (GO:0045454). OZ727 activation was blocked by pre-treatment (30 min) of live parasite cultures with 10 μ M of the cysteine protease inhibitor, E64d, followed by incubation with OZ727 for an additional 1 h (black). Competition of OZ727 binding to target proteins, was achieved by co-incubation with 300 nM of OZ727 and either an equivalent (300 nM, light grey) or excessive (900 nM, dark grey) concentration of OZ03 for 1 h. The extent of alkylation of redox homeostasis proteins is expressed relative to levels obtained after treatment with 300 nM of OZ727 alone for 1 h. In (A) and (B) data represents the mean \pm SEM of at least three biological replicates.

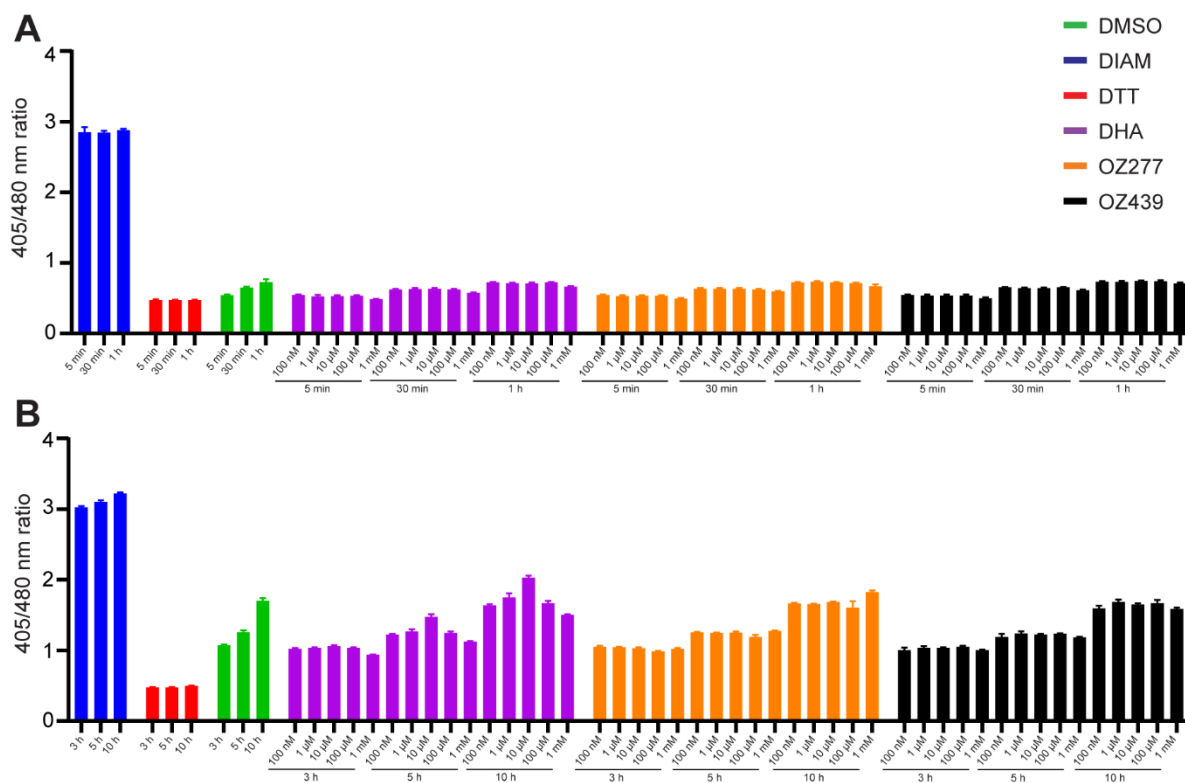


Figure S6. Effects of peroxide antimalarial on reduced hGrx1-roGFP2. Reduced recombinant hGrx1-roGFP2 was incubated with peroxides (DHA, OZ277 and OZ439) at increasing concentrations (100 nM, 1 μM, 10 μM, 100 μM and 1 mM). The redox ratio of the recombinant protein (405/480 nm) was measured using a plate reader after 5 min, 30 min, 1 h (A), 3 h, 5 h, and 10 h (B). 10 mM of DTT and 1 mM DIAM was used to fully reduced and oxidized respectively the recombinant protein. Data represents the mean \pm SD of two biological replicates, with three technical replicates.

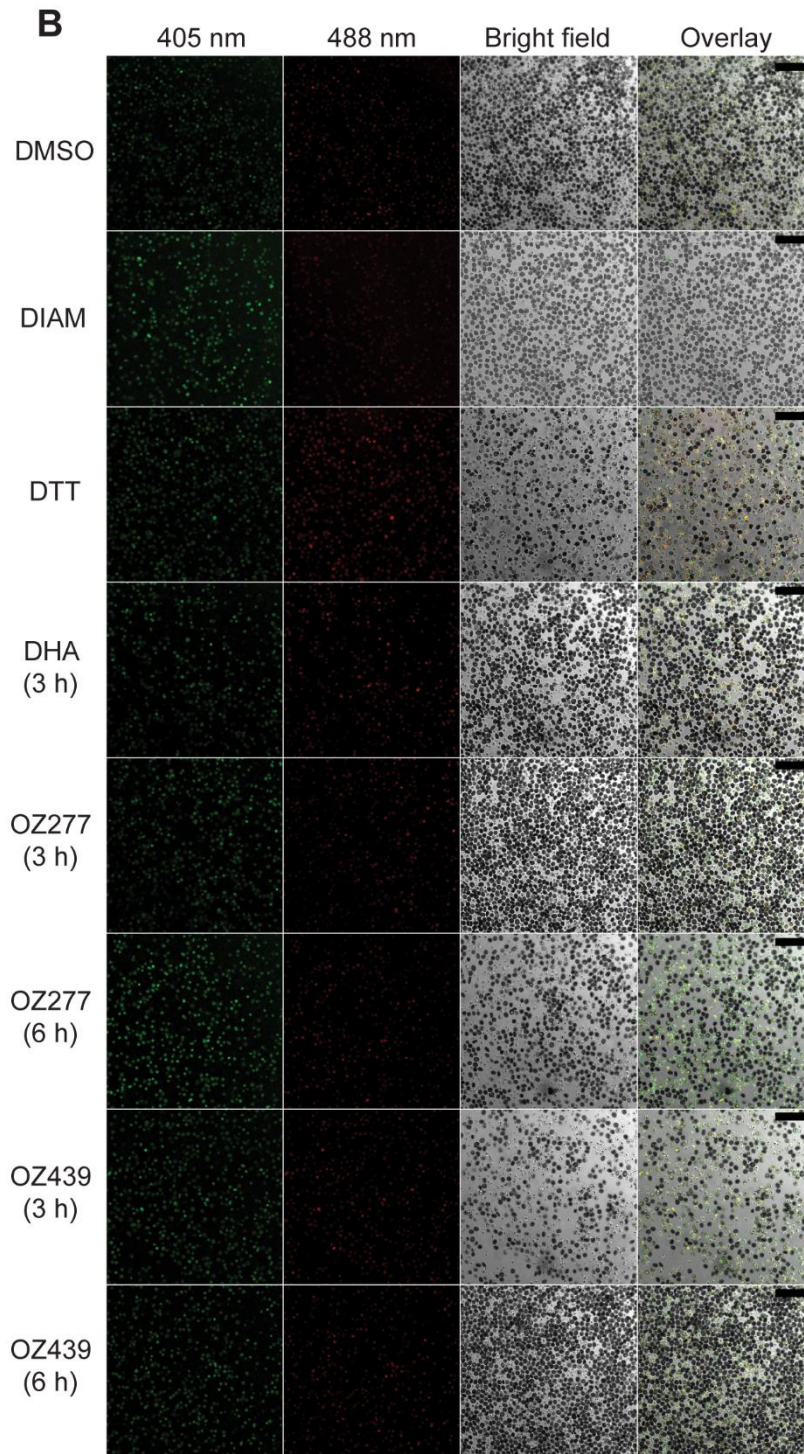
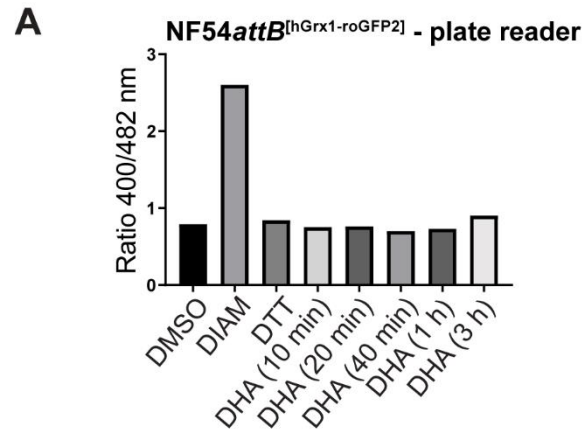


Figure S7. Effect of peroxide antimalarials on the redox ratio of *P. falciparum* NF54attB^[hGrx1-roGFP2] parasites. (A) Effects of 100 nM DHA (short incubation - 10, 20, 40 min, 1 and 3 h) on the redox ratio of *P. falciparum* NF54attB^[hGrx1-roGFP2] parasites. The fluorescence ratio showed that short durations of DHA does not affect the redox sensor within the parasite. Fluorescence was measured using the plate reader and data is from one biological replicate. (B) Parasites were treated with DHA, OZ277 and OZ439 at 300 nM and samples were taken at 3 h post treatment for DHA, OZ277 and OZ439 and at 6 h for OZ277 and OZ439 for the measurement of fluorescence ratio of the redox sensor using CLSM. Fluorescence ratio of the redox sensor increased for parasite treated with OZ277 and OZ439 for 3 and 6 h treatment, while DHA treatment did not change the redox ratio. The black magnification bar represents 50 μ m. For both measurements (A and B), DMSO treated parasites at the longest drug duration acted as control, DTT at 10 mM (5 min treatment) was the fully reduced control, while DIAM at 1 mM (5 min treatment) was the fully oxidised control.

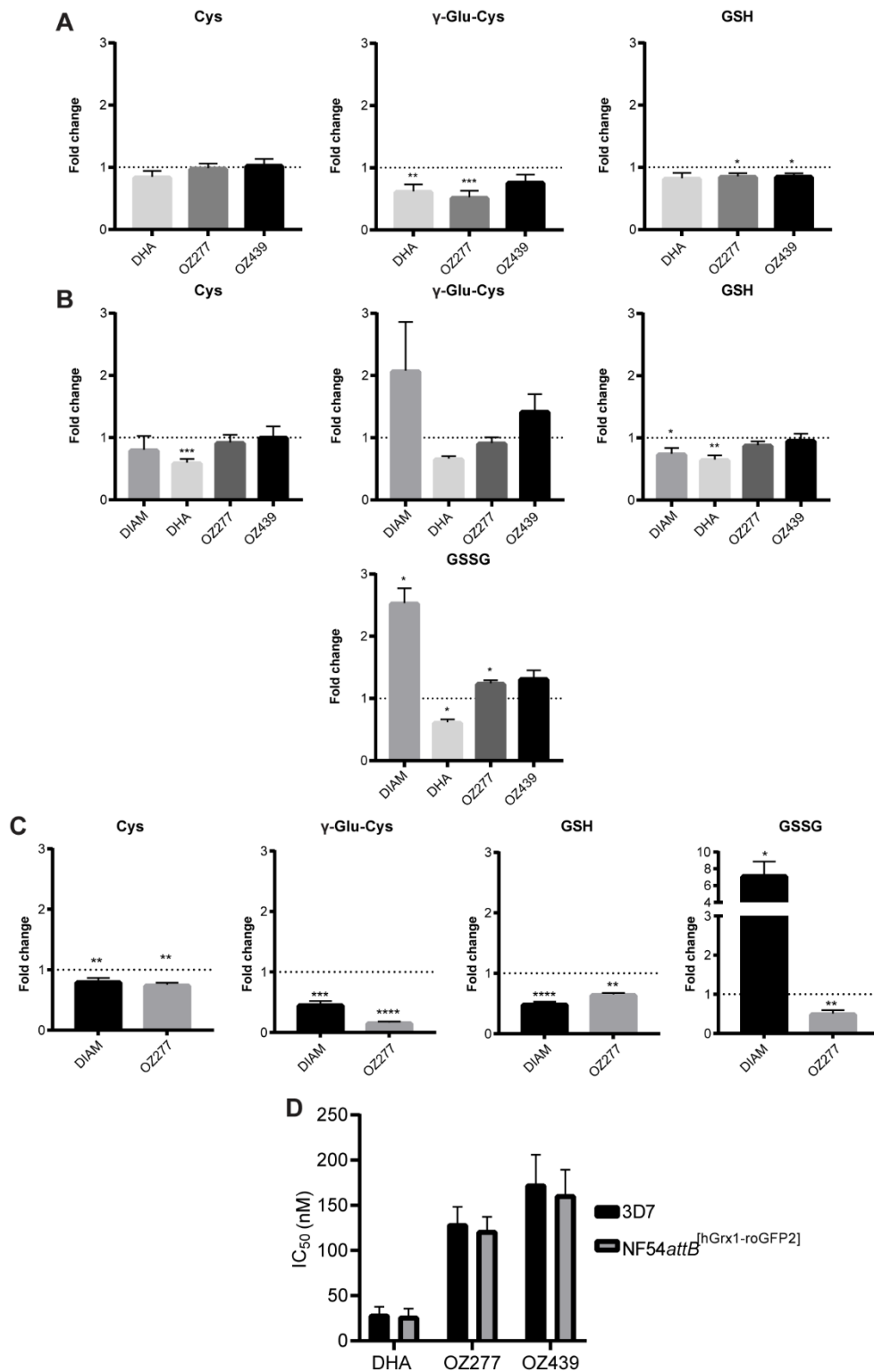


Figure S8. Effect on thiols (using different sample preparation methods) and activity of peroxide antimalarials in *P. falciparum* 3D7 and NF54attB^[hGrx1-roGFP2] parasites. (A, B and C) Determination of relative levels of derivatised cysteine (Cys), γ -glutamyl-cysteine (γ -GluCys), GSH and GSSG compared to DMSO control in *P. falciparum* 3D7 and NF54attB^[hGrx1-roGFP2] parasites. Treatment with DHA (100 nM, 3 h treatment), OZ277 (300 nM, 3 h treatment), OZ439 (300 nM, 6 h treatment) using method 1 (A) and method 2 (B) in 3D7 parasites, or method 3 (C) in NF54attB^[hGrx1-roGFP2] parasites.

DTT (10 mM, 5 min treatment) was the fully reduced control, while DIAM (1 mM, 5 min treatment) was the fully oxidised control. Bars represent the relative abundance (mean \pm SEM) compared to DMSO control. Total thiol measurements in 3D7 parasites are from two biological replicates (with three technical within each biological) for method 1, and three biologicals replicates (with four technical within each biological) for method 2. Thiol measurement in NF54*attB*^[hGrx1-roGFP2] parasites (method 3) is from one biological replicate (with three technical). P-value was calculated using one-way ANOVA, (*, $p < 0.05$; **, $p < 0.01$, $p < 0.01$; ***, $p < 0.0001$; ****). (D) Activity of peroxide antimalarials on magnet harvested *P. falciparum* 3D7 and NF54*attB*^[hGrx1-roGFP2] parasites. Bar graph showing mean \pm SEM IC₅₀ values for DHA, OZ277 and OZ439 tested in a 3 h (DHA and OZ277) or 6 h (OZ439) pulse assay with 90% enriched magnet harvested 3D7 and NF54*attB*^[hGrx1-roGFP2] parasites (0.12% Hct). Assays were conducted on three independent occasions in triplicate. There was no significant difference in the measured IC₅₀ values for the two parasite lines.

References

1. Ismail, H. M.; Barton, V.; Phanchana, M.; Charoensutthivarakul, S.; Wong, M. H. L.; Hemingway, J.; Biagini, G. A.; O'Neill, P. M.; Ward, S. A., Artemisinin activity-based probes identify multiple molecular targets within the asexual stage of the malaria parasites *Plasmodium falciparum* 3D7. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113* (8), 2080-2085. DOI: 10.1073/pnas.1600459113.
2. Wang, J.; Zhang, C.-J.; Chia, W. N.; Loh, C. C.; Li, Z.; Lee, Y. M.; He, Y.; Yuan, L.-X.; Lim, T. K.; Liu, M.; Liew, C. X.; Lee, Y. Q.; Zhang, J.; Lu, N.; Lim, C. T.; Hua, Z.-C.; Liu, B.; Shen, H.-M.; Tan, K. S. W.; Lin, Q., Haem-activated promiscuous targeting of artemisinin in *Plasmodium falciparum*. *Nat. Commun.* **2015**, *6*, 10111. DOI: 10.1038/ncomms10111.