A Click Chemistry-Based Proteomic Approach Reveals that 1,2,4-Trioxolane and Artemisinin Antimalarials Share a Common Protein Alkylation Profile

Hanafy M. Ismail, Victoria E. Barton, Matthew Panchana, Sithivut Charoensutthivarakul, Giancarlo A. Biagini, Stephen A. Ward, and Paul M. O’Neill*

Abstract: In spite of the recent increase in endoperoxide antimalarials under development, it remains unclear if all these chemotypes share a common mechanism of action. This is important since it will influence cross-resistance risks between the different classes. Here we investigate this proposition using novel clickable 1,2,4-trioxolane activity based protein-profiling probes (ABPPs). ABPPs with potent antimalarial activity were able to alkylate protein target(s) within the asexual erythrocytic stage of Plasmodium falciparum (3D7). Importantly, comparison of the alkylation fingerprint with that generated from an artemisinin ABPP equivalent confirms a highly conserved alkylation profile, with both endoperoxide classes targeting proteins in the glycolytic, hemoglobin degradation, antioxidant defence, protein synthesis and protein stress pathways, essential biological processes for plasmodial survival. The alkylation signatures of the two chemotypes show significant overlap (ca. 90%) both qualitatively and semi-quantitatively, suggesting a common mechanism of action that raises concerns about potential cross-resistance liabilities.

Despite concerns about the recent emergence of drug-resistance,[1] the artemisinins (1a–c, Figure 1) remain frontline agents for the treatment of malaria.[2] Understanding the mechanism of action of such an important class has been the subject of intense research over the last two decades.[2] The proposed mechanism of bioactivation of the class involves the cleavage of the endoperoxide bridge by a source of Fe²⁺ or heme. This cleavage results in the formation of oxy-radicals that rearrange into primary or secondary carbon centered radicals (or electrophilic carbocations through single-electron transfer oxidation) (Scheme 1).[3]

These reactive intermediates are proposed to alkylate proteins and form adducts with essential parasite macromolecules that result in the rapid death of the parasite. However, the detail of these important alkylation reactions are sparse and the underlying hypothesis remains controversial.[2] The debate has broadened with the development of highly active fully synthetic endoperoxides based on the pharmacophore of artemisinin namely the trioxolanes (2a)[4] and the tetraoxanes.[5] From the perspective of the underlying chemical mechanism of activation of peroxides and from a cross-resistance risk it is important to establish if these different...
endoperoxide chemotypes share a common mechanism of action or not.

A study of 1,2,4-trioxolanes using monoclonal antibodies has demonstrated parasite protein alkylation with both OZ277 (2b) and OZ439 (2c).[10] However, the methods employed in this work were unable to definitively identify the targeted proteins.

In a recent study, Wang et al.[9] used a non-optimised ART-alkyne activity-based protein-profiling probe that via click-chemistry reactions was associated with some 124 P. falciparum proteins.[7] In this study, a further 125 proteins are reported as being identified in single replicate experiments only, raising concerns of the specificity of the approach.[7] Concurrently, using both ART-alkyne and azide optimized probes we were able to identify 59 P. falciparum proteins with high confidence that were specifically alkylated by ART pointing towards a pleiotropic mechanism of drug action.[9] In our study we deployed probes with reduced linker length and lipophilicity compared to the probe used by Wang et al., and we used both copper-dependent and copper-free reactions over a shortened incubation time (optimized to 1 h as originally described,[9] cf. Wang et al., reaction time of 3 h) together with control non-peroxide probe partner equivalents. These methodological differences help to improve the specificity and pharmacological relevance of the alkylated proteins identified. Significantly in our study only 6 proteins were identified in less than two replicate experiments (cf. 125 in Ref.[7]) with an azide probe, demonstrating the improved specificity of our approach using optimized active and control probe based methodology.

Here, using our refined approach, we describe the rational design of potent activity-based protein-profiling probes (ABPPs) based on a 1,2,4-trioxolane antimalarial core in order to characterize their malaria parasite protein alkylation fingerprint. Significantly, we show that these synthetic 1,2,4-trioxolanes and a semi-synthetic ART share an overlapping parasite protein-alkylation signature suggestive of a common mechanism of action for the endoperoxide class of antimalarial.

The probes were designed with the alkyne/azide click handle sited within the adamantane ring system since this is the site (Scheme 1) of reactive C-radical/carbocation generation post activation by FeIII. We also deployed a bio-orthogonal copper-free “click” methodology via the use of an azide analogue along with its negative control Figure 2. The azide probes were included in our analysis to demonstrate that the protein alkylation was solely due to iron mediated activation with no role for the copper in peroxide activation during sample work-up as discussed previously.[9] This Cu-free click reaction possesses comparable kinetics to the Cu-catalyzed reaction and proceeds within minutes in live cells with no apparent cytotoxicity issues.[9b] The complementary reporter tags used in our study can either be sourced commercially or synthesized by literature procedures (Figure S1 in the Supporting Information).

Scheme 2a provides an overview for the synthesis of alkyne probe P1 (6a) and azide probe P2 (7a) along with control probes CP1 (6b) and CP2 (13b). The first step in the synthesis of the trioxolane probes involved the Koch–Haaf carbonylation of hydroxyl adamantanone to give the methyl ester 3. Co-oxidation of oxime 4 with 3 provided trioxolane 5; hydrolysis of the methylester function of 5 followed by EDC-mediated coupling of propargyl amine provided probe P1 (6a) in good overall yield. Coupling of 3-azido-1-propylamine to 6 provided the azide probe P2 (7a) as shown in Scheme 1. The control probe CP1 (6b) was made by a similar approach using diol 9 in a cyclisation reaction with 3 to produce the corresponding carba ester analogue 10. Hydrolysis of 10 and coupling of the resultant acid with propargyl amine afforded 6b in good yield (70%). Azide 7b was made in a similar manner by hydrolysis and coupling as shown in Scheme 2b.

The active probes containing azide/alkyne functionality retained potent antimalarial activity as determined by their IC50 in vitro against P. falciparum 3D7 parasites (Figure S2 and Table S1). The non-peroxide negative control probes CP1 (6b) and CP2 (7b) had no appreciable activity (IC50 > 10 μM) in these assays,[9b] confirming the essentiality of the endoperoxide-bridge for drug activity and further validating...
our probe plus control pair strategy for biologically relevant target protein identification.

As a next step, in vitro cultures of *P. falciparum* 3D7 were incubated with 1 μM of the alkyne trioxolane based probe P1 (6a) or its corresponding control CP1 (6b), for 6 hours, a time already shown to be pharmacologically relevant, causing irreversible parasite toxicity. Following incubation with P1 (6a) alkylated proteins were extracted from erythrocyte free parasites and tagged with Alexa Fluor 488 azide via a click reaction. This was processed for 1D-Gel analysis as described in the supporting information section. After fluorescence imaging of the 1D-Gel, the strongest labeling was observed in the region of 12–75 KDa (Figure S2c).

Importantly, labeling was not observed for the corresponding “negative control” probe CP1 (6b) samples. Additional experiments were carried out to investigate the lowest concentration of reporter azide required to obtain maximum labeling with minimum background on SDS gels (data not shown). As depicted in Figure S2c, the Alexa Fluor 488 azide at a concentration of 20 μM was able to distinguish the P1 (6a) labeling profile from its corresponding control CP1 (6b).

To rule out the possibility that Cu(I) may have led to parasite independent (artifact) protein alkylation and to further validate the results a more stringent bio-orthogonal copper-free “click” methodology was employed using cyclooctyne reporter tags as depicted in Figure 3. Replacement of an alkyne with an azide group in P2 (7a) had no detrimental effect on the antimalarial potency of the trioxolane azide probe as indicated in Figure S2, Figure 3 and Table S1. However, the labeling profile intensity with P2 (7a) was much higher compared to P1 (6a) (Figure 3c,d) suggesting greater efficiency of the copper-free click reaction.

After validating the importance of the endoperoxide bridge for protein alkylation using a 1D gel, we excluded the gel electrophoresis step and advanced the method to direct analysis of the alkylated protein matrix captured using an “on-bead” trypsin digestion protocol as shown in Figure 3b and Figure S3. Overall, multiple proteins critical to parasite life were identified as trioxolane targets with the P2 (7a) probe (Figure S3). No labeling was evident with CP2 (7b) the
negative control analogue or with the DMSO control (Figure S3). Having completed the analysis of the 1,2,4-trioxolane proteome we carried out a head-to-head comparison of an analogous ART activity-based profiling probe (Figure 4).[9]

Remarkably, as depicted in Figure 4, both the trioxolanes probe P2 (7a) and ART probe P3 (11a) share strongly overlapping protein-labeling profiles both qualitatively and semi-quantitatively. From a total of 62 proteins confidently identified with the two probes 53 of the proteins were tagged with both P2 (7a) and P3 (11a) (Figure 4a), with no labeling observed for control probes CP2 (7b) and CP3 (11b) (Figure S3). From a mechanistic perspective it is important to note that ca. 70% of the tagged proteins can be readily glutathionylated (Figure 4b), a post translational modification that can effect redox regulation and signal transduction.[11] For instance, EXP1, the membrane glutathione S-transferase identified with both P2 (7a) and P3 (11a) (Figure 4a), with no labeling observed for control probes CP2 (7b) and CP3 (11b) (Figure S3). From a mechanistic perspective it is important to note that ca. 70% of the tagged proteins can be readily glutathionylated (Figure 4b), a post translational modification that can effect redox regulation and signal transduction.[11] For instance, EXP1, the membrane glutathione S-transferase identified with both P2 (7a) and P3 (11a) (Figure 4a), with no labeling observed for control probes CP2 (7b) and CP3 (11b) (Figure S3). From a mechanistic perspective it is important to note that ca. 70% of the tagged proteins can be readily glutathionylated (Figure 4b), a post translational modification that can effect redox regulation and signal transduction.[11]

Moreover, the global analysis of protein alkylation generated through P2 (7a), for both classes, that is, ART and trioxolanes, is consistent with the “cluster bomb” hypothesis,[7,9,15] whereby Fe^{2+}/heme-activated drug alkylates multiple redox-susceptible protein targets functioning in multiple cellular pathways (Figure S4) including the food vacuole, as it is considered important for iron dependent activation, and also in the cytosol (Figure S5). To conclude, a chemical proteomic approach has for the first time enabled formal identification of the key proteins that are alkylated by the 1,2,4-trioxolane class of antimalarial. Significantly, the proteomic profile of 1,2,4-trioxolanes is similar to the artemisinins suggesting that 1,2,4-trioxolanes

Figure 4. Mass spectrometry experiments with azide trioxolane azide probe (P2 (7a)) vs. artemisinin azide probe (P3 (11a)). a) Venn diagram demonstrating overlap between proteins identified with the endoperoxide probes, P2 (7a) and P3 (11a) respectively. (b) Percentage of the glutathionylated proteins, which contains the GSH binding motif that was identified with endoperoxides probes P2 (7a) and P3 (11a) in light of Kehr et al.[11] (c) Head to head comparison between proteins identified with P2 (7a) vs. P3 (11a). Proteins sorted according to their molecular weight from high to low. Errors bars represented the standard deviation for protein quantity in each treatment calculated by dividing the exponentially modified protein abundance index (emPAI)[16] for each protein by the total emPAI values (each treatment contain two replicate, for accuracy each replicate is the average of four injections into the Orbitrap LC-MS/MS instrument).
are multi-targeting like artemisinin and it remains to be seen if a similar stress response and accumulation of ubiquinated proteins occurs for this class of antimalarial in \( P. falciparum \) resistant parasites.\[^{17}\]

Clearly, our data raises concerns of the potential cross-resistance\[^{19}\] between these two different antimalarial chemotypes. Our optimised endoperoxide-ABPPs strategy has generated a specific and robust set of tools to study potential protein targets of the endoperoxide class of antimalarials.\[^{19}\] We are currently further refining this approach to accommodate a broader range of peroxide-based antimalarial chemotypes. Work is also underway to establish the lifecycle-dependent “endoperoxome” patterns in asexual and sexual stages of \( P. falciparum \) parasite isolates with well-characterized artemisinin drug resistance phenotypes to assist in our understanding of this worrying clinical phenomenon.

## Acknowledgements

This work was funded by the Wellcome Trust through its Strategic Support Fund award, Medical Research Council Confidence in Concept funding, and through Mahidol–London PhD Scholarships to M.P. and S.C. The work was also supported from grants from the BBSRC (UK) (P.O.N., V.B., S.A.W.: BB/C006321/1, BBS/B/S/05508, BBS/O/O/2004/06032, and BBS/S/P/2003/03535) and in part by the EU (Antimalarial FP6 Malaria Drugs Initiative).

**Keywords:** antimalarial · artemisinin · chemical biology · probes

**How to cite:** Angew. Chem. Int. Ed. 2016, 55, 6401–6405 Angew. Chem. 2016, 128, 6511–6515

---


[19] This work was first presented at the Royal Society of Chemistry Biological & Medicinal Chemistry Sector Meeting on new perspectives in DMPK held at The Chemistry Centre, Burlington House, in London, February 2014 see http://blogs.rsc.org/books/2014/03/06/best-poster-rsc-bmcs-meetin/.