Molecular & Biochemical Parasitology 179 (2011) 37–41

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Short communication

Transcript-level responses of *Plasmodium falciparum* to thiostrepton

Sarah J. Tarr[1], R. Ellen R. Nisbet[2], Christopher J. Howe*

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, Cambridgeshire CB2 1QW, United Kingdom

ARTICLE INFO

Article history:
Received 15 March 2011
Received in revised form 3 May 2011
Accepted 11 May 2011
Available online 19 May 2011

Keywords:
Plasmodium
Malaria
Thiostrepton
Retrograde signalling
Gene expression

ABSTRACT

The antimalarial activity of the antibiotic thiostrepton has long been attributed to inhibition of apicoplast protein synthesis through binding of apicoplast ribosomal RNA. However, the kinetics of parasite death upon thiostrepton treatment differ from those seen for other inhibitors of apicoplast housekeeping functions. We have analysed global changes in gene expression of the malaria parasite, *Plasmodium falciparum*, in an attempt to shed light on the responses of the parasite to this drug. Our results indicate a delay in gene expression profiles of thiostrepton-treated parasites. A small number of genes appear to be regulated outside of this trend; our data suggest a response from genes encoding components of the mitochondrial translational machinery, while little response is seen from genes encoding apicoplast-targeted proteins. Our findings are consistent with an effect of thiostrepton on mitochondrial protein synthesis, and thus warrant a re-evaluation of the target of thiostrepton in *Plasmodium*. They also provide some suggestion of mitochondrion–nucleus signalling in the parasite.

Plasmodium species belong to the phylum Apicomplexa. These parasitic protozoa share a common 'apical complex' of organelles necessary for the invasion of new host cells. Also common to apicomplexans is a relic plastid (apicoplast) that has been shown to be the site of a number of important metabolic pathways representing a number of anti-malarial drug targets [1].

The apicoplast contains its own 35 kb circular genome with chloroplast-like genes and organisation [2,3]. The apicoplast genome includes genes in an inverted repeat configuration for small and large subunit rRNAs, as well as numerous genes for ribosomal and other proteins. Based on their prokaryotic ancestry, apicoplast ribosomes are believed to be the targets of many antimalarial antibiotics. For example, tetracyclines target the 30S rRNA subunit and clindamycin targets the 23S rRNA peptidyltransferase domain [4]. However, there are few cases where a direct effect of such drugs on apicoplast translation has been demonstrated. An interesting phenomenon associated with some apicoplast-targeting drugs is the 'delayed-death' phenotype, whereby drug treatment does not affect parasite survival until the second generation i.e. the progeny of treated parasites invade and establish infection in the new host cell normally, but fail to produce viable daughter cells themselves. It has been suggested that delayed-death stems from the inhibition of processes such as transcription and translation in the apicoplast that are required for its long-term maintenance ('housekeeping'), whereas direct inhibitors of metabolic pathways such as isoprenoid synthesis lead to an immediate effect on parasite survival [5]. However, in *P. falciparum*, while clindamycin and doxycycline both lead to delayed-death, treatment with the 23S rRNA GTPase domain inhibitor thiostrepton leads to death that is not delayed. Apicoplast ribosomes are believed to be the primary target of thiostrepton [4,6], although it has been suggested that if thiostrepton also targets mitochondrial or cytosolic protein synthesis, this would account for the lack of delay in death due to thiostrepton treatment [7]. The morphologies of both the apicoplast and mitochondrion are affected by thiostrepton treatment. The drug causes the apicoplast (which would normally elongate, branch and divide) to remain undivided and spherical, while the mitochondrion remains undivided and slightly elongated. It was noted that these organelle morphologies could result from slowed lifecycle progression, as at high concentrations of thiostrepton parasites grow slowly and do not progress past the trophozoite stage [4]. Thiostrepton has been previously shown to inhibit the growth of *P. falciparum* 3D7 with an LD_{50} of 1.8 μM [8]. Apicoplast transcription is inhibited by thiostrepton, presumably due to inhibition of expression of the *rpoB/C* genes (for RNA polymerase subunits) present in the apicoplast genome [8].

A crucial site for 23 S rRNA binding by thiostrepton is the A1024 site of the *P. falciparum* 23 S rRNA, which is equivalent to the A1067 site of the *E. coli* 23 S rRNA. While the presence of A1067 confers sensitivity of *E. coli* ribosomes to thiostrepton, introduction of an A1067G mutation in *E. coli* did not completely alleviate thiostrepton-mediated inhibition of protein synthesis [9].

* Corresponding author. Tel.: +44 01223 333688.
E-mail address: ch26@mole.bio.cam.ac.uk (C.J. Howe).
[1] Present address: Research Department of Structural and Molecular Biology, University College London, Gower Street, London, WC1E 6BT, United Kingdom.
[2] Present address: Sansom Institute for Health Research, University of South Australia, Adelaide, SA 5000, Australia.

0166-6851/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2011.05.004

Furthermore, introduction of the equivalent mutation into an apicoplast 23 S rRNA sequence expressed *in vitro* did not fully inhibit binding of the rRNA by thiostrepton [6]. Since both the *Plasmodium* cytosolic and mitochondrial rRNA sequences contain G at this position, these observations raise the question of whether thiostrepton could also affect either or both the mitochondrial and cytosolic ribosomes [7]. Additional targets of thiostrepton have also been proposed; thiostrepton and its derivatives were recently shown to cause an accumulation of polyubiquitinated protein in *Plasmodium*, and it was suggested that the drug may also target the parasite proteasome [10].

In many eukaryotes, plastid or mitochondrial stresses lead to alterations in expression of nuclear genes encoding organelle-targeted proteins. These protective responses occur through what are known as retrograde signalling pathways [11,12]. In the case of chloroplast–nucleus signalling, nuclear genes encoding ribosomal proteins, rubisco and light-harvesting complex components are subject to regulation [13,14]. Similarly, mitochondrion–nucleus signalling functions to maintain the cell's supply of glutamate in the face of a dysfunctional tricarboxylic acid cycle [12]. It is not known whether such signalling pathways exist in *Plasmodium*. Our own searches for homologues of plastid–nucleus and mitochondrion–nucleus retrograde signalling pathway components have suggested that typical retrograde signalling mechanisms may be absent from the parasite. Nonetheless, given that both the apicoplast and mitochondrion are essential for parasite survival, it is possible that the parasite harbours divergent or distinct signalling pathways allowing the regulation of expression of nuclear genes encoding organelle proteins. Furthermore, these pathways may be expected to be highly divergent and their components representative of novel drug targets.

In *Arabidopsis*, inhibition of chloroplast translation using lincomycin was sufficient to induce chloroplast–nucleus retrograde signalling [13]. Therefore, in order to investigate the presence of such pathways in *P. falciparum*, we have attempted to induce stress (and therefore, retrograde signalling) to the apicoplast by inhibiting apicoplast translation using thiostrepton. We undertook a global analysis to investigate the transcript-level gene expression responses of the parasite to the drug. An LD_{70} concentration of thiostrepton (2 μM as established in our lab) was chosen for global gene expression profiling of the transcript-level responses of the parasite to the drug, as we sought to disrupt apicoplast function in order to induce signalling, rather than killing the parasites outright. Synchronous parasites were studied across one full lifecycle under LD_{70} thiostrepton treatment (Supplementary Fig. 1); after 52 h, ring stage parasites were evident in the thiostrepton-treated cultures (see arrows in Supplementary Fig. 1) suggesting that, at this concentration, the cultures were not completely arrested. Indeed, at 24 h of treatment, thiostrepton-treated parasites appeared phenotypically indistinct from the untreated controls. RNA was extracted from synchronous *P. falciparum* cultures (in triplicate) treated for 24 h (from the ring- to trophozoite-stage) with LD_{70} thiostrepton, as well as DMSO-treated controls. (All work involving human blood was carried out in accordance with the Human Tissue Act (2004)). Using this concentration and timeframe, we sought to stress the parasites without killing the entire population such that transcript-level changes reflected responses to drug treatment rather the gene expression profiles of dying parasites. RNA was labelled and hybridised to Affymetrix *Plasmodium*/*Anopheles* GeneChips by Geneservice (Nottingham). Microarray data analyses were carried out using Bioconductor [15] and analysed for differential gene expression using GCRMA and Linear Models for Microarray Analysis [16]. The raw and normalised microarray data were deposited as a Gene Expression Omnibus accession (http://www.ncbi.nlm.nih.gov/geo/; GSE28701). Differentially expressed genes were sorted by Bonferroni–Holm adjusted

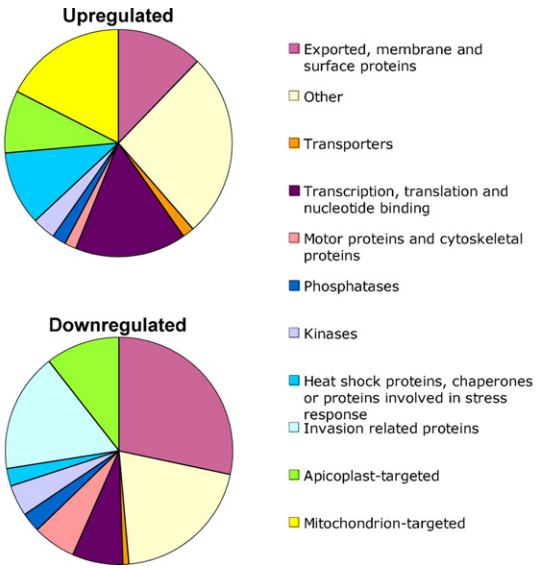

Fig. 1. Functional categorisation of genes differentially expressed in *P. falciparum* upon treatment with thiostrepton (excluding unknown proteins).

p-values; an adjusted *p*-value threshold of 0.05 defined 243 genes as differentially expressed. Independent qRT-PCR analysis of nine genes (PF14_0641, PF13_0040, PF14_0133, PFL1550w, PFI0735c, PF10_0334, MAL13P1.185, PFB0570w and PFB0665w) showed a strong positive correlation with the microarray data (Spearman's $\rho = 0.875$, p-value = 0.0134) allowing more general conclusions to be drawn from the data as a whole. Differentially expressed genes were assigned to functional categories. Genes of unknown function will not be discussed here. Of the differentially expressed genes, 94 were upregulated and 149 were downregulated (Fig. 1; also see Supplemental Tables 1 and 2). Fold changes in expression were small; the median upregulation was 1.39-fold and median downregulation was 0.44-fold. While these fold changes are small, they are in line with the small gene expression responses observed for *Plasmodium* in other studies [17,18].

The identities of differentially expressed genes were studied for signs of a general stress response to drug treatment. Although the analysis suggested that few proteins involved in stress responses were regulated at the transcript level, genes encoding a protease and a heat shock protein (HslV and a DnaJ protein, respectively), two chaperonin-like proteins, an ubiquitin-like protein and an ubiquitin hydrolase were upregulated. HslV has been identified as a homologue of a prokaryotic protease involved in protein turnover [19]. Three genes encoding DnaJ proteins (PF14_0013, PF11_0513 and PFA0110w i.e. RESA) were downregulated upon thiostrepton treatment according to the microarray analysis. All of these exhibited reductions in expression of less than 0.5-fold. Together, these observations suggested only a limited transcript-level response of stress response genes.

Genes encoding proteins involved in cellular signalling were also studied for perturbation in response to thiostrepton treatment. Few kinases or phosphatases were seen among the genes differentially expressed, indicating that (on a transcript level) intracellular signalling was not key to the drug response. Furthermore only a single transporter gene was upregulated, indicating that the parasite did not attempt to increase expression of transporters in order to remove the drug from the cell.

Given the previously observed effects of thiostrepton on apicoplast and mitochondrial morphology [4], we were surprised to observe that only a small proportion of the differentially expressed genes encoded proteins targeted to these organelles. The genes encoding apicoplast-targeted leucine-tRNA ligase, pseudouridine