CHAPTER 5

CONCLUDING DISCUSSION

At a time when antimalarial drug resistance is critical and the need for compounds with novel modes of action is beyond the point of urgency, cytostatic drugs should be considered in drug screening and development programmes. Cytostatic drugs have proven therapeutic potential in protozoan diseases, e.g. DFMO, which is still the safest alternative in the treatment of West African sleeping sickness [78]. Even the acclaimed antimalarial, artesunate, has a partial cytostatic effect [299]. However, the cytostatic mechanism resulting in growth arrest of treated cells and normal progression of untreated controls requires special consideration for basic comparisons of response in terms of assay methodology used and data analysis. This is particularly important when studying multistage organisms such as *P. falciparum*, which constantly develop during the IDC, such that growth arrest compared to normal progression will result in significant differences merely due to stage. This critical principle was kept in mind throughout the entire investigation presented here, from the drug sensitivity assays up to the functional genomics investigations, and gave rise to the relative t_0 strategy, which was an integral part of the analyses performed.

The global aim of the study was to perform functional genomics analyses of polyamine metabolism (specifically during polyamine depletion resulting in cytostasis) via the co-inhibition of the bifunctional PfAdoMetDC/ODC. This is the first report of such a comprehensive functional genomics approach to the response of the malaria parasite to environmental perturbation. In order to achieve this, the study was preceded with drug sensitivity assays to determine the appropriate inhibitor concentrations (including characterisation of the interaction between the two inhibitors used), radio-labelled substrate assays to ensure complete enzyme inhibition, membrane-integrity assays to ascertain that the high dosages used did not cause chemical cytotoxicity and parasite morphology studies to determine the most appropriate sampling times. The experimental design of the functional genomics investigations was carefully considered to be most informative. A time course experiment was performed in duplicate (two biological replicates) on synchronous parasites, with samples taken at three time points (just before and during cytostasis) such that the expression period of PfAdoMetDC/ODC was spanned. For the transcriptomics investigation, a reference design microarray experiment was conducted to enable normalisation across all the arrays for easy consolidation of replicates and cross sample comparison during differential abundance analysis. The master image compiled during the proteomics data analysis (Fig. 4.1) is analogous to the reference design of the microarray. Three independent functional genomics investigations (transcriptomics, proteomics and metabolomics) were performed. Raw data quality was carefully assessed by visual inspection and diagnostic analyses, where appropriate, to ensure

proper data correction and normalisation. Data quality assurance formed a crucial part of data analysis, since the quality of the data indirectly determines the reproducibility/reliability of the derived differential abundance lists and the validity of the biological conclusions [232].

Global expression profiling after treatment with cytostatic drugs has been performed in cancer [233, 234], but not in multistage organisms such as *P. falciparum*. One of the major finds of this investigation was the demonstration that transcriptional arrest precedes the growth arrest induced by polyamine depletion. It is the first time to our knowledge that this preceding event and mechanism of cytostasis was elucidated, and it could be a more general phenomenon also pertaining to other organisms/systems. The transcriptional arrest was particularly evident due to the multistage nature of *P. falciparum* and the arrest of treated and normal transcriptional progression of untreated parasites were easily visualised when the microarray data were ordered according to gene peak expression times within the IDC. In addition, the approximate time of the transcriptional arrest was estimated to occur at about 15 - 16 hpi of the IDC, thus late ring/early trophozoite stage, which correlates with the start of PfAdoMetDC/ODC expression. At the dosages used, complete enzyme inhibition occurred soon after protein expression, which further underscored the enzyme-specific inhibitory effects of DFMO and MDL73811 [153, 154]. The exact mechanism by which polyamine depletion results in transcriptional arrest is currently not clear, but the importance of this in macromolecular synthesis (including RNA and proteins e.g. transcription factors) [88], optimal ribosome function [236] and the association of the main fraction of polyamines with RNA [64] is well established. On the transcript level, differential abundance and GO analysis indicated the increase of transcription factors and ribosomal components and on the protein level the increase of proteins involved with protein synthesis was observed. These could indicate a physiological attempt by the parasite to induce transcription and translation to overcome the transcriptional arrest, as opposed to other processes, such as DNA replication and energy metabolism, which were clearly suppressed.

Despite the cytostasis and generalised transcriptional arrest, the abundance of 10.1% (538/5332) transcripts, 10.6% (53/500) protein spots and 26% (24/92) metabolites were differentially affected as a result of the perturbation compared to the relative t_0. However, many metabolites in the untreated controls were similarly affected. The transcriptomics fold changes range between maximum 3.2-fold up and 5-fold down, similar to other transcriptome reports of perturbed *Plasmodium* where relatively small amplitude transcriptional responses were detected [189, 196]. Yet, in contrast with several other studies [194, 196, 197] perturbation-specific, compensatory responses could be detected in the plasmodial transcriptome after PfAdoMetDC/ODC co-inhibition with the increase in the transcripts for LDC and OAT and the decrease in that for AdoMet synthetase. The additional differential co-regulation of transcripts located adjacently on the chromosomal level after polyamine depletion (e.g. chromosome 10, PF10_0014 to PF10_0025), reiterates the perturbation-specific effect of polyamine-depletion on the transcriptome. The confirmation of the compensatory

transcriptional induction of OAT and suppression of AdoMet synthetase on the protein and metabolite level is quite remarkable compared to other studies of *Plasmodium* [194, 196, 197] where such perturbation-specific transcriptional responses could not be demonstrated. However, in most of these reports the use of unsynchronised cultures and parallel time point comparison could have masked the generally small transcriptional changes seen in *Plasmodium* upon perturbation, compared to the >3-fold transcriptional changes commonly detected during perturbations of *M. tuberculosis* [138]. The limited evidence of compensatory feedback and small amplitude of transcriptional responses have been attributed to the dominant role of post-transcriptional mechanisms of gene regulation in *Plasmodium* [128, 194, 195]. However, the co-ordinated compensatory responses specific to polyamine and methionine metabolism as detected in the transcriptome, proteome and metabolome in this investigation support the role of transcriptional control in response to environmental perturbation (specifically polyamine depletion) in the parasite. Furthermore, current evidence from other plasmodial perturbations [179, 189, 190, 198, 199], the recent discovery of various *cis*-regulatory motifs [118] and experimental evidence for an expanded family of DNA-binding proteins [122, 123] demands re-assessment of the role of transcriptional control in *Plasmodium* [118].

Moreover, the results obtained illustrate the potential of microarray transcriptional profiling in investigations of the physiological response of *Plasmodium* to environmental perturbation. Despite previous reports [194, 196, 197], generalised stress (host-parasite interaction transcripts e.g. surface antigens), as well as perturbation-specific compensatory responses, could be detected in the transcriptome, but it required careful experimental design, as discussed above. However, in this investigation the exact drug target was known and specific inhibitors of the bifunctional complex were applied, in contrast to several other reported environmental perturbations of *P. falciparum* (e.g. glucose deprivation [189], heat-shock [190] and even chloroquine treatment [179, 196]) for which the exact target was not known. Therefore, whether microarray technology has the ability to reveal the mode of action of novel antimalarial compounds, as demonstrated for *M. tuberculosis* [138], remains to be seen, but it will most certainly be influenced by the specificity of such compounds for single targets, as well as the proper functional annotation of the genes/transcripts within the biochemical pathways involved.

Future experiments to confirm the proposed transcriptional control on the expression of LDC, OAT and AdoMet synthetase could include promoter studies, i.e. the upstream sequences of these genes could be tested for their ability to drive expression of reporter genes (e.g. luciferase) [300, 301] upon polyamine depletion. If true regulation at the level of transcription initiation and not due to alteration of mRNA stability or other post-transcriptional means [302, 303] exists for these genes, then PfAdoMetDC/ODC co-inhibition should cause the reporter gene expression to increase with the LDC and OAT promoters and to decrease with the AdoMet synthetase promoter.

It appears as if distinct biological processes in *Plasmodium* involve specific combinations of regulatory mechanisms. Some cellular processes may be predominantly controlled by transcription factor binding and recruitment or by post-transcriptional regulation, whereas others may require the additional involvement of epigenetic regulatory processes, e.g. *var* gene expression and switching [118]. In this investigation some genes (OAT and AdoMet synthetase) showed coordinated levels in the transcriptome and the proteome and appear to be regulated at the transcriptional level, whereas another showed translational delay (LDH) and others (e.g. falcipain-2 and pdx1) resulted in incremental changes at the transcriptional level compared to significant changes on the protein level, which could be attributed to post-transcriptional regulation of these particular enzymes. The transcript for PfAdoMetDC/ODC was decreased ~2-fold with DFMO/MDL73811-treatment in accordance with the transcripts for the proteins DHFR/TS and DHPS/PPPK when targeted by pyrimethamine and sulphadoxine [197]. DHFR/TS expression is regulated by the binding of the protein to its own mRNA, thus acting as a negative feedback to control its own translation [242]. A similar mechanism could apply to PfAdoMetDC/ODC, but this needs to be elucidated, since the protein was too large to penetrate the 2D-gels and protein levels could not be assessed in this study. This potential protein-RNA interaction could be investigated by ChIP (chromatin immunoprecipitation)-chip analysis or electrophoretic mobility shift assays. Alternatively, polyamines may also be required to stabilise the transcript of PfAdoMetDC/ODC, resulting in its instability and degradation upon polyamine depletion.

LDC induction as compensatory mechanism for polyamine depletion in *Plasmodium* could not be confirmed, since the protein could not be detected by 2D-GE or cadaverine with LC-MS/MS or protein activity with radio-labelled substrate assays. The possible inhibition of the induced LDC via DFMO as in *S. ruminantum* [73] cannot be excluded at the current time, but demonstration of an increased protein concentration via Western blot analysis will indisputably confirm or reject the compensatory role of LDC during polyamine depletion of *P. falciparum*. The simultaneous expression of the transcripts for LDC and PfAdoMetDC/ODC at 24 – 25 hpi is indicative of this and although parasite growth was not restored at the treatment dosage the increased transcript abundance of LDC may indicate a potential resistance mechanism, should PfAdoMetDC/ODC be clinically targeted in the future. Additional experiments to confirm the compensatory role of LDC could include PfAdoMetDC/ODC co-inhibition of stable or transient LDC overexpressing parasites, followed by IC_{50} determination. If LDC indeed has the capacity to restore polyamine depletion to some extent, the IC_{50}s of DFMO and MDL73811 are expected to increase under conditions of LDC overexpression.

This study presented the first description of a comprehensive metabolite analysis (92 metabolites with reliable data) of perturbed *Plasmodium*. However, due to the large number of metabolites simultaneously analysed, global metabolomics do not take into account basic enzyme kinetics or metabolic flux, which are fundamental to any study of metabolism [304]. Interpretation of global metabolomics data in isolation without supporting evidence (e.g. from the transcriptome and proteome) is extremely difficult and can easily result in inaccurate

conclusions [304]. Therefore, biological interpretation of the metabolomics data in this investigation was cautiously limited to confirmation of transcriptional and proteomic responses, yet interesting results were obtained. Metabolic homeostasis was maintained despite the transcriptional arrest and corresponding decreased proteome. The most compelling differences between the metabolomes of treated and untreated parasites were downstream of the co-inhibited PfAdoMetDC/ODC with the perturbation-specific decrease of putrescine, spermidine and 5-methylthioinosine, indicating a halt of metabolism downstream of the enzymatic blockade. Yet, upstream of PfAdoMetDC/ODC, metabolic homeostasis was preserved and ornithine and AdoMet levels were maintained despite the complete inhibition of PfAdoMetDC/ODC. Thus, on a biochemical level, regulatory mechanisms were demonstrated in this study. This includes the role of OAT in the maintenance of ornithine concentration in *P. falciparum.* Moreover, we propose feedback inhibition of AdoMet on the AdoMet synthetase transcript and protein levels, in contrast to the poorly-regulated trypanosomal enzyme [157, 198]. The proposed difference in the regulation of AdoMet synthetase between Trypanosoma and Plasmodia could be the reason for the efficacy of MDL73811 in *T. brucei rhodesiense*-infected mice [184] and failure of MDL73811 in *P. berghei*-infected mice [153], resulting in hypermethylation with polyamine depletion in the first case and possibly only polyamine depletion in the second. This requires further investigation in these animal models, but AdoMet synthetase promoter studies in the presence of high levels of AdoMet, as discussed above, could be particularly useful to confirm true transcriptional regulation of this enzyme.

Methylation assays as performed here indicated that MDL73811 does not result in hypermethylation of *P. falciparum* gDNA, but histone-methylation can also result in transcriptional repression [125] and was not investigated. However, in contrast to other reports [124, 126, 127], the presence of 5mC was detected in both treated and untreated *P. falciparum* gDNA via South-Western immunoblotting, despite the low GC content of the genome [96]. 5mC-Methylation appeared to increase during development independent of PfAdoMetDC/ODC co-inhibition, which may be explained by the expression of the putative DNA(cytosine-5)-methyltransferase (MAL7P1.151) early in the trophozoite stage. The proposed stage-dependent gDNA methylation (as opposed to perturbation-dependent methylation) was further substantiated by the fact that AdoMet levels in both treated and untreated parasites were maintained during the time course.

The ultimate objective of the investigation was to validate PfAdoMetDC/ODC as a potential drug target for antimalarial therapeutic intervention. In *S. cerevisiae* it was shown that the transcriptional changes associated with target deletion or under-expression theoretically mimic the effect of chemically inhibiting that target [188]. Since genetic manipulation of *P. falciparum* is technically challenging [130] and essential gene knock-out mutants are not viable [including ODC knock-out and silent mutants (C. Wrenger, unpublished data)] and can therefore not be studied, the functional genomics approach was followed. The induction of perturbation-specific compensatory responses to circumvent the detrimental effects of polyamine depletion corroborates

the importance of this pathway to the malaria parasite. In the absence of polyamines transcriptional arrest and cytostasis occurs, which interrupts the IDC and prevents schizogony and parasite proliferation. Therefore, polyamines are essential molecules for parasite survival and PfAdoMetDC/ODC can be regarded as a valid drug target for antimalarial drug development.

There is often scepticism about the clinical significance of polyamine metabolism as drug target in the treatment of malaria, since even upon complete inhibition of biosynthesis and success of treatment *in vitro,* the parasite is still able to salvage and thus maintain its polyamines when it resides *in vivo* [145]. However, our laboratory is currently involved in an active search for the protein responsible for polyamine transport within the parasite. Once it is elucidated and both polyamine biosynthesis and transport can be rationally inhibited, as demonstrated with the combination of DFMO and the bis(benzyl)polyamine, MDL27695 [161], this target may hold promise for antimalarial treatment with a novel mechanism radically different from those currently employed clinically. A follow-up investigation could perhaps aim at designing a single molecule that is able to destabilise the protein as a whole (e.g. by binding to the hinge region, which connects the two catalytic sites), thereby inactivating both the active sites of ODC and AdoMetDC simultaneously. Such a compound, in combination with an inhibitor of the yet evasive polyamine transporter, could form the predecessors of the next generation of antimalarial drugs.

Every day more than 3000 people die of malaria in Africa [13], which is more than the number of people who tragically died in the 9/11 terrorist attacks in the United States. This number is 1.6% of the annual 57 million deaths that occur globally, yet malaria accounts for only 0.4% of the world's biomedical research [305]. Therefore, research on this intriguing parasite should have the overall aim ultimately to relieve the enormous burden of the disease, e.g. by contributing information leading to novel drug targets or leads that can eventually result in clinical treatment, prevention or vector control. The investigation presented here contributed to this long-term goal by indicating the most appropriate drug sensitivity methods to employ in screening efforts to avoid potentially excluding cytostatic drug leads, by shedding light on the basic mechanism resulting in cytostasis (i.e. transcriptional arrest), by providing evidence of compensatory mechanisms in parasite polyamine metabolism, which supports the role of transcriptional regulation and contributes to the validation of PfAdoMetDC/ODC as an antimalarial drug target, and by revealing a potential resistance mechanism (i.e. compensatory LDC induction) against polyamine depletion as a treatment strategy. It is sincerely hoped that this knowledge, even in the smallest possible way, will contribute to eventually finding a solution against this devastating disease.