CHAPTER 6

Concluding discussion

"We don't have perfect tools, but the tools we do have today if fully scaled up will have a profound effect"

Robert Newman (Director WHO Global malaria program)

Malaria is a killer disease transmitted by the protozoan parasite, *Plasmodium*. With the spread of increasing resistance to currently used drugs (Noedl *et al.*, 2008), insecticide resistance (Tatem *et al.*, 2006), the effect of global warming, global travel as well as the identification of enzoonotic species that are now able to infect humans (Bronner *et al.*, 2009), the need for new drugs are more urgent than ever. Unique differences between *P. falciparum* and eukaryotic cells must be exploited in order to identify novel drug targets. One such potential drug target is polyamine metabolism, which differs significantly from that of humans and is essential to parasite survival. *Plasmodium* polyamine metabolism includes a uniquely bifunctional AdoMetDC/ODC complex that regulates the biosynthesis of polyamines within the parasite. Functional genomics, as applied in this study to AdoMetDC inhibited malaria parasites, is an integral part of drug discovery to investigate the therapeutic potential of this bifunctional enzyme as an antimalarial drug target.

The general objective of this study was to determine the biological relevance and consequences of the inhibition of Plasmodial AdoMetDC with MDL73811 in order to chemically validate *Pf*AdoMetDC as a drug target. This question was answered with the use of a functional genomics approach in which both transcriptomics and proteomics were utilised in order to provide a picture of the global response of the parasite to AdoMetDC inhibition. Ideally, it is hoped that the use of the technologies associated with functional genomics will allow the ability to obtain the mode-of-action of drugs.

Due to the multistage life cycle of the Plasmodial parasite careful consideration were given to experimental design in order to obtain maximal information from the transcriptomic study. A reference design was employed that enabled the determination of the differential abundance of any sample in relation to the other samples (Kerr & Churchill, 2001). Three time points were investigated in the ring stage (16 HPI), early trophozoite stage (20 HPI) and the late trophozoite stage (26 HPI) in order to obtain the exact point of transcriptional arrest. The early time points used in this study allowed a direct comparison of the transcriptome and therefore negated the use of the t_0 reference strategy (van Brummelen *et al.*, 2009). Pearson correlations and hierarchical clustering confirmed the use of the direct comparison employed within this study and also confirmed that the

differentially affected transcripts are representative of drug-specific effects and not stage specific effects. A total of 549 transcripts were differentially affected by the inhibition of AdoMetDC with MDL73811.

The proteome does not always mimic the transcriptome, therefore to obtain a global picture of the response of the parasites to AdoMetDC inhibition the proteome were investigated using 2-DE. Due to the complexity and notorious nature of Plasmodial proteins, the proteins were solubilised in a potent lysis buffer. The nature of the lysis buffer negates the use of any traditional methods of protein quantification which is problematic for the determination of differentially regulated proteins. In order to overcome this problem, the existing Plasmodial 2-DE protocols (Nirmalan *et al.*, 2004a, Makanga *et al.*, 2005) were optimised for use in our laboratory. Optimisation included the use of the 2-D Quant kit for protein quantification to ensure that similar amounts of protein were used for both the treated and untreated samples, therefore enabling differential abundance analysis. The 2-DE protocol was also optimised to reduce hemoglobin contamination in the 14 kDa and pI 7-9 range by the addition of extra wash steps and softer sonication methods. Finally, the use of the fluorescent stain Flamingo Pink enabled a quantitative measure of proteins within the 2-DE gels. Application of this methodology to the ring and trophozoite stages of the parasite enabled the MS-identification of 125 protein spots. Interestingly, protein isoforms were a prominent feature of the spots that were identified and accounted for ~28% of the total number of Plasmodial protein spots identified in the ring and trophozoite stages. This clearly illustrates the prominent role of protein isoforms during Plasmodial protein regulation and the use of PTM's as a regulatory mechanism within the parasite. Furthermore, a comparison between the ring, trophozoite and schizont stages (Foth *et al.*, 2008) revealed that only 9 proteins were shared in all 3 stages, therefore indicative of stage specific protein production. This is similar to other MS-based studies on the various life stages of the parasites (Lasonder *et al.*, 2002, Florens *et al.*, 2002) in which stage specific production of proteins were also observed.

The optimised method described in Chapter 2 proved robust and reproducible in various applications of the Plasmodial proteome. This was demonstrated in Chapter 3 in which the proteome of AdoMetDC inhibited parasites were investigated and resulted in good spot detection and spot identification with MS. The consistency of this method was also demonstrated with the co-inhibition of AdoMetDC/ODC in which 400 spots were detected in each of the 3 time points investigated (Van Brummelen, 2009). The same optimised proteomics protocol was also used in 2 separate herbicide studies on the Plasmodial proteome (J. Verlinden MSc thesis in preparation, J. Snyman MSc thesis in preparation) in which good spot separation was achieved. Overall, the

optimised 2-DE methodology proved robust and is repeatable for different parasite applications. The established proteomic methodology was applied to the proteome of inhibited AdoMetDC to obtain a snapshot of the proteome at two time points (16 HPI and 20 HPI). Complementary proteomic techniques were employed that made use of both 1-D SDS-PAGE as wells as 2-DE to obtain maximal information from the proteome. This approach paid dividend in that 11 proteins were identified using the SDS-PAGE gels that would normally fall outside the 2-DE gel range and would have remained unidentified.

Unlike the transcriptome in which at least half of the transcripts are classified as hypothetical, this was not the case for the AdoMetDC inhibited proteome. Only 9% (4/46 proteins) of the total proteome that was identified was regarded as hypothetical proteins with unknown functions. This was probably due to the small portion of unique proteins that were identified (46 proteins) in comparison to the AdoMetDC inhibited transcriptome that contained 549 differentially expressed transcripts (Chapter 4). Another reason for the small number of hypothetical proteins was probably due to the fact that 2-DE was only representative of high abundance proteins and therefore the majority of these have already been characterised. Of the 46 identified proteins that were differentially expressed 18% were associated with glucose metabolism. Some of the other groups that were highly represented included protein folding (11%), polyamine metabolism (11%), proteolysis (15%), translation (13%) and oxidative stress (5%). These groups were also prominent in the differentially affected transcripts from the transcriptome, in which oxidative stress (3%), translation (6%) and polyamine metabolism (3%) were represented (Chapter 4). Therefore, AdoMetDC inhibition does affect certain key pathways that seem to be polyamine related or dependent on the presence of polyamines.

Evidence as to the possibility of post-transcriptional regulation was supported in this study in that 17% (55/325) of the ring and 24% (64/272) of the trophozoite proteome were differentially regulated which is in contrast to the transcriptome in which little regulation were detected within the first two time points. It should however be considered that the transcriptome and proteome samples were harvested independently from each other and therefore a possible time window exists. Ideally, the samples for the transcriptome and the proteome analysis should be harvested simultaneously to eradicate possible time errors that may develop and should be considered for all future functional genomics experiments.

Even though, a combination of proteomic gel-based techniques were employed relatively few proteins were identified in comparison to the transcriptome (549 differentially affected transcripts).

In total, 61 unique Plasmodial proteins were identified with the combination of 1-D SDS-PAGE and 2-DE. This re-iterated the use of complimentary proteomic techniques to obtain differentially expressed proteins (Nirmalan *et al.*, 2007). The use of MudPIT-based technologies would have allowed the identification of more proteins within the study, but with the disadvantage of the loss of protein isoforms. PTM's are employed as a mechanism to regulate protein activity during the parasite's life cycle (Nirmalan *et al.*, 2004a) and certain proteins are predicted to act as controlling nodes that are highly interconnected to other nodes and thus results in a highly specialised interactome (Wuchty *et al.*, 2009, Birkholtz *et al.*, 2008b). It is therefore of utmost importance to identify protein isoforms within the proteome and determine their regulatory functions. The use of 2-DE enabled the identification of various protein isoforms. Ideally, these protein isoforms should be investigated further in order to be able to distinguish between the different protein isoforms based upon their different PTM's. The identification of these different PTM's will also provide more clarity on the function of the protein and whether the isoform is an active protein or an inactive protein. In order to obtain more protein spots on the 2-DE gels fractionation techniques could also be considered. The proteome can be fractionated into different pI fractions before running the fraction on a 2-DE gel (Nirmalan *et al.*, 2007, Nirmalan *et al.*, 2008). This fraction will enable enhanced spot and protein determination within a specified pI range and provide a more detailed picture of the proteome. Organellar fractionation can also be considered to obtain proteins associated with a specific compartment of the parasite. These approaches will produce more protein spots and will also eliminate high abundance proteins therefore enabling the determination of low abundance protein profiles. This approach should be considered for future experiments, which will then enable the identification of both high (current approach) and low (fractionation) abundance proteins.

Both the transcriptome and the proteome of AdoMetDC inhibited *P. falciparum* parasites revealed inhibitor-induced differences. These differentially expressed genes and proteins include polyamine-related pathways, possible compensatory mechanisms, down-stream pathways as well as regulated proteins in other essential pathways within the parasite. Essential pathways associated with AdoMetDC inhibition included the folate pathway, oxidative stress and redox metabolism as well as cytoskeleton biogenesis. Polyamine metabolism is essential to parasite survival and depletion of the polyamines induced transcriptional arrest within the parasite.

Down-stream metabolic pathways that were severely affected by AdoMetDC inhibition included the decreased transcript abundances of adenosine deaminase, PNP and HPPRT. The protein of PNP was also decreased in abundance which confirmed the transcript levels. The decreased abundances

of these 3 transcripts are probably as a result of the decreased MTI due to the decreased dcAdoMetDC as a result of AdoMetDC inhibition. These 3 transcripts are therefore polyamine dependent and an absence of polyamines will result in their decreased abundances and will also impact on DNA and RNA metabolism.

Polyamine-related transcripts also revealed a decrease in transcript and protein abundance upon inhibition of AdoMetDC and include AdoMet synthase and adenosylhomocysteinase that are associated with methionine recycling. Despite the decreased transcript and protein abundances of both AdoMet synthase and AHC there were no alteration of the AdoMet levels. This is indicative of homeostasis and tight regulation of the AdoMet levels within the parasite. Trypanosomal AdoMet synthase is not feedback regulated by AdoMet which results in the significant increase in AdoMet levels and subsequent hypermethylation within Trypanosomal parasites and consequent parasite death (Muller *et al.*, 2008, Goldberg *et al.*, 2000, Yarlett *et al.*, 1993). It has been reported that Plasmodial AdoMet synthase is similar to the Trypanosomal AdoMet synthase and is not feedback regulated. The data that were obtained with the inhibition of *Pf*AdoMetDC is indicative of the contrary. The decreased transcript and protein abundances of AdoMet synthase and the lack of change in the AdoMet and AdoHcy metabolite levels and subsequent lack of hypermethylation with MDL73811 treatment suggests that AdoMet synthase plays an essential role during polyamine metabolism and is tightly regulated. It is therefore essential to determine the possible transcriptional regulatory mechanisms that may be involved with AdoMet synthase.

Phosphoethanolamine N-methyltransfease was identified as a unique transcript to AdoMetDC inhibition. Both the transcript and the various protein isoforms of PEMT were decreased in abundance with AdoMetDC inhibition. From the comparisons that were made it seems that PEMT is dependent on spermidine and spermine since the mono-functional inhibition of ODC did not identify PEMT as a differently regulated transcript. An interesting observation that was made from the polyamine-related transcripts that were identified is that it seems that these transcripts are under post-transcriptional control. The transcripts and proteins of AdoMet synthase, AHC, PNP and PEMT were all decreased and is therefore indicative of transcriptional and post-transcriptional control mechanisms. It may be that the transcripts of these proteins are stabilised by the presence of polyamines, therefore exerting transcriptional control on both the transcripts and the proteins.

The transcript of AdoMetDC was not differentially regulated by inhibition with MDL73811, which is in contrast to the co-inhibition of AdoMetDC/ODC that resulted in two-fold decreased abundance of the transcript (van Brummelen et al., 2009). An interesting observation would have been to

determine if the protein abundance of AdoMetDC was affected by the irreversible inhibition with MDL73811. This was attempted by western blot analysis of treated and untreated parasites but unfortunately the antibody demonstrated some unspecific binding and therefore no conclusive results could be made. A new antibody is currently under development from our laboratory, and once this task has been completed the AdoMetDC antibody could be used to determine if the protein abundance of AdoMetDC is differentially regulated with inhibition. This is an important validation step that is still lacking. Previously, it was determined that upon inhibition of the folate pathway the transcript levels remained constant, but that enzyme activity increased with a possible increase in the synthesis of new protein to combat the effect of the drug (Nirmalan *et al.*, 2004b).

Polyamine-related transcripts with increased transcripts included lysine decarboxylase and calcium/calmodulin-dependent protein kinase 2 (PFL1885c), and is indicative of possible compensatory mechanisms within the parasite as a response to polyamine depletion. Due to the large size of LDC the protein could not be detected on the 2-DE gels. It is assumed that during normal growth of the parasite lysine is converted to cadeverine by LDC although the precise role of cadaverine within the parasite remains unclear. Evidence in *Vibrio vulnificus* suggests an increase in LDC and cadaverine may enable protection against oxidative stress while its product; cadaverine acts as a radical scavenger of superoxide (Kim *et al.*, 2006, Kang *et al.*, 2007). In a polyamine-depleted environment cadaverine may also be utilised by SpdS therefore freeing the remaining spermidine for eIF5A synthesis and subsequent protein synthesis (Pegg *et al.*, 1981, Park *et al.*, 1991). It is therefore assumed that a similar situation exists in Plasmodial parasites in which the increased transcript abundance of LDC may be indicative of an attempt by the parasite to preserve protein synthesis by freeing spermidine for utilisation by eIF5A or that the increased transcript abundance of LDC and possible increase in cadaverine may provide protection against oxidative stress or possibly both.

The protein of eIF5A was decreased in abundance and would therefore result in decreased protein synthesis. The decreased protein abundance of eIF5A may suggest that cadaverine is not utilised by eIF5A but rather acts as a radical scavenger, but this needs confirmation within the Plasmodial parasite. The protein levels of pyrroline 5-carboxylate reductase increased in the treated samples and may therefore also be a polyamine-related response. The increased protein abundance of pyrroline 5-carboxylate reductase may suggest an attempt by the parasite to relieve the excess build-up of ornithine as a result of AdoMetDC inhibition and may also be a possible compensatory mechanism.

Calcium/calmodulin-dependent protein kinase 2 were increased as well as various FIKK kinases, calcium dependent protein kinase 1 and cAMP dependent protein kinase regulatory subunit were decreased in transcript abundance. In *P. falciparum*, calcium signalling pathway is able to control vital functions within the parasite especially the cell cycle, which corroborate with the increase in calcium throughout the life cycle of the parasite. Host melatonin regulates both calcium and cAMP which acts as second messengers in the Plasmodial life cycle. A rise in calcium will result in an increase in cAMP production which will further induce calcium release, although the exact mode-of-action of the calcium influx pathway remains elusive to date. This may indicate a role of the calcium/calmodulin-dependent protin kinase 2 and the various FIKK kinases in the regulation of the cell cycle and needs further investigation in the future.

Three key pathways seem to be affected by the inhibition of AdoMetDC and in comparison to other perturbation studies seems to be unique to polyamine perturbations. These pathways include the folate pathway, oxidative stress and cytoskeleton biosynthesis (Figure 6.1). Upon polyamine depletion a general decrease in folate-related transcripts was determined which may possibly result in a polyamine- and folate-depleted environment within the parasite. The connection between polyamine metabolism and folate synthesis has been established previously (Bistulfi *et al.*, 2009). Further investigation of folate and polyamine depletion revealed that in a folate and polyamine depleted environment the IC_{50}'s of both MDL73811 and PYR were decreased. The possibility of synergism between polyamine depletion and folate depletion was investigated and revealed an additive effect rather than a synergistic drug interaction. Polyamine and folate depletion in human ovarian cancer revealed a synergistic killing effect (Marverti *et al.*, 2010) which is in contrast to the results obtained for the Plasmodial parasites. This may be as a result of the differences between mammalian and Plasmodial polyamine metabolism, as well as the regulation of AdoMet which seem to exist within the parasite. Although it should be noted that the synergistic studies between PYR and MDL73811 needs further investigation. The advantage of eliminating 2 pathways is that it will reduce the possibility of resistance. Therefore the decreased transcript abundances obtained for both pathways and the additive effect of polyamine-and folate-depletion may indicate co-regulation of the pathways, but needs further investigation.

Another consequence of polyamine metabolism seems to be the interaction with oxidative stress (Figure 6.1). In the transcriptome various oxidative stress-related transcripts were all decreased possibly resulting in increased oxidative stress within the parasite. The thioredoxin interactome revealed that OAT, AdoMet synthase and AHC were all interacting partners of thioredoxin (Sturm *et al.*, 2009). Similarly thioredoxin reductase is also a binding partner of AdoMet synthase which

may ultimately result in a possible link between the tight regulation of the AdoMet cycle as well as regulation of the polyamine pathway (Sturm *et al.*, 2009, Wuchty *et al.*, 2009). The decreased transcript and protein abundance of AdoMet synthase may have an influence on thioredoxin reductase activity or protein expression, which may result in regulation of the redox status of the parasite. The increased transcript of LDC which may result in increased levels of cadaverine may also provide a clue as to the attempt of the parasite to relieve the induced oxidative stress by other pathways than the conventional redox metabolism. Two of the proteins associated with oxidative stress (2-cys peroxiredoxin and GST) revealed an increase in protein abundance over time despite the transcripts having decreased abundance, indicative of post-transcriptional control mechanism within the parasite. Another possibility is that the proteins can be resistant to degradation, or the protein lag behind the transcript and the decrease in protein abundance will be seen later (Foth *et al.*, 2008). This may also be similar to *Pf*DHFR-TS which is translationally regulated as it is able to bind to its own mRNA, therefore initiating the inhibition of its own translation (Zhang & Rathod, 2002). The influence of polyamine depletion on oxidative stress prompts further investigation to determine the oxidative status of the parasite after AdoMetDC inhibition. The functional genomics results indicate that AdoMetDC inhibition may result in an increased oxidative state within the parasite which may therefore reveal a clue as to the mode-of-action of MDL78311 within the Plasmodial parasite.

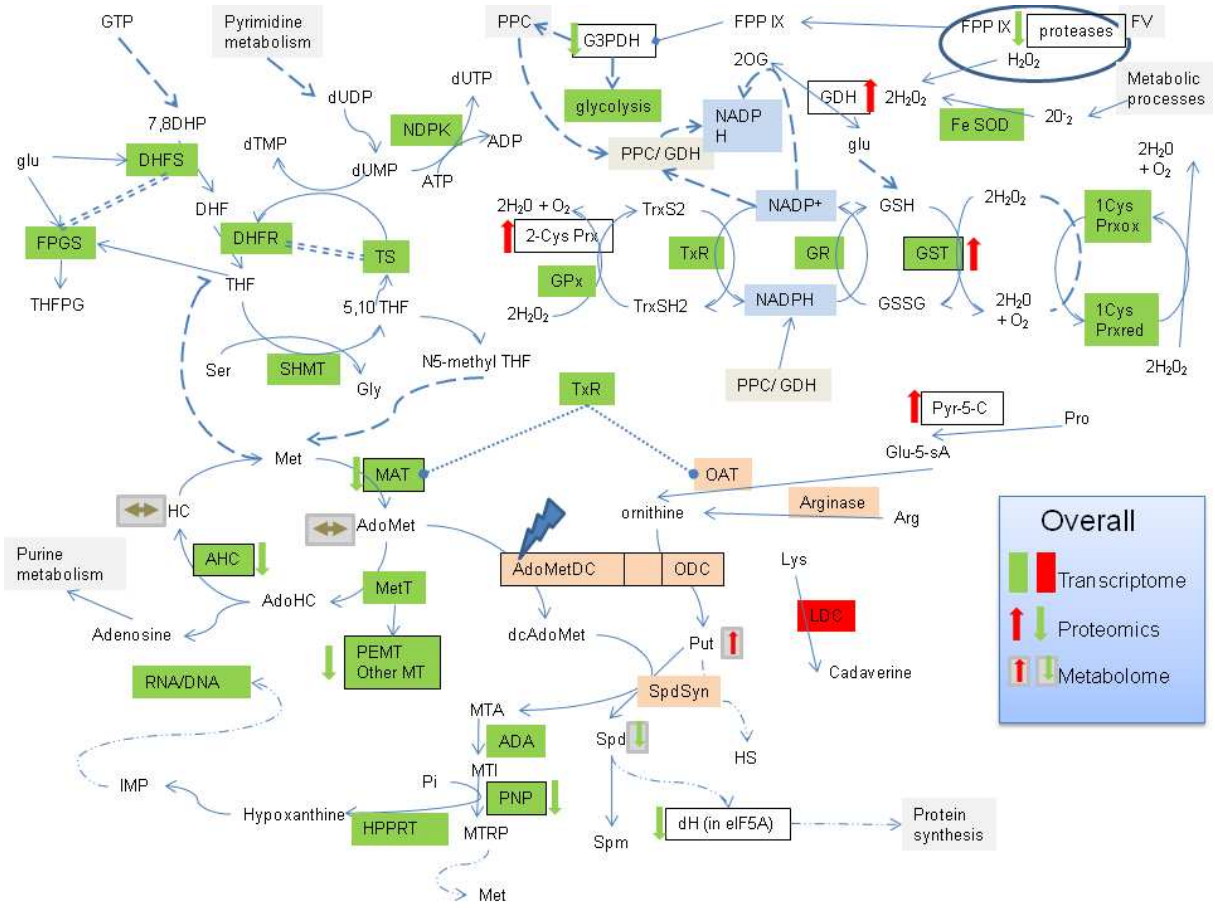

Figure 6.1: Functional consequences of polyamine depletion induced by AdoMetDC inhibition.
Green in indicative of corresponding decrease in transcript or protein while red is indicative of a corresponding increase in transcript or protein abundance. Names that are blocked with a black line is indicative of the change in protein, while transcripts are not blocked. For example: PNP denotes a decrease in both transcript and protein abundance, while HPPRT denotes a decrease in only the transcript.

Various transcripts involved in cytoskeleton organisation and biogenesis was also unique to the AdoMetDC dataset re-iterating the recent link that was established between polyamines and microtubules (Savarin *et al.*, 2010). The transcript abundance of actin and tubulin were severely decreased with AdoMetDC perturbation. The disruption in tubulin may suggest a link to cell cycle arrest in the G1-phase of the parasite. It may therefore be assumed that polyamines may play a role in stabilisation of these transcripts involved in the cell cycle and that upon polyamine depletion the majority of these transcripts may become destabilised resulting in parasite arrest. Investigations on the cell cycle are currently underway in our laboratory. It would be interesting to determine the role of polyamine depletion on microtubule formation and possible apoptosis within the parasite.

In conclusion, transcriptomics and proteomics as part of a functional genomics strategy provided the tools needed to investigate the global response of AdoMetDC inhibition on the parasite. Indeed, these tools are not perfect, but as shown throughout this study if they are scaled up and used

correctly, functional genomics can provide a profound effect on the drug discovery efforts. Here, it was demonstrated that inhibition of AdoMetDC does have a unique transcriptomic fingerprint. Furthermore, unique compensatory pathways were identified that provided clues as to the global effect of polyamine depletion on the parasite. Severely affected pathways like folate biosynthesis, oxidative stress and cytoskeleton biogenesis can be exploited further in combination with polyamine depletion to provide a more pronounced effect on the parasite. Another pathway that may also be considered for targeting in combination with polyamine depletion is protein kinases that were severely increased in abundance and may play an essential role in the cell cycle. Integration of functional genomics and systems biology were critical in the determination of uniquely affected pathways and possible regulatory mechanisms. Fortunately, the target was known within this study and it remains to be seen if a functional genomic approach in malaria parasites will be able to elucidate the mode-of-action of a compound with the same success as seen in tuberculosis and antibacterial research. This study revealed an in depth investigation into the transcriptome and proteome of AdoMetDC inhibited parasites and provided a novel contribution to the ongoing fight against malaria.