

# CHAPTER 2

## A CRITICAL EVALUATION OF ANTIMALARIAL DRUG SENSITIVITY METHODS FOR TESTING CYTOSTATIC DRUGS

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### **2.1 INTRODUCTION**

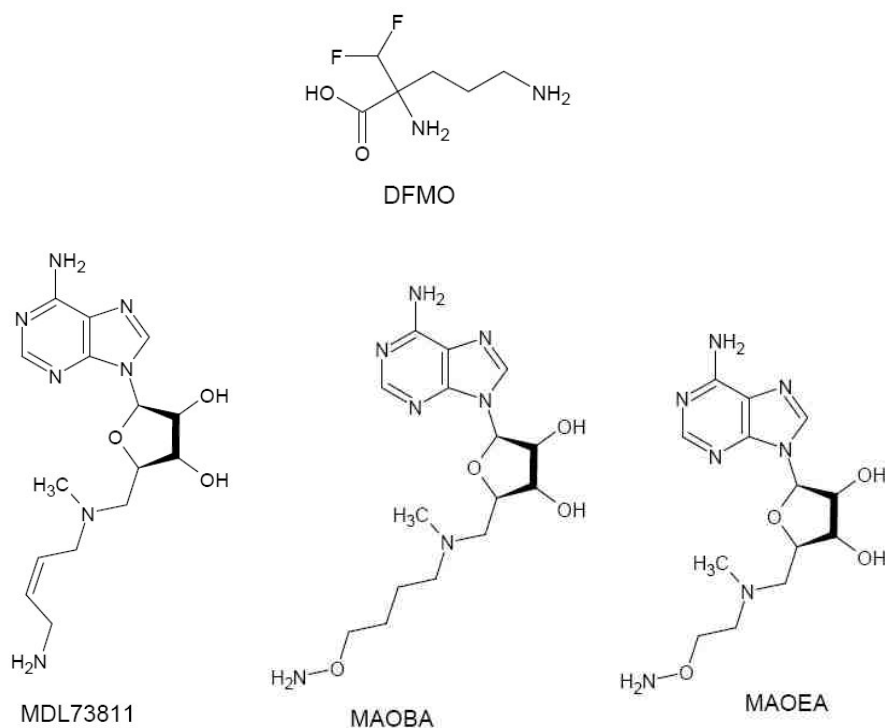
Continuous drug-screening efforts for new antimalarial therapeutics, including target-based and lead-based endeavours, is of critical importance since most antimalarials last only ~10 years in the clinical setting before drug resistance develops [55]. One way of delaying the development of drug resistance is by using compounds in combination (e.g. artemether/lumefantrine and the historically used chloroquine/proguanil and pyrimethamine/suphadoxine) because the parasite then has to mutate at several sites simultaneously to become resistant to the treatment regimen, which is much less likely than the occurrence of a single mutation conferring resistance to a single drug [55]. A drug combination selectively targeting polyamine and methionine metabolism, i.e. inhibition of both polyamine biosynthesis and transport, may be a useful therapeutic alternative to delay the development of drug resistance [81, 145].

#### **2.1.1 Polyamine biosynthesis inhibitors**

DFMO (eflornitine, Fig. 2.1) is one of the most studied polyamine biosynthesis inhibitors that was initially synthesised by Metcalf and colleagues [146]. DFMO is an enzyme-activated, irreversible inhibitor of ODC, which decreases putrescine levels [90], usually resulting in cytostasis [147]. It is currently being tested as cytostatic, antineoplastic agent in several anticancer clinical trials [148, 149], but it is clinically used only to treat West African trypanosomiasis (sleeping sickness) due to *T. brucei gambiense* [150]. East African trypanosomiasis due to *T. brucei rhodesiense* is tolerant to DFMO due to higher specific ODC activity and faster enzyme turn-over in these parasites [151].

*In vitro*, the compound halts the plasmodial IDC in the trophozoite stage [152] in a cytostatic manner, meaning that it causes growth arrest due to polyamine depletion without cytotoxicity, although the parasite eventually dies after 67 h exposure to the drug [153]. The effects of DFMO in *Plasmodium* can be alleviated by the addition of putrescine or spermidine [90, 153] as well as high concentrations of the diamine, cadaverine (0.4 mM) [154], but there are contradictory reports on the efficacy of spermine to restore growth after DFMO inhibition [90, 153, 154]. A concentration of 5 mM DFMO was demonstrated to inhibit ODC of the chloroquine-resistant FCR-3 by more than 99%, whereas 10 mM DFMO ensured complete growth arrest *in vitro* [154].

However, *in vivo* a 2% solution of DFMO only limited *P. berghei* erythrocytic schizogony without increasing the survival time of the infected mice [155].



**Fig. 2.1** Chemical structures of DFMO, MDL73811, MAOBA and MAOEA.

Another classic inhibitor of polyamine and methionine metabolism is the irreversible AdoMetDC inhibitor, 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (MDL73811 or AbeAdo, Fig. 2.1) [156]. Similar to DFMO, MDL73811 also arrests the plasmodial IDC in the trophozoite stage [153] due to a decrease of spermidine and spermine, but not putrescine. Instead, putrescine increases 3- to 4-fold [153]. MDL73811 inhibition can thus be reversed by the addition of spermidine or spermine, but not putrescine [153]. The cis-butenyl isomer of MDL73811 is the active form, whereas the trans-butenyl isomer (MDL74391) is about 15 - 20 times less efficient in inhibiting AdoMetDC [153]. After 48 h treatment of *P. falciparum* cultures *in vitro* the  $IC_{50}$  of MDL73811 varied between 1 and 3  $\mu$ M for chloroquine-sensitive (D6 and NF54) and chloroquine-resistant (W2, FCR-3 and ITG2) strains, respectively [153]. However, similar to DFMO, MDL73811 had no effect on mice infected with *P. berghei* [153], but it was very effective against both *T. brucei brucei* and *T. brucei rhodesiense*-infected mice [157].

Other AdoMet analogues and irreversible AdoMetDC inhibitors include 5'-deoxy-5'-[N-methyl]-N-[2-(aminooxy)ethyl]amino]adenosine (MAOEA, Fig. 2.1) [158] and 5'-deoxy-5'-[N-methyl]-N-[2-(aminooxy)butyl]amino]adenosine (MAOBA, Fig. 2.1). These analogues have tertiary nitrogens instead of sulfonium atoms (as in AdoMet) and have side chains of variable length ending in reactive groups, e.g.

aminoxygroups [159]. MAOEA was initially tested in murine leukaemia L1210 cells and was found to decrease the cellular content of dcAdoMet, 5-methylthioadenosine, spermidine and spermine [160]. MAOEA was also demonstrated to be active against *T. brucei brucei* ( $IC_{50} = 1.3 \mu M$ ), but increasing the length of the side chain (e.g. MAOBA) or swapping the methyl group (attached to the nitrogen) for an ethyl group caused reduced potency [159]. According to the literature MAOEA has not yet been tested in malaria (nor MAOBA), but because of its activity in trypanosomiasis, it was anticipated that this compound would also inhibit the growth of Plasmodia.

Bis(benzyl)polyamine analogues such as N',N'-bis{3-[(phenylmethyl)amino]propyl}-1,7-diaminoheptane (MDL27695) are spermine analogues with elongated central methylene chains and benzyl-substituted terminal amines [161]. Their antimalarial activity increases when the central methylene chain length is increased. This increase in potency is related to higher lipid solubility and uptake of the compounds into host erythrocytes, compared to the more water-soluble free-amine analogues that are more than 1000 times less potent [161]. Treatment of rat hepatoma cells with  $1 \mu M$  MDL27695 repressed ODC and AdoMetDC by 50% after 8 h [162]. In *Leishmania donovani*,  $10 \mu M$  MDL27695 inhibited ODC by 36% and AdoMetDC by 58% with accumulation of putrescine and spermidine [163]. *In vitro* treatment of *P. falciparum* with MDL27695 resulted in an  $IC_{50}$  of  $3.0 \mu M$  for the chloroquine-sensitive strain D6 and a 15-fold higher  $IC_{50}$  for the chloroquine-resistant strain, FCR-3 [161]. However, MDL27695 at its *in vivo*  $IC_{90}$  of 15 mg/kg suppressed only 70% of the *P. berghei* parasitaemia in subsequent experiments and had a cure rate of only 21% (3/14) [161].

MDL73811 is approximately 1000 times more active than DFMO against *P. falciparum in vitro* [153], but neither of these on their own, nor the potent bis(benzyl)polyamine analogue, MDL27695, cures *P. berghei in vivo* [153, 155, 161]. The potent *in vitro* antimalarial activity and the absence of *in vivo* efficacy of these compounds can be explained by exogenous polyamine salvage from the host [145]. However, the combination of MDL27695 and DFMO, which inhibits ODC, AdoMetDC and exogenous polyamine import, cured 100% (14/14) of *P. berghei*-infected mice [161]. A polyamine transporter has not yet been identified in the *P. falciparum* genome, but a drug combination selectively inhibiting both polyamine biosynthesis and transport may provide a promising antimalarial strategy [145].

### **2.1.2 Antimalarial drug sensitivity methods**

Drug sensitivity methods are indispensable tools for the surveillance of drug resistance and for establishing therapeutic guidelines. Antimalarial drug efficacy depends primarily on the ability of these compounds to kill malaria parasites by interfering with essential metabolic processes. This reduces their multiplication and allows the immune system to remove them from the circulation. Drug efficacy varies according to the sensitivity of each parasite clone within the natural population, which is referred to as drug sensitivity [12]. In addition to

surveillance and prevention of drug resistance, drug development programmes rely on accurate measurement of drug sensitivity to identify the most suitable candidates for further characterisation.

There are several approaches to assess the susceptibility of *P. falciparum* to antimalarial drugs. The most classical method is determining the therapeutic response or efficacy *in vivo*, which was originally defined in terms of parasite clearance as sensitive (S) or three degrees of resistance (RI, RII and RIII) [12]. *In vivo* tests nowadays include molecular analysis to distinguish true recrudescence (i.e. reappearance of disease after it has been quiescent) from re-infection and pharmacokinetic tests to identify host-related factors, such as poor absorption [12]. A different approach is to measure the drug sensitivity *in vitro* since this allows for the complete exclusion of host-related factors such as host immunity and provides a more objective perspective of the inherent drug sensitivity than the *in vivo* tests [12].

Traditional *in vitro* tests measure the effect of the antimalarial drugs on the growth and development of malaria parasites. This can be determined by several methods, e.g. the World Health Organisation (WHO) microtest, isotopic assays, parasite lactate dehydrogenase (LDH) assay, double-site enzyme-linked LDH immunodetection (DELI) assay and the histidine- and alanine-rich protein 2 (HRPII) assay [12]. In all these tests the growth of drug treated cultures is measured relative to drug-free control, but the parasite density and haematocrit (i.e. inoculum) and developmental stage of the parasites have to be controlled since these parameters can have a significant impact on the outcome of the assays [12]. The WHO microtest [164] uses schizont maturation as a measure of parasite growth [12], but in analogy, parasite growth can also be measured by determining the increase in the percentage of infected erythrocytes (parasitaemia) during a culture period of 48 to 96 h. Reading the test results via microscopy is particularly labour-intensive and prone to variability of interpretation, but methods have been developed that allow the automated reading of results, e.g. via a flow cytometer or various indirect measurements of parasitaemia. The tritium-labelled hypoxanthine incorporation assay determines the ability of antimalarial compounds to inhibit parasite growth by causing the reduced uptake of this radio-labelled nucleic acid precursor by the parasite [165]. The technique allows for a high degree of automation and is considerably faster to perform than tests based on morphological assessment. However, isotopic assays have mostly been replaced with other methods because of restrictions on the use of radioactive material [12].

LDH was one of the first plasmodial enzymes that were shown to be electrophoretically, immunologically and kinetically distinct from the host and was initially used primarily as an indicator of the presence of malaria parasites for diagnostic purposes. The levels of parasite LDH correspond to the parasite density and rapidly decrease upon initiation of treatment and the resulting lowered parasite densities [12]. Parasite LDH is distinguishable from the host LDH due to its requirement for the 3-acetyl pyridine adenine dinucleotide (APAD), which is an analogue of nicotinamide adenine dinucleotide. Makler and colleagues used this

knowledge to develop an assay that determines parasite growth inhibition profiles by measuring the enzymatic activity of the parasite homologue as an indirect indication of parasite growth [166]. However, the assay requires initial parasite densities of 1 – 2% and was found to be insensitive for field application, which led to the development of a new LDH assay. The new LDH or DELI assay measures enzyme levels via immunodetection using two monoclonal antibodies that specifically recognise parasite LDH [167], which makes the assay considerably more sensitive than the original method [12].

Another addition to the list of *in vitro* drug sensitivity methods is the HRP<sub>II</sub> enzyme-linked immunosorbent assay (ELISA) [168], which measures the production of HRP<sub>II</sub> by *P. falciparum* during the course of its growth and multiplication [12]. HRP<sub>II</sub> levels are closely correlated to parasite density and development. The assay also uses a double-site sandwich ELISA and is ~10 times more sensitive than the isotopic assay [12].

In this chapter, several AdoMetDC inhibitors were considered for use in combination with the established ODC inhibitor, DFMO, to obtain complete inhibition of both catalytic sites of PfAdoMetDC/ODC for subsequent functional genomics investigations (Chapters 3 and 4). These compounds were evaluated by performing several biochemical assays, including calculating their IC<sub>50</sub>s under the particular laboratory conditions and determining the appropriate dosages to be used in combination. In order to achieve these objectives, several established antimalarial drug sensitivity methods were critically evaluated to determine the most appropriate assay for testing cytostatic drugs, such as the polyamine biosynthesis inhibitors.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 *In vitro* cultivation of asexual *P. falciparum* cultures**

Continuous *P. falciparum* asexual cultures of the chloroquine-sensitive 3D7 strain were maintained *in vitro* according to a modified Trager and Jensen method [169], which supports intracellular parasite development. The erythrocyte preparation, culture thawing and general maintenance are described below.

Type A+ blood was collected in ethylenediamine tetra-acetic acid (EDTA) vacuum-tubes, transferred to centrifuge tubes using aseptic technique and centrifuged at 2500 g for 5 min at room temperature (Hermle Z320 centrifuge). The serum and buffy-coat were aspirated and an equal volume of wash medium [RPMI-1640 (SIGMA, Missouri, USA) supplemented with 0.4% (w/v) D-glucose (SIGMA), 88 mg/l hypoxanthine (SIGMA), 48 mg gentamycin (SIGMA), buffered with 12 mM HEPES (SIGMA) and 21.4 mM sodium bicarbonate (Merck, Darmstadt, Germany) per litre MilliQ (distilled, de-ionized, 0.22 filter sterilised) H<sub>2</sub>O], was added. The erythrocytes were resuspended and centrifuged at 2500 g for 5 min, after which the supernatant was aspirated. The wash procedure was repeated four to five times to reduce the presence of leukocytes. Washed erythrocytes were resuspended in an equal volume of wash medium and stored at 4°C. The wash medium was aspirated and replaced three to four times per week to preserve the erythrocytes.

A glycerol-frozen aliquot of asexual *P. falciparum* 3D7 parasites was removed from liquid nitrogen storage and quickly thawed in a water bath at 37°C. The thawed aliquot was transferred to a centrifuge tube under sterile conditions in a laminar flow cabinet. The osmotic potential of the thawed stock was gradually reduced by the drop-wise addition of 200 µl 12% (w/v) sodium chloride (NaCl) solution, followed by pipette-mixing for ~10 - 20 s and then the drop-wise addition of 1.8 ml 1.6% NaCl, again followed with mixing for ~10 - 20 s. The parasites were collected by centrifugation at 2500 g for 5 min at room temperature. The supernatant was aspirated and 10 ml culture medium [wash medium with 0.5% (m/v) Albumax II (purified lipid-rich bovine serum albumin, Invitrogen, Paisley, UK)], preheated to 37°C, was added. This was followed by 500 µl freshly collected, washed, type A<sup>+</sup>, packed erythrocytes to establish a ~5% haematocrit culture. The erythrocytes were suspended and transferred to 75 cm<sup>2</sup> Cellstar culture flasks (Greiner bio-one, Frickenhausen, Germany) and gassed for 30 s with a special gas mixture containing 90% nitrogen, 5% oxygen and 5% carbon dioxide (Afrox, Johannesburg, South Africa). The flasks were sealed air-tight and incubated at 37°C.

Parasite growth was monitored daily by visual microscopic inspection of thin smears, which were fixated by methanol (MeOH) and stained with a 10% (v/v) Giemsa stock solution (Merck) in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4). Parasitaemia was calculated by counting the equivalent of 1000 erythrocytes using the Miller technique for reticulocyte counting [170] at a 1000 times enlargement with a Nikon Labophot microscope. This counting technique requires an evenly distributed erythrocyte smear across the microscope field, which is then divided into equal-sized squares, i.e. four in the case of the Nikon Labophot. The total number of erythrocytes (infected and uninfected) in one of the four squares was counted, but the infected erythrocytes across the whole field, i.e. all four squares, were counted without moving the field. The same procedure was followed in randomly adjacent fields until a total of 250 erythrocytes were counted in the single squares. By extrapolation, the equivalent of 1000 erythrocytes was inspected for parasites (250 erythrocytes x 4 blocks). The number of parasites per 1000 erythrocytes was then divided by 10 for the percentage parasitaemia, which was generally maintained at 3 to 5%. Culture medium was replaced daily and the parasites were gassed with the special gas mixture before incubation at 37°C. Once thawed, cultures were not grown for longer than 3 months to prevent genetic alteration. Ring-stage (5 – 10% parasitaemia) parasite-infected pellets were cryopreserved with an equal volume of freezing medium (28% glycerol/wash medium) on a regular basis and stocks were preserved at -180°C in liquid nitrogen.

### **2.2.2 Sorbitol synchronisation of growth stage**

Parasites were synchronised once a week in the ring stage with the Lambros and Vanderberg method [171] with minor modifications. Cultures of 20 ml were transferred to a centrifuge tube and centrifuged at 2500 g for 5 min at room temperature. The supernatant was aspirated, 4 ml of 15% sorbitol solution (preheated to 37°C) was added, mixed well and incubated for 5 min at 37°C. The suspension was centrifuged at 2500 g for 5 min,

the supernatant removed and 8 ml of 0.1% (w/v) glucose solution (preheated to 37°C) was added and mixed well. The suspension was incubated for 5 min at 37 °C and centrifuged at 2500 g for 5 min at room temperature. The supernatant was aspirated and the infected erythrocytes were resuspended in culture medium to restore the ~5% haematocrit (including the lysed erythrocytes). The culture was divided into two culture flasks and 5 ml culture medium and 250 µl packed erythrocytes were added to each to replace the erythrocytes lysed during synchronisation. The cultures were gassed as mentioned and incubated at 37°C.

### **2.2.3 Parasite growth and drug sensitivity assays**

Drug treatment in 96-well plate format was performed on synchronised, early ring-stage parasites and samples for direct and indirect parasitaemia quantitation assays were treated in parallel (duplicate plates) as described below.

#### **2.2.3.1 Drug treatment and plate storage until analysis**

Stock solutions of the ODC inhibitor, DFMO, and the AdoMetDC inhibitors MAOBA, MAOEA and MDL73811, as well as chloroquine (cytotoxic drug control), were prepared by dissolving the compounds in PBS followed by sterilisation with a 0.22 µM Minisart syringe filter (Sartorius Stedim Biotech, Göttingen, Germany). Aliquots were stored at -20°C and were diluted just before use in culture medium to achieve the desired concentrations. DFMO was kindly provided by P. Woster (Wayne State University, Michigan, USA), MAOEA and MAOBA were gifts from J. Secrist (Southern Research Institute, Alabama, USA), MDL73811 was obtained from Sanofi-Aventis (USA) and chloroquine diphosphate from SIGMA.

Drug dilution series consisting of eight two-fold dilutions were prepared for each drug and 50 µl of each concentration was pipetted in quadruplicate into sterile 96-well flat-bottomed culture plates with lids (Greiner bio-one Cellstar). The same volume of culture medium was aliquoted into eight wells for quadruplicate uninfected and untreated controls. Duplicate plates were prepared to enable method comparison under exactly the same culture and treatment conditions. After preparing the drug containing plates, the cultures were removed from the incubator, thin smears were prepared and the parasitaemia calculated as discussed in section 2.2.1. The haematocrit was estimated after centrifugation at 2500 g for 5 min in graduated tubes (100 µl increments). The parasitaemia was adjusted to 1% with uninfected A<sup>+</sup> erythrocytes and the haematocrit to 10% with culture medium. A volume of 50 µl synchronised, early ring-stage cultures were then pipetted into all the wells (excluding the uninfected control wells) for a final haematocrit of 5%, i.e. an inoculum (parasitaemia x haematocrit) of 5. The haematocrit of uninfected A<sup>+</sup> erythrocytes was also adjusted to 10% and 50 µl volumes were pipetted into the remaining wells. The plates were covered and placed inside a disinfected gas chamber, sealed, gassed with a special gas mixture for 5 min and grown at 37°C in a humid atmosphere for a complete life cycle of 48 h.

After the incubation period, the success of the treatment was evaluated by microscopical inspection of Giemsa-stained thin smears of the wells containing the untreated, highest and lowest drug concentration before continuing with the drug sensitivity assays. With successful treatments one plate was sealed and frozen at -20°C for the parasite LDH (PF13\_0141) [166] and HRPII (pHRPII\_830) ELISA assays [168] and the other was fixed (see below) for fluorescence activated cell sorting (FACS) [172].

Plates destined for FACS analysis were fixed with an equal volume of 4% (w/v) D-glucose/10% (v/v) Tris-saline (10 mM Tris, 150 mM NaCl, 10 mM NaN<sub>3</sub>)/10% (v/v) formaldehyde and adjusted to a final pH of 7.3 with sodium hydroxide (NaOH) [172, 173]. A ~10% (v/v) formaldehyde solution was prepared from 3 in 10 parts 35% (v/v) formaldehyde (SIGMA). Erythrocyte lysis occurred if all the components of the fixing solution were not prepared fresh and therefore the solution was prepared just before use and tested on an uninfected erythrocyte sample of 5% haematocrit before addition to the plates, to prevent lysis compromising the experiment. Fixed plates were stored at 4°C for FACS analysis.

### **2.2.3.2 Indirect parasitaemia quantitation assays**

#### **2.2.3.2.1 Lactate dehydrogenase (Malstat) assay**

The LDH assay measures the parasitic conversion of L-lactate to pyruvate resulting in the reduction of APAD. The reduced APAD in turn reduces nitroblue tetrazolium (NBT) and forms a blue formazan product, which is measured spectrophotometrically [12]. The LDH assay was performed according to Makler and colleagues [166], as follows: The Malstat reagent (pH 9) was prepared [0.2% (v/v) Triton-X 100 in 0.1 M Tris-HCl followed with the addition of 2.1% (w/v) lithium-lactate and 0.022% (w/v) APAD] and stored at 4°C. A volume of 100 µl Malstat reagent was pipetted into the wells of a non-sterile 96-well plate. The thawed, drug treated culture plates were resuspended and 6 µl of culture lysate was transferred to its corresponding well in the Malstat-containing plate. The drug treated plates were afterwards returned to storage at -20°C for subsequent HRPII ELISA analysis. NBT/phenazine ethosulphate (PES) was mixed 20 to 1 in PBS and 25 µl was added to the wells as chromogen. The plates were foil-covered and incubated in the dark at room temperature for 20 - 30 min until the reaction was terminated by the addition of 30 µl of 10% acetic acid. The concentration of the blue formazan product was determined with a Multiskan Ascent ELISA scanner (Thermo LabSystems, Massachusetts, USA) at an absorbance of 650 nm. The percentage response was calculated relative to that of the untreated parasite controls (Eq. 2.1) and the IC<sub>50</sub> was calculated with concentration response curves as described in section 2.2.3.4.

$$\text{Percentage response} = \frac{(\text{drug treated response} - \text{background response})}{(\text{untreated response} - \text{background response})} \times 100 \quad \dots\dots\text{Equation 2.1}$$



### 2.2.3.2.2 Histidine-rich protein II (HRPII) ELISA

The HRPII assay is a double-site sandwich ELISA i.e. binding of a mouse primary anti-HRPII antibody to the HRPII antigen forms a complex that is recognised by an anti-mouse horseradish peroxidase (hrp)-conjugated secondary antibody (hrp-conjugate). Excess unbound antibodies are removed and the conjugated hrp of the bound secondary antibody catalyses the oxidation of o-phenylenediamine by hydrogen peroxide, resulting in a yellow chromogenic product, 2,3-diaminophenazine, which is measured spectrophotometrically [168, 174]. The assay was performed with two commercially available monoclonal antibodies kindly provided by M. Bubb from the National Bioproducts Institute (Pinetown, South Africa) according to the protocol of H. Noedl (<http://malaria.farch.net>) [168]. 96-Well plates were coated overnight with 100 ng of anti-HRPII IgM capture antibody at 4°C and then blocked with 2% (w/v) bovine serum albumin (BSA, Roche) in PBS for 2 h at room temperature. The plates were washed three times with 0.05% Tween 20 in PBS in a Wellwash 4 ELISA washer (Labsystems, Helsinki, Finland), air dried, sealed in aluminium-plastic covers and stored with dessicant at -20°C until used.

On the day of the analysis, the drug treated culture plates (treated as discussed in section 2.2.3.1 and used for the LDH assay prior to the HRPII ELISA) were freeze-thawed twice to ensure lysis of the red blood cells and membranes. Cell lysates (initial inoculum = 5) were diluted 1/40 with PBS to prevent antigen overload (determined assay capacity < 0.15 initial inoculum) and 100 µl was transferred to each well of the pre-coated anti-HRPII IgM plates. The plates were incubated for 1 h at room temperature and subsequently washed three times with 0.05% Tween 20 in PBS. After the wash step, 100 µl of 1/3000 diluted, hrp-conjugate in 2% (w/v) BSA/1% (v/v) Tween 20/PBS (pH = 7.4) was added and incubated for a further 1 h. [This conjugate dilution was determined to provide optimal signal (linear to the inoculum size) for the particular batch of IgM with a 1/40 sample dilution, but had to be titrated with every new batch. Such titration was performed with a checkerboard design: the 96-well plate was coated with two concentrations of anti-HRPII IgM (usually 50 and 100 ng) and a sample dilution series (e.g. initial inoculum of 5 to 0.02) was distributed on the horizontal axis of the plate and different conjugate dilutions (e.g. 1/2000, 1/4000 and 1/6000) on the vertical axis]. After incubation with the anti-mouse secondary antibody, the plates were washed again three times and 100 µl of substrate (1 mg/ml o-phenylenediamine/0.8 mg/ml hydrogen peroxide in 0.1 M trisodium citrate/citric acid buffer, pH 4.5) was added as chromogen. After 10, 20 and 30 min incubation in the dark, the absorbance of the resulting yellow product was read at 450 nm with a Multiskan Ascent ELISA scanner. HRPII readings were corrected by subtracting the average HRPII value of the culture at the beginning when drug treatment was initiated [time zero ( $t_0$ )]. Triplicate readings were used to compile dose response curves.

### **2.2.3.3 Direct parasitaemia quantitation assays**

#### **2.2.3.3.1 FACS analysis**

Drug treatment of cultures with an initial inoculum of 5 was performed for 48 h as discussed in section 2.2.3.1. The success of the treatment was first evaluated via thin smears before continuing with plate fixation as discussed in section 2.2.3.1. Fixed plates were stored at 4°C and were stable for weeks to months until FACS analysis was performed.

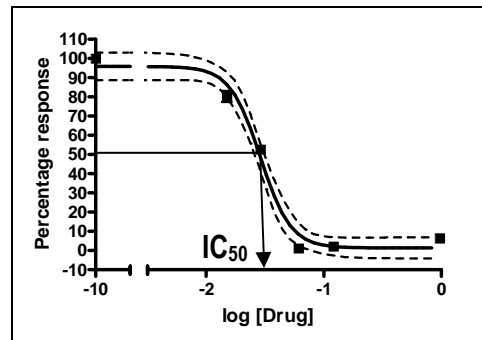
Samples were subsequently resuspended and 50 µl was transferred to 1 ml PBS inside 5 ml FACS tubes (Beckman Coulter, California, USA). The samples were stained with 0.525 µM of the membrane-permeable, fluorescent dye thiazole orange (SIGMA) that intercalates into DNA and RNA, thus differentiating the parasitised erythrocytes from the anuclear uninfected erythrocytes [172, 175]. To prevent quenching of the dye, samples were protected from light until the completion of the analysis. The samples were incubated for 1 h at room temperature and afterwards kept on ice until analysis with an Epics XL.MCL flow cytometer (Beckman Coulter) with laser excitation at 488 nm and fluorescence detection at 525 nm. The flow cytometer was initially calibrated with a series of fixed ring (8.6%, 4.3%, 2.15%, 1.08%, 0.54%, 0.27% parasitaemia) and trophozoite (6.4%, 3.2%, 1.6%, 0.8%, 0.4%, 0.2% parasitaemia) infected erythrocytes and the instrument was set to differentiate between uninfected, ring infected and trophozoite infected erythrocytes according to the size (forward scatter) and granularity (side scatter) of the detected fluorescence. At the start of every FACS run, uninfected erythrocytes and untreated parasite controls of known parasitaemia and parasite stage were analysed to ensure that the instrument settings were correct. In view of the cost of the analysis all samples were analysed in duplicate and not triplicate as with the other drug sensitivity assays, but a total of 50000 fluorescent events (cells) were counted to reduce the variation between replicates. Pearson correlation of the parasite counts by FACS and microscopy was calculated in Excel. The Pearson correlation is a measure of similarity i.e. the strength of the linear relationship between two variables or datasets and ranges between -1 (anti-correlated) and 1 (perfectly correlated).

#### **2.2.3.4 Concentration-response curves**

GraphPad Prism v. 4.0 software (GraphPad Software Inc., California, USA) was used to construct sigmoidal concentration-response curves according to the four-parameter logistic equation (Hill equation), including the baseline response (Bottom), the maximum response (Top), the curve slope (Hill slope) and the IC<sub>50</sub>, i.e. the concentration that elicited a response halfway between the curve maximum and the baseline, as presented in Equation 2.2 [176].

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogIC}_{50} - X) \cdot \text{HillSlope}}}$$

.....Equation 2.2 [176]



**Figure 2.2** Sigmoidal concentration-response curve used by GraphPad Prism 4.0 software to calculate the median inhibitory concentration ( $IC_{50}$ ). The 95% confidence zone around the best-fit curve is indicated with a dotted line [176].

The software compiles concentration-response curves by performing mean background (uninfected control) subtraction, log transformation of the X-values (concentrations in  $\mu\text{M}$  or  $\text{mM}$ ), normalisation of the Y-values (response) against the mean untreated control as a percentage and non-linear regression to obtain a sigmoidal concentration-response curve with variable slope (Fig. 2.2).

### **2.2.3.5 Propidium iodide (PI) membrane integrity assay**

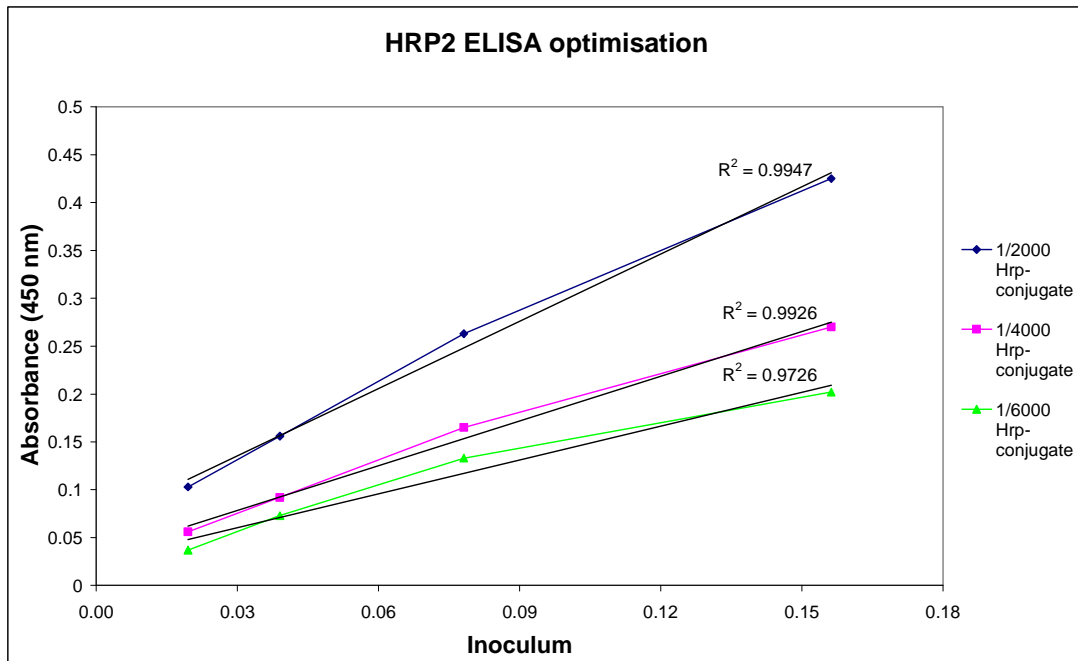
To exclude potential cytotoxicity at the high concentrations of DFMO and MDL73811 used to prevent parasites escaping cytotaxis and causing asynchrony [154], drug treatment at 5 mM DFMO, 5  $\mu\text{M}$  MDL73811 and the combination was performed and membrane integrity was assessed with propidium iodide (PI). This DNA-binding probe does not pass through intact membranes and nuclear staining indicates membrane permeabilisation [177], which is an indirect measure of chemical cytotoxicity [178]. PI fluorescence was detected with FACS using similar methodology as described in section 2.2.3.3.1. An Epics XL.MCL flow cytometer with laser excitation at 488 nm and fluorescence detection at 585 nm was set to count at least 50000 fluorescent events or 300 s. Samples were assayed immediately after drug treatment as formaldehyde fixing was found to cause membrane perforation. Samples were resuspended and 25  $\mu\text{l}$  was transferred to 1 ml PBS inside 5 ml FACS tubes. These were incubated in the dark, on ice for 15 min with 0.04  $\mu\text{M}$  PI (SIGMA), before FACS analysis. Positive controls of membrane damage were prepared by exposing samples to 0.05% saponin for 1 min, followed by PI addition and immediate analysis. As opposed to the general FACS procedure where it was required to differentiate between uninfected and different stages of parasitised erythrocytes, the aim here was to assess membrane damage and the FACS instrument had to be recalibrated for PI penetration and fluorescence. To test the efficacy over a wide range of parasitaemias, chicken erythrocytes, which are nucleated as opposed to human erythrocytes, were saponin-lysed to simulate 100% parasitaemia and were used for instrument calibration purposes.

## **2.3 RESULTS**

For functional genomics investigations of polyamine-depleted *P. falciparum* (Chapters 3 and 4) DFMO was chosen as the inhibitor of choice for ODC since it is a selective and specific enzyme-activated, irreversible inhibitor of this enzyme [147] and one of the most widely studied and characterised polyamine biosynthesis inhibitors [146]. For co-inhibition of both the catalytic sites of the bifunctional PfAdoMetDC/ODC, three alternative AdoMetDC inhibitors were considered and tested to be used with DFMO, namely MDL73811 [156], MAOEA [158] and MAOBA (Fig. 2.1 B-D). To determine the IC<sub>50</sub>s of these compounds, three different drug sensitivity assays were evaluated for suitability for screening such cytostatic drugs. Serial dilutions of the compounds were prepared and 3D7 *P. falciparum* parasites were treated as described (section 2.2.3.1) for 48 h. The same sample sets were analysed in parallel with the LDH assay, HRPII ELISA and FACS to enable comparison of the results obtained.

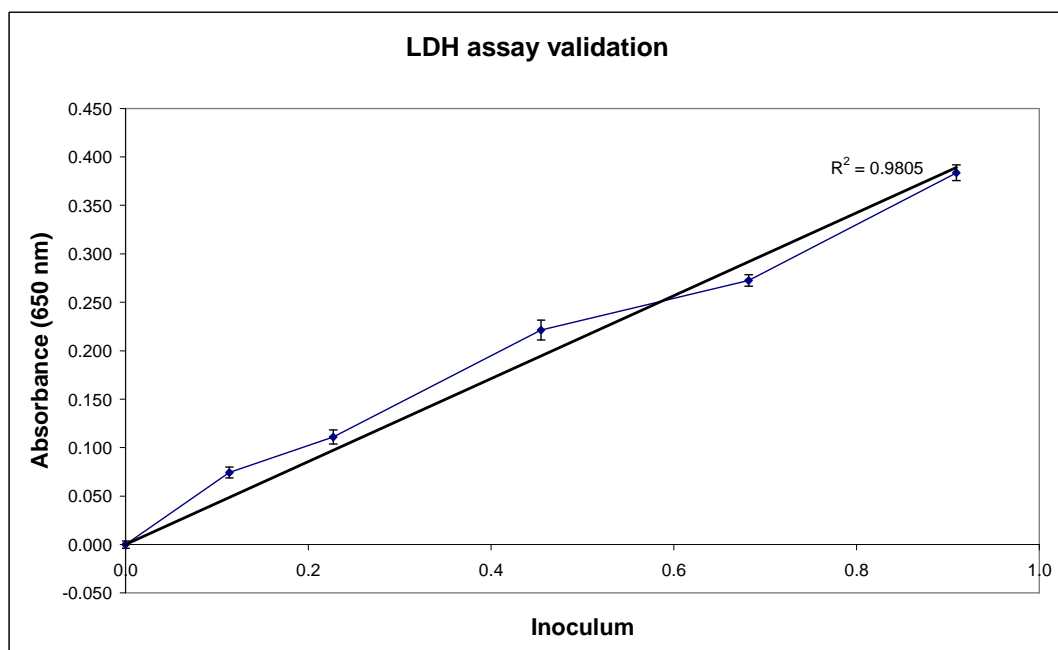
### **2.3.1 Validation of signal to inoculum linearity**

Linearity of absorbance versus parasite inoculum (parasitaemia x haematocrit/sample dilution) was carefully optimised for the two colorimetric assays, the HRPII ELISA and the LDH assay. For the HRPII assay, the quantity of anti-HRPII IgM and hrp-conjugate, the optimal culture dilution and colour reaction time was determined with a checkerboard design (described in section 2.2.3.2.2) to prevent overloading the capacity of the assay with too much antigen. This optimisation had to be repeated for every new batch of IgM and hrp-conjugate obtained, but in general the combination of 100 ng anti-HRPII IgM, 1/2000 to 1/4000 diluted hrp-conjugate and a sample inoculum dilution  $\leq 0.15$  was used, resulting in a signal that was linear with the sample inoculum when incubated for 20 - 30 min. A conjugate dilution of 1/3000 was subsequently used (Fig. 2.3). The HRPII assay was by far the most sensitive of the three assays tested and could detect an inoculum of as low as 0.025 (parasitaemia of 0.005% with a 5% haematocrit).



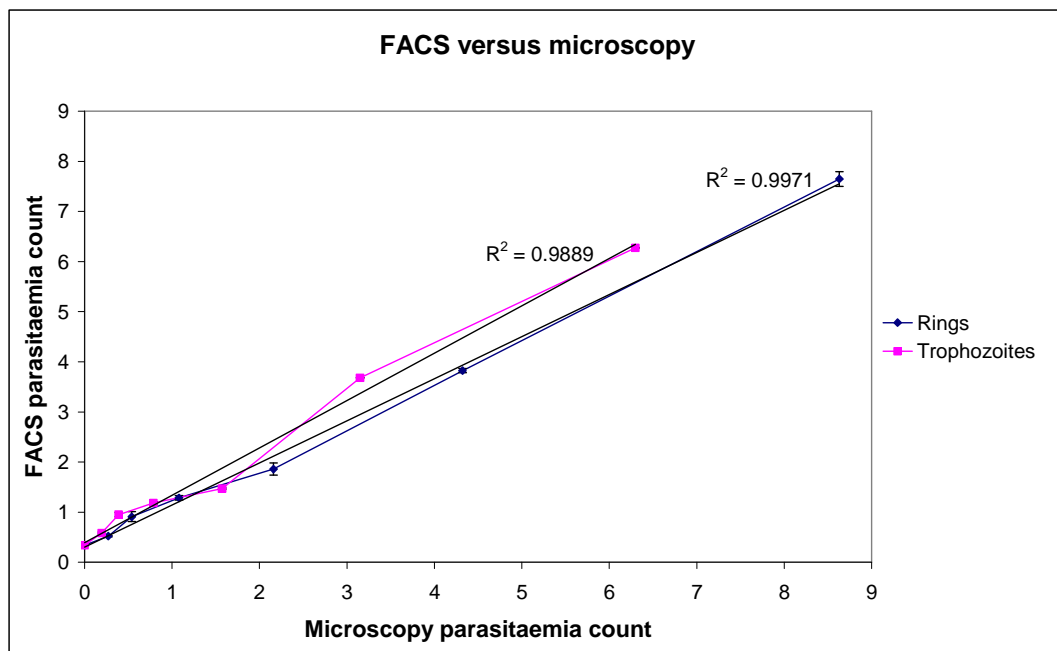
**Fig. 2.3** HRPII ELISA optimisation of the hrp-conjugate dilution and sample inoculum.

Similarly, the linearity of the LDH assay was determined by measuring the LDH activity of a series of inocula (varying parasitaemia/haematocrit and sample dilution) (Fig. 2.4). This enzymatic assay (versus the antigen-antibody HRPII assay) has a higher capacity for larger inocula (higher than 2.0 for ring stage parasites, Fig. 2.9), and the upper plateau was not reached in any of the assays performed. Samples with an initial inoculum of 5 were routinely diluted to an inoculum of 0.2 to prevent haemoglobin interference with the colour reaction.



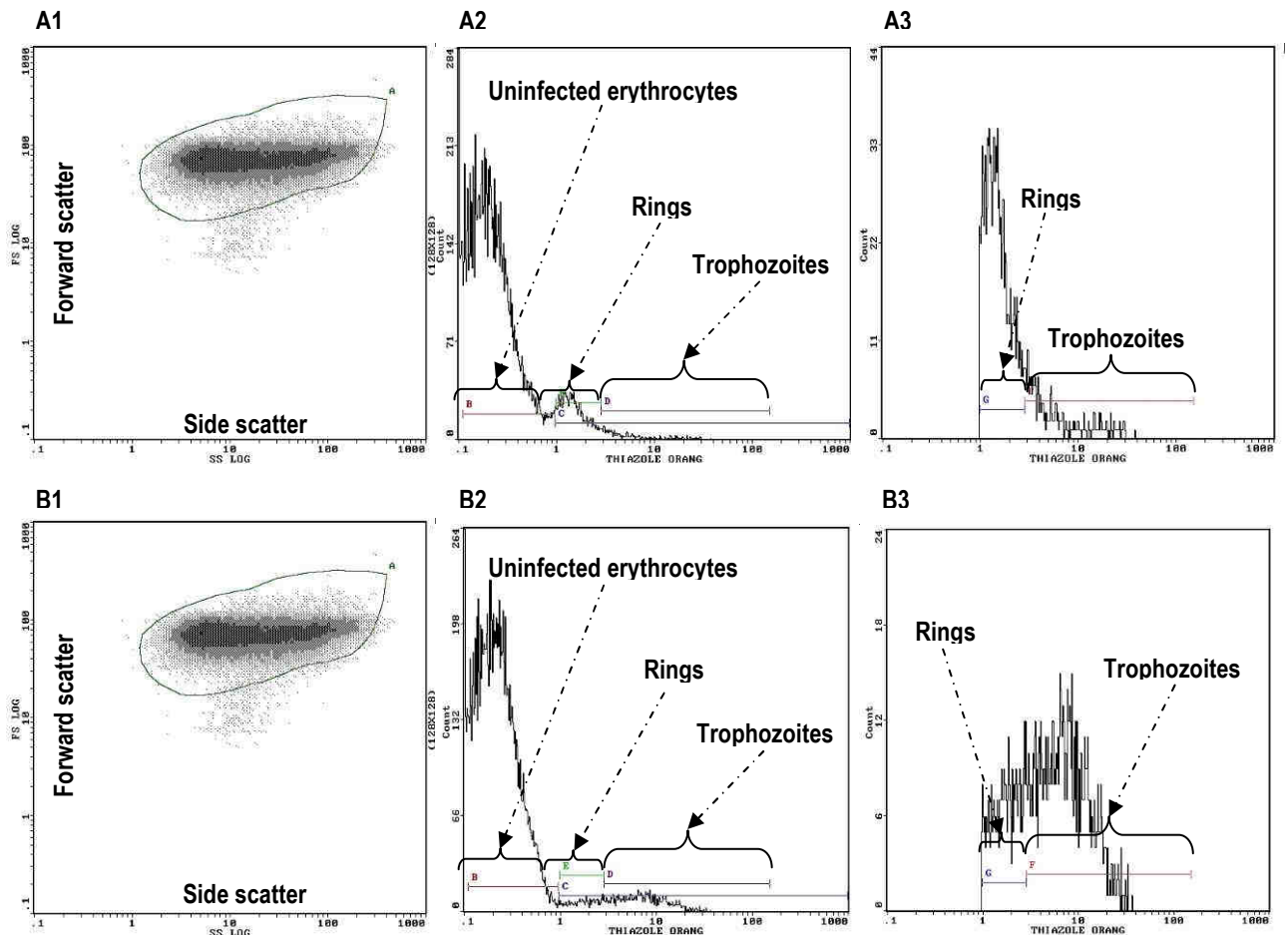
**Fig. 2.4** Validation of signal to inoculum linearity of the LDH assay.

The accuracy of the FACS instrument was validated by a series of ring and trophozoite-infected erythrocytes with known parasitaemia that were prepared by microscopy (Fig. 2.5). The thiazole orange fluorescent dye used for FACS analysis, intercalates with DNA, resulting in a fluorescent signal for parasitised erythrocytes, but not with anuclear, uninfected erythrocytes [175]. Similar to previous reports [172, 175] there was excellent correspondence between the fluorescent parasite counts (FACS) and the Giemsa-stained parasite counts (microscopy, Fig. 2.5). FACS and microscopical data had a Pearson correlation  $> 0.99$  for both rings and trophozoites. However, FACS analysis had a sensitivity of only about 0.5% parasitaemia.



**Fig. 2.5** Linearity of parasite counts by FACS analysis versus microscopy

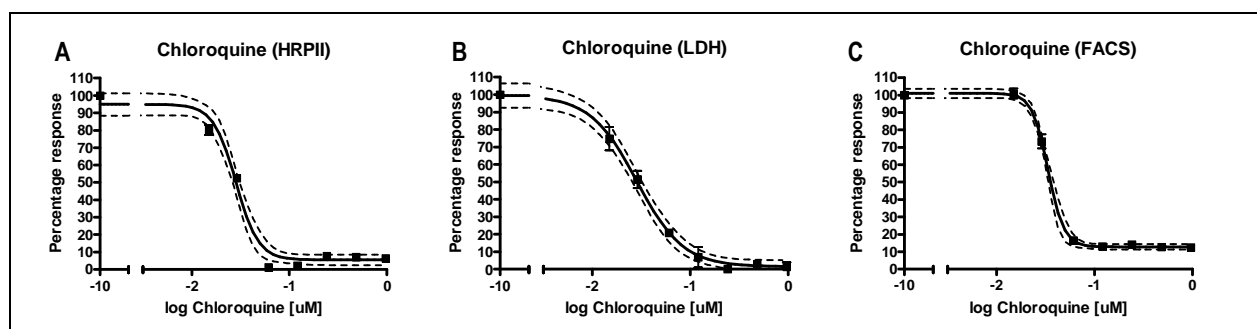
Based on the size (forward scatter) and granularity (side scatter) of the fluorescence, uninfected and parasitised erythrocytes containing different parasite stages could be differentiated (gating) [172]. When forward (Y-axis) and side scatter (X-axis) of fluorescence was plotted, the signal of the uninfected erythrocytes (least granular) lay furthest to the left, followed by the rings (moderately granular) and the signal of the trophozoites (most granular) appeared furthest to the right on the horizontal axis (Fig. 2.6). The method was not set to differentiate schizonts from the other parasite stages, but the signal of such mature parasites would be expected at the far right on the horizontal axis because of their high granularity.



**Fig. 2.6** Typical FACS images illustrating the forward (size) and side scatter (granularity) of the fluorescence, which was used to differentiate between uninfected, ring and trophozoite-infected erythrocytes (gating). Note that the sample in A contained mainly ring-stage parasites (**A3**), whereas the sample in B contained both rings and trophozoites (**B3**).

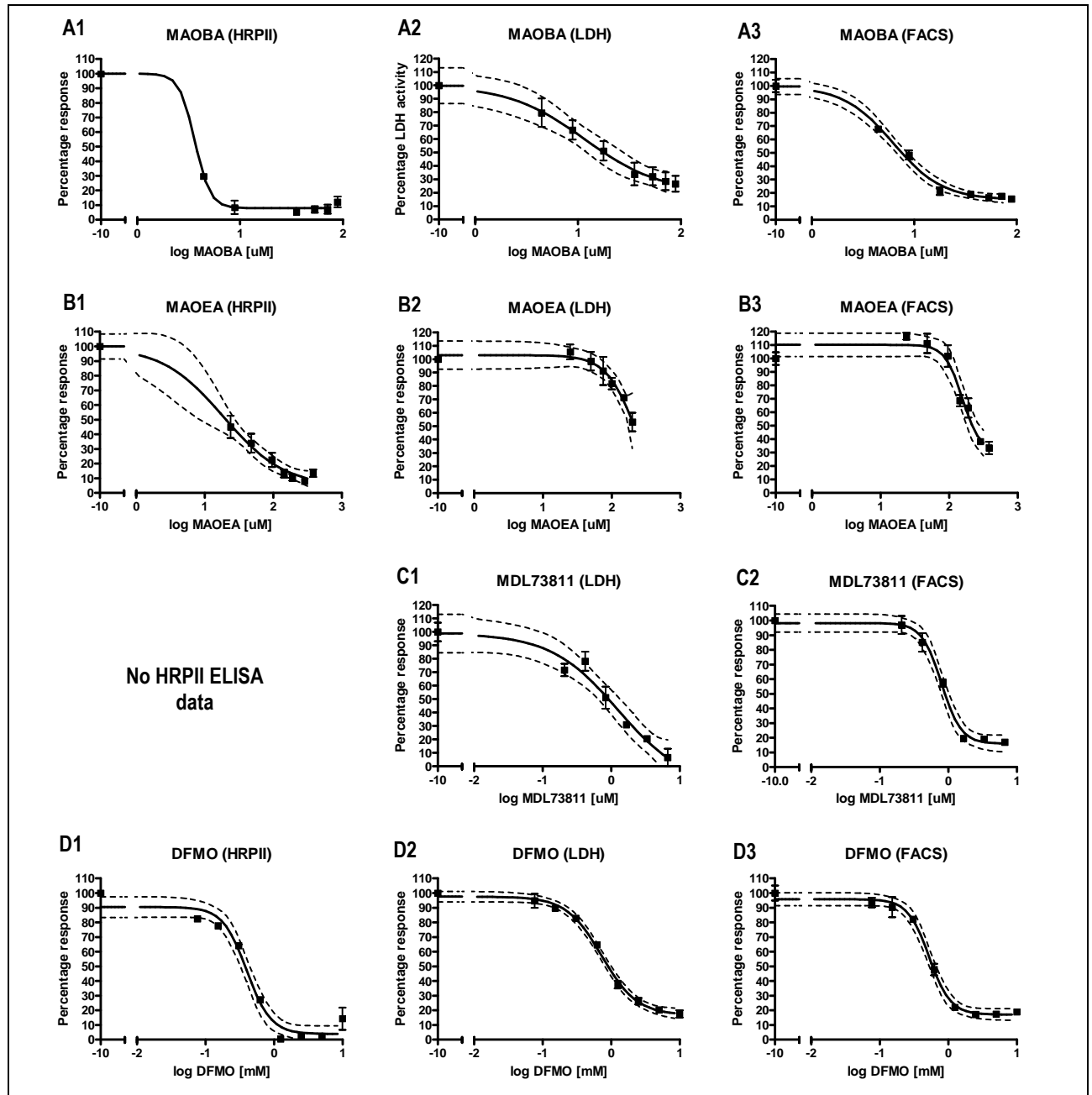
### 2.3.2 Concentration-response curves and $IC_{50}$ s

The data obtained from the LDH assay, HRPII ELISA and FACS were used to compile concentration-response curves of the four cytostatic drugs in order to calculate their  $IC_{50}$ s. Chloroquine was always included as drug treatment control, albeit cytotoxic. The shape of the concentration-response curves obtained for chloroquine was always sigmoidal (S-shaped, Fig. 2.7) and the  $IC_{50}$ s across the different assays were relatively consistent (28 - 34 nM) and comparable with the published value of 50 nM for the chloroquine-sensitive 3D7 strain (Table 2.1) [179].



**Fig. 2.7** Concentration-response curves of chloroquine from data derived of **A)** HRPII ELISA, **B)** LDH assay and **C)** FACS analysis. The 95% confidence intervals around the best-fit curves are indicated. All three curves are truly sigmoidal with little variation.

However, despite the general success of the drug treatment and dosage ranges used, as evaluated by microscopy, the curves obtained for the cytostatic drugs were often not truly sigmoidal (Fig. 2.8).



**Fig. 2.8** Concentration-response curves of cytostatic compounds **A)** MAOBA, **B)** MAOEA, **C)** MDL73811 and **D)** DFMO using indirect (HRPII ELISA and LDH assay) versus direct (FACS) parasitaemia quantitation assays. The 95% confidence intervals around the best-fit curves are indicated, except for A1.

This was especially true for the concentration-response curves obtained for MAOBA and MAOEA from the two colorimetric assays and their resulting  $IC_{50}$ s (Table 2.1) varied significantly across replicates with large 95% confidence intervals, whereas the curves compiled from the FACS data were usually truly sigmoidal and the  $IC_{50}$ s had acceptable variation. The graphical 95% confidence interval of the MAOBA HRPII ELISA data could not be calculated by the software (Fig. 2.8A1). The concentration-response curve compiled from the LDH



assay data of MDL73811 was also not truly sigmoidal, but HRPII ELISA data were not generated due to the unavailability of the assay at the time when the drug was obtained.

It should be kept in mind that the software algorithm attempts to force the data into a sigmoidal shape, but that the data points should ideally be distributed uniformly on the curve to be regarded as truly sigmoidal. Since the three assays were performed on exactly the same samples, comparable results were expected. The IC<sub>50</sub>s for DFMO across the different assays varied between 400 and 730 μM, which is in the same order as the published value of 1.3 mM for chloroquine-sensitive strains of *P. falciparum* [90, 153]. The values for MDL73811 varied between 0.58 and 0.80 μM, which is comparable with the published IC<sub>50</sub> of 1 μM for chloroquine-sensitive parasites. No published values are available for MAOBA and MAOEA, since these have not previously been tested in Plasmodia.

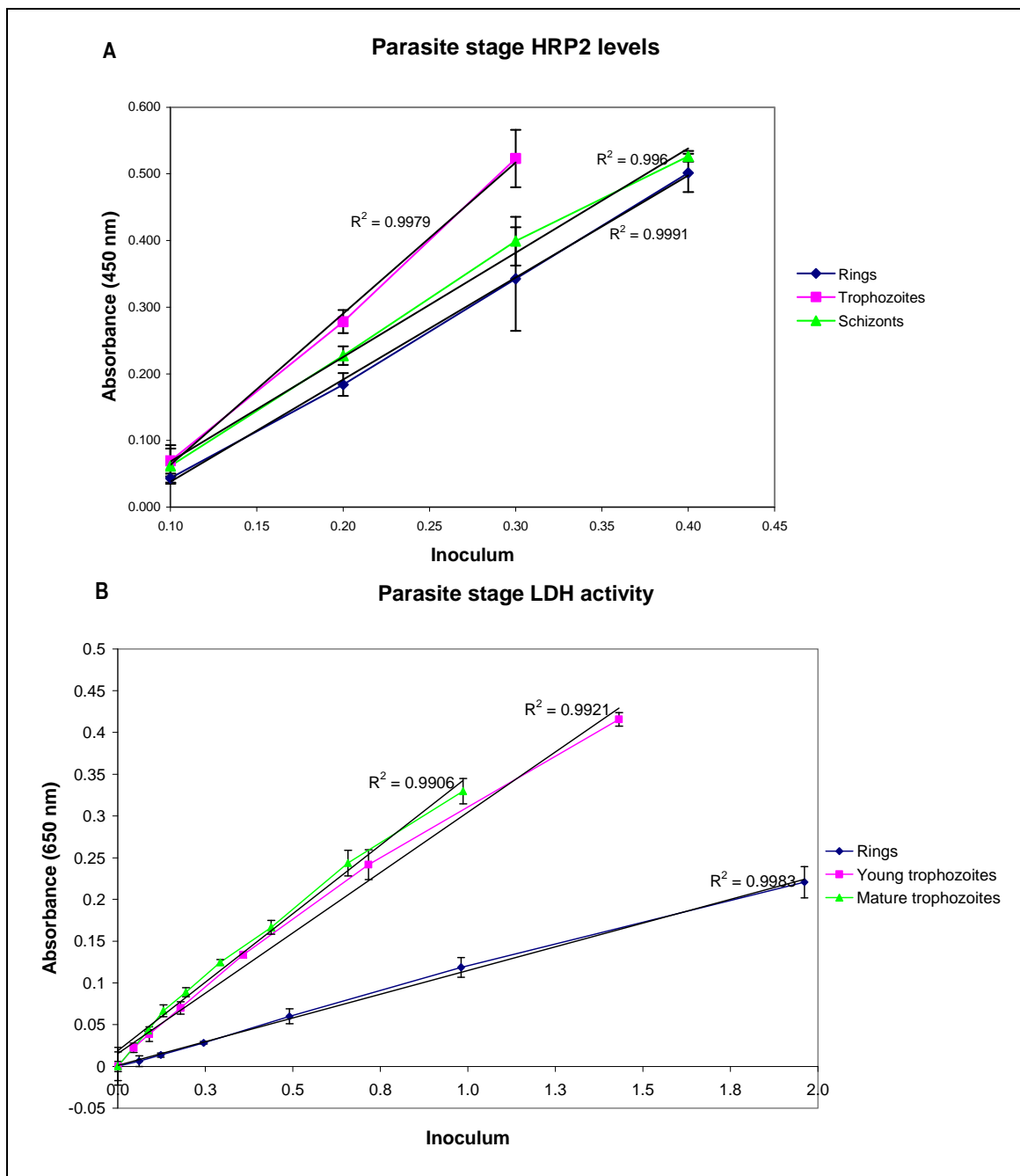
**Table 2.1 IC<sub>50</sub> values obtained with three different drug sensitivity assays**

Compound	IC <sub>50</sub> values (μM) with 95% confidence interval		
	HRPII ELISA (Indirect method)	LDH (Indirect method)	FACS (Direct method)
Chloroquine	0.028 (0.026 to 0.031)	0.028 (0.024 to 0.033)	0.034 (0.032 to 0.036)
MAOEA	18.83 (12.22 to 29.02)	433.5 (5.667 to >1000)	156.3 (122.6 to 199.3)
MAOBA	3.635 (1.899 to 6.955)	11.6 (5.226 to 25.77)	6.228 (5.323 to 7.286)
MDL73811	-	0.577 (0.391 to 0.852)	0.798 (0.690 to 0.923)
DFMO	400.6 (331.9 to 483.5)	729.6 (633.7 to 840.1)	526.4 (461.8 to 600.0)

The inconsistencies obtained for some of the cytostatic drugs with the colorimetric assays versus FACS compared to the generally reliable data of chloroquine were subsequently proposed to be due to the difference in mechanism and stage of action of the cytotoxic chloroquine compared to the cytostatic compounds. Chloroquine kills parasites rapidly in the ring stage and when parasitaemias of drug treated parasites and untreated controls are compared 48 h later to calculate the percentage response, the parasites that survived have developed into rings again and their HRPII content or LDH activities are compared to those of the ring controls. However, the cytostatic drugs cause parasite growth arrest in the trophozoite stage [153, 154] and depending on the concentration (within the series of drug dilutions), alter the synchronisation of the parasite population due to arrest of the affected parasites compared to the normal progression of the unaffected parasites. In order to calculate the IC<sub>50</sub> after treatment for 48 h, varying degrees of asynchronous trophozoites (arrested) and subsequent ring stage parasites are compared with the untreated, synchronised ring controls (Eq. 2.1). This is not problematic when the parasites are physically counted (microscopy or FACS), but indirect quantitation of parasitaemia based on metabolism (HRPII content and LDH activity), will lead to data

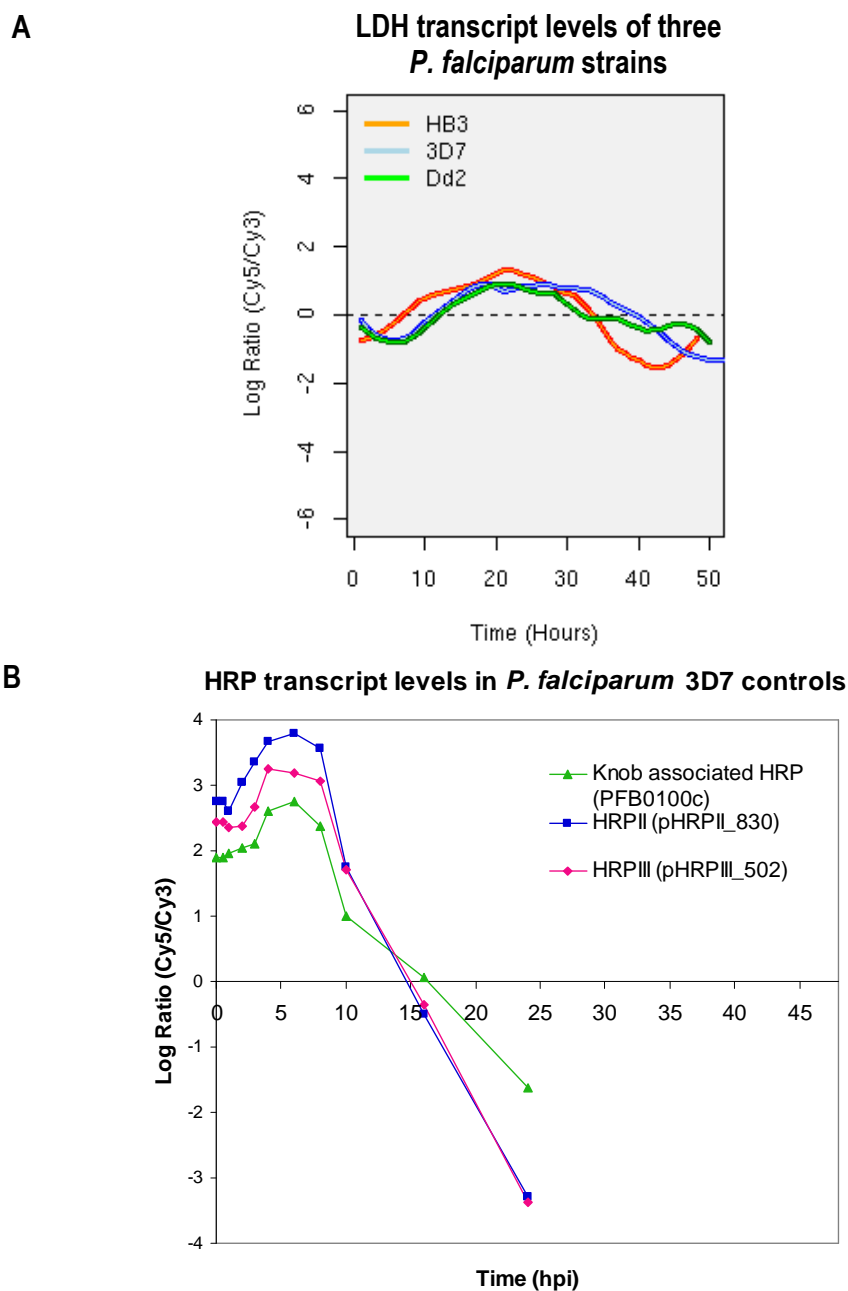
inconsistencies and reproducibility problems. This is due to the fact that parasite metabolism is not equal across the IDC, but reaches a maximum in the trophozoite stage [29, 89].

To prove this hypothesis, the HRP2 content and LDH activity across different (synchronised) parasite stages were investigated. As demonstrated earlier, both the HRP2 content and LDH activity were linear with the parasite inoculum, but the slope of these straight lines was very different depending on the parasite stage (Fig. 2.9A, B). These results confirm the conundrum entered into when comparing metabolic activities across different parasite stages after growth arrest of the treated parasites versus normal progression of untreated controls.



**Fig. 2.9** Parasite stage-specific levels of **A)** HRP2 as detected by HRP2 ELISA assay and **B)** LDH activity according to the LDH assay.

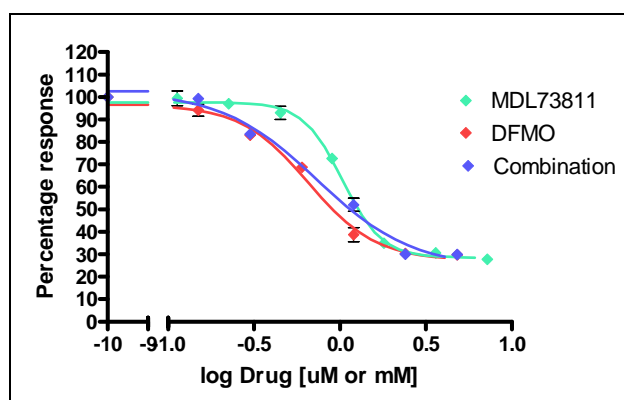
The LDH enzyme activity increased with development from rings to young and subsequently mature trophozoites, whereas the HRP<sub>II</sub> content of the trophozoites was the highest and the contents of rings and schizonts were very similar. However, LDH transcripts peak at 18 h post-invasion (hpi) according to the 3D7 IDC transcriptome (Fig. 2.10A, <http://malaria.ucsf.edu>) [91] and the proteins have delayed translation as described in section 4.3.1.2. HRP<sub>II</sub> transcripts peak at 6 hpi (Fig 2.10B). The transcript half lives and translation times of the histidine-rich proteins are not known, but HRP<sub>II</sub> was previously reported to be mainly secreted during the second half of the IDC with a marked rise during schizont development and rupture [180]. This is in contrast with the results presented here and peculiar considering the time of peak expression (6 hpi) and just-in-time transcriptional mechanism of *P. falciparum* [29].



**Fig. 2.10** Transcript levels of **A)** LDH of strains HB3, 3D7 and Dd2 during the complete IDC (<http://malaria.ucsf.edu>) [91] and **B)** the three histidine-rich proteins of 3D7 parasites during the first 24 h of the IDC (M. Llinás, unpublished data).

### 2.3.3 DFMO and MDL73811 interaction during PfAdoMetDC/ODC co-inhibition

The IC<sub>50</sub>s of DFMO and MDL73811 were determined as 0.53 mM and 0.8 μM (from FACS data) for the particular laboratory conditions as described in section 2.3.2. However, the effect of the drug combination during PfAdoMetDC/ODC co-inhibition could be additive (1+1=2), synergistic (1+1>2) or antagonistic (1+1<2). Synergistic or antagonistic interactions would have necessitated dose alterations for treatment with the combination. To investigate this, the fixed-ratio method of investigating drug interaction [181] was applied whereby a dilution series of DFMO and MDL73811 was prepared in a fixed-ratio combination of their IC<sub>50</sub>s (0.53 mM versus 0.8 μM). The highest concentration of the combination series contained eight times the IC<sub>50</sub> of both the drugs, which was then two-fold diluted until 0.0625 times the IC<sub>50</sub>s. A 2-fold dilution series starting from 16 times the IC<sub>50</sub>s of the individual compounds was also prepared and assayed in parallel. The concentration-response curves obtained for DFMO, MDL73811 and the combination are presented in Fig. 2.11.



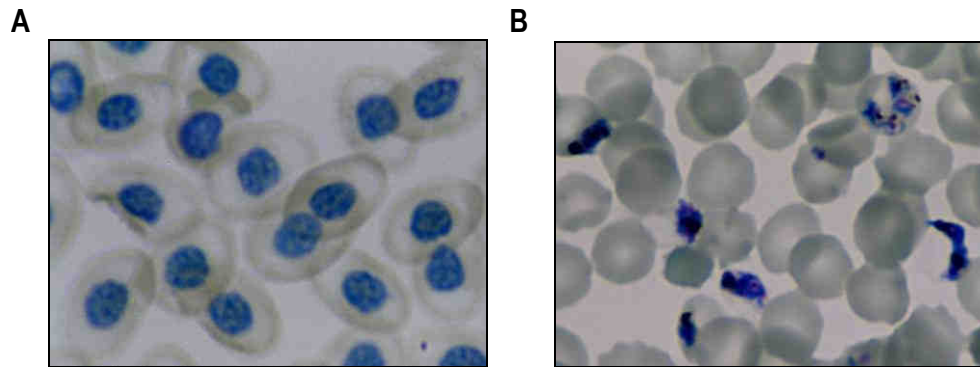
**Fig. 2.11** Concentration-response curves of MDL73811 (concentration in μM), DFMO (concentration in mM) and the combination compiled with GraphPad Prism v.4 from FACS data.

The curves compiled for the individual drug treatments correlates well with those of the combination of DFMO and MDL73811, which means that the total effect of the combination is the sum of that of the individual treatments i.e. additivity (Fig. 2.11). This corresponds with previous reports [153, 154]. Antagonism would have caused the position of the combination curve to shift towards the right of the MDL73811 and DFMO curves and with synergism it would have shifted towards the left.

### 2.3.4 PI membrane integrity assay of PfAdoMetDC/ODC co-inhibition

Both DFMO and MDL73811 cause growth arrest of *P. falciparum* in the trophozoite stage due to polyamine depletion, but the effects are cytostatic [154]. However, to ensure that dosages as high as five times the IC<sub>50</sub> of both drugs in combination remained cytostatic for use in the subsequent functional genomics investigations (Chapters 3 and 4), parasites were incubated with PI following drug treatment [178]. This DNA-binding probe does not pass through intact membranes and nuclear staining thus indicates membrane permeabilisation [177], which is an indirect measure of chemical cytotoxicity [178]. In contrast with the FACS procedure for drug

susceptibility testing (section 2.2.3.3.1), which differentiated between the uninfected and different stages of parasitised erythrocytes (Fig. 2.6), the PI assay was aimed to assess membrane damage and the FACS instrument had to be recalibrated to detect PI penetration and fluorescence. To test the procedure over a wide range of parasitaemias, chicken erythrocytes, which are nucleated as opposed to human erythrocytes, were saponin-lysed and used as positive control to simulate membrane-damaged, parasitised erythrocytes (Fig. 2.12).



**Fig. 2.12** Giemsa-stained thin smears of **A)** chicken erythrocytes, which contain nuclei and **B)** 3D7 *P. falciparum*-infected human erythrocytes.

In the absence of saponin, the chicken erythrocytes were not detected, but saponin treatment allowed PI penetration and nuclear fluorescence, resulting in a 96.2% “parasitaemia” (Table 2.2.). However, anuclear human erythrocytes were not detected despite saponin treatment. Similarly, parasitised erythrocyte controls were not detected without saponin treatment. The procedure was subsequently applied to assess erythrocyte membrane integrity after treatment with the combination of 5 mM DFMO and 5  $\mu$ M MDL73811, but parasites could not be detected above the background, indicating that PI did not penetrate and that the membranes of parasitised erythrocytes remained intact despite the high dosages used for treatment (Table 2.2).

**Table 2.2** PI assay and FACS analysis of PfAdoMetDC/ODC co-inhibited *P. falciparum*

Sample	% Parasitaemia
Chicken erythrocytes + PI	0.03
Chicken erythrocytes + saponin + PI	96.2
Human erythrocytes + saponin + PI	0
4-5% <i>P. falciparum</i> trophozoite controls + PI	0.25
4- 5% <i>P. falciparum</i> trophozoite controls + saponin + PI	4.74
5 mM DFMO-treated <i>P. falciparum</i> + PI	0.09%
5 $\mu$ M MDL73811-treated <i>P. falciparum</i> + PI	0.10%
DFMO/MDL73811 combination + PI	0.08%

## 2.4 DISCUSSION

In this chapter, three AdoMetDC inhibitors were considered for use in combination with DFMO in functional genomics investigations of PfAdoMetDC/ODC co-inhibited *P. falciparum*. The IC<sub>50</sub>s of these cytostatic drugs had to be determined and in the process three high-throughput drug sensitivity methods (HRPII ELISA, LDH

assay and FACS) were evaluated to determine the most appropriate method for testing cytostatic compounds. HRPII levels and LDH activity are closely associated with parasite density and development [12], whereas FACS analysis physically counts the number of infected erythrocytes based on their DNA contents and fluorescence [175]. Of the three methods tested, the HRPII ELISA assay was the most sensitive and could detect inocula as low as 0.025 (e.g. 0.005% parasitaemia, 5% haematocrit), but the assay capacity was easily overloaded with antigen and sample dilution was required.

The accuracy of drug sensitivity assays such as these three are critical in resistance-surveillance and drug discovery programmes. These assays measure the proliferation or growth of drug treated parasites relative to a drug-free control. Since variables such as parasite inoculum (haematocrit and parasitaemia) and the stage-dependent action of the drugs tested can influence the outcome of the assays, it is important to control these factors (including parasite stage) when the assays are performed [12]. However, cytostatic drugs, such as the polyamine biosynthesis inhibitors, cause growth arrest in the trophozoite stage [153, 154], whereas the untreated controls progress as usual according to the IDC. Thus, despite starting with a synchronised culture, the cytostatic action of these compounds will cause arrest of a proportion of the parasites and normal progression of the balance, resulting in varying degrees of synchronisation depending on the drug concentration within the dilution series. When stage-independent, direct counting methods such as microscopy or FACS analysis are used to measure parasitaemia, these cytostatic effects on synchronisation have no influence. However, when indirect measures of parasitaemia based on metabolic activity (HRPII content or LDH activity) are applied, inconsistent and variable results may be obtained due to the stage-dependence of these metabolism-based assays. This was especially true for MAOEA and MAOBA. It is well established that parasite metabolism is not equal across the IDC, but reaches a maximum in the trophozoite stage [89]. Therefore, drug discovery efforts employing only methods based on indirect assessment of parasitaemia may obtain inaccurate results and potentially miss novel compounds with cytostatic modes of action, which is far from ideal at a time when every potential antimalarial should be considered. Cytostatic drugs are known to have therapeutic potential in parasitic diseases and DFMO is currently the safer, clinically used alternative in the treatment of West African trypanosomiasis [78]. It is, therefore, suggested that more than one method (direct and indirect measures of parasitaemia) be used in parallel for drug screening to avoid missing potential hits.

Previously,  $IC_{50}$ s for DFMO, MDL73811 and various 3-aminoxyl-1-aminopropane derivatives [90, 153, 154] were determined with the tritium-labelled hypoxanthine incorporation assay, which monitors DNA synthesis as an indication of parasite growth [165]. Drug treatment is usually performed in the early ring-stage for 24 h, after which  $^3H$ -hypoxanthine is added for 18 h [165]. The assay therefore also measures parasitaemia indirectly based on DNA synthesis, but the assay is terminated at ~42 hpi, i.e. before schizogony. For these cytostatic drug treatments, DNA synthesis of untreated schizont controls would be compared to varying degrees of

arrested trophozoites and schizonts, which may have similar DNA metabolic activities considering that DNA metabolism peaks during the late trophozoite/early schizont stages [29]. Therefore, this assay did not give reason to doubt the methodology used for any of the cytostatic compounds [90, 153, 154], but if performed over 48 h and schizogony occurred, similar problems could have been experienced as with the colorimetric assays used in this investigation.

Different levels of synchronisation and parasite stage are well-known problems in susceptibility surveillance for *P. vivax*, which is predominantly asynchronous compared to *P. falciparum* isolates that are mostly synchronous. Recent *P. vivax* surveillance studies of various drugs in Papua Indonesia with the schizont maturation test demonstrated that *in vitro* susceptibility was correlated to the initial parasite stage and isolates that were predominantly in the trophozoite stage had a 2-fold higher IC<sub>50</sub> than those predominantly in the ring stage [182]. Furthermore, the surveillance studies also indicated a correlation between the *in vitro* susceptibility and the duration of the assays [182]. Since the HRPII ELISA, LDH assay and FACS were performed in parallel on the same samples in this study, the standard 48 h incubation period used for most drug sensitivity assays was used. However, longer incubation times are often recommended to test slow-acting drugs, e.g. pyrimethamine/sulphadoxine [12], that primarily exert their effects in the schizont stage [183]. Longer incubation times, such as 72 h, may theoretically also solve the conundrum faced in the case of the polyamine biosynthesis inhibitors, since the arrested trophozoites would then be compared with controls that progressed to the subsequent trophozoite stage and not the ring stage as with 48 h incubation, resulting in comparable metabolic measurements (incl. HRPII and LDH levels). It is currently not clear why the results obtained with the colorimetric analyses of MAOEA and MAOBA were so highly variable, whereas the IC<sub>50</sub>s of DFMO and MDL73811, determined with the same assays, were relatively acceptable.

Due to the inconsistent results obtained for MAOEA and MAOBA, and better characterisation of ML73811 in the literature [153, 157, 184, 185], the latter drug was selected as the AdoMetDC-inhibitor of choice to be used in the functional genomics investigations. The combination of 5 mM DFMO and 5 µM MDL73811 was shown to be additive and to remain cytostatic at these high dosages. However, the inclusion of MAOEA and MAOBA in the analysis revealed opposite activities in *P. falciparum* compared to what was previously reported for *T. brucei brucei* [159]. MAOEA had an IC<sub>50</sub> of 1.3 µM in *T. brucei brucei* and analogues with longer side chains had reduced potency [159]. However, the results in *P. falciparum* showed that MAOBA with its 4-carbon-aminoxy side chain (Fig. 2.1) was 25 times more active (FACS IC<sub>50</sub> = 6.2 µM) than MAOEA with its 2-carbon-aminoxy chain (FACS IC<sub>50</sub> = 156.3 µM, Fig. 2.1). Apart from potential pharmacokinetic differences of these compounds in *Plasmodium*, the contradictory results between the two species could indicate structural differences in the active site of the *P. falciparum* versus the *T. brucei brucei* AdoMetDC. To investigate this possibility, docking studies of MAOEA and MAOBA in the active site of AdoMetDC were performed with a three-dimensional model of the AdoMetDC domain of PfAdoMetDC/ODC [186] (results not shown). These

studies revealed 15 potential conformations for MAOBA on the plasmodial active site compared to only one for MAOEA, which indicated a significantly higher probability of the first being in the correct conformation to inhibit the enzyme than with MAOEA.

In conclusion, the cytostatic mechanism of polyamine biosynthesis inhibitors resulting in growth arrest of the treated cells and normal progression of the 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 a multistage organism such as *P. falciparum*, which constantly develops during the IDC (Fig. 1.2) such that growth arrest compared to normal progression will result in significant differences merely due to stage. This should be kept in mind with any comparative analysis of such an organism treated with these compounds, including drug sensitivity assays. In addition, the same principle also applies to the relative quantitation of RNA, proteins and/or metabolites as is required in functional genomics investigations, hence the relative  $t_0$  strategy applied in the following chapters.