Development and evaluation of a novel and innovative drug sensitivity assay for *Plasmodium falciparum*

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Abstract

Background: Malaria is still the most relevant human parasitic infection in the world. Antimalarial resistance is what most hampers malaria control; sensitivity testing is hence crucial for resistance detection and further establishment of public health guidelines for malaria treatment. There are several sensitivity tests for *Plasmodium falciparum* – the deadliest human malaria species – but no official global guidelines. Haemozoin is a crystal with birefringent and paramagnetic properties, produced by the *Plasmodium* parasite while it is developing inside the host red blood cells. This study aimed at the evaluation of a new magneto-optical method, which makes use of the detection of haemozoin, to test the sensitivity of *Plasmodium* to antimalarials.

Methods: The amount of haemozoin in samples of *P. falciparum* 3D7 or Dd2 incubated with dihydroartemisinin (DHA), piperaquine (PQ), chloroquine (CQ), and pyrimethamine (PYR) was measured over time; growth curves were produced and 50% inhibitory concentrations calculated. Several confirmation assays were performed with DHA, PQ, and CQ. Subsequent tests were performed, to investigate what introduced most variability to the results.

Results and discussion: Parasite maturation and inhibition effects were easily observed; there was some variability among replicates, and the variability tests showed this was probably due to the protocol of the drug assays.

Conclusion: Although the assay protocol needs improvement in order to obtain less variable results, this method has great potential for sensitivity testing – even field-wise.

Keywords: malaria, antimalarial resistance, sensitivity tests, magneto-optical test

Background

Malaria has been noted since ancient history: it is believed to be the main cause of death among primates previous to *Homo sapiens*. But this disease is still the most relevant human parasitic infection in the world, being a public health concern to this day. [1-5]

According to the World Health Organization (WHO), there were 214 million worldwide last year – almost 90% of them in the African region. [6]

Malaria is caused by the protozoan *Plasmodium*, which is transmitted by the bite of a female mosquito from the genus *Anopheles*. The infection in humans is caused by one or more of the species *P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale* and *P. knowlesi*, the first being the cause for almost all malaria deaths and severe disease. [1,2,4,9]

During the blood stage of its life cycle, the parasite produces a dark pigment called haemozoin, in its vacuole.[1,4,8,9,10] This pigment is not exclusive to malaria as other parasites – such as *Haemoproteus*, *Schistosoma*, and *Rhodnius* – also synthesize haemozoin. [7,12] Reports of this pigment are even more ancient than the study of malaria itself, since it has been used to detect the disease for a very long time. [1,4,8,10]

The parasite digests most of the host's haemoglobin and this proteolysis releases reduced haem and globin; while globin is digested by enzymes, the reduced haem is extremely reactive and thus readily oxidized to its ferric form haematin, with consequent production of H_2O_2, a potentially very toxic molecule. [8,10,11,14,15-17,20-22]

Since haem can cause the disruption of biological membranes, inhibit enzymatic processes, and initiate the chain of oxidative metabolism it might also be potentially toxic to the parasite. As malaria parasites lack haem oxygenase (an enzyme which catalyses haem degradation in all vertebrates), they are unable to cleave haem and thus it is not excreted from the cell. [8-11,13,14]

To prevent its accumulation and bypass this toxicity, the parasite must maintain it as an insoluble compound and thus readily transforms free haem into haemozoin via a process of biocrystallisation. [8-11,13-14]

The presence of haemozoin in a malaria parasite marks the evolution of a young-trophozoite (ring-form) with almost no pigment to a densely pigmented schizont. This means the amount of pigment inside a parasite increases with its development within the host's red blood cell. [8-11,13,19-22]

Therefore, haemozoin might be utilised as an indirect maturation indicator for the parasite itself. This means when haemozoin is being detected for purposes of studying parasite development in certain conditions, parasite maturation might be assessed as well, in order to study the parasite's susceptibility to certain antimalarial drugs. [8-11,18-22]

Malaria treatment has been a fast-developing subject; not very long ago, the standard treatment was 4-aminoquinolines and sulfa compounds and less than 20 years ago, these started to fail due to emergent resistance. *P. falciparum* has now developed resistance to almost all classes of clinically used antimalarial drugs. [1,23,24,26]

In the past few years, resistance to the new first-line treatment, the artemisinins, has been a popular concern, since the parasites have shown to clear slower from the blood of patients.

Despite any debate on whether malaria parasites are acquiring resistance to the current first-line treatment, antimalarial drug resistance is a major public health concern. It prevents malaria control by enabling perpetuation of transmission, besides increasing morbidity and mortality due to malaria infections. [26]

It is hence of high importance to detect this resistance beforehand as to prevent the further spread of malaria and consequent increased mortality. For that, knowledge of the parasite's susceptibility to currently used antimalarial drugs is essential in deciding appropriate treatment and establishing adequate therapeutic guidelines.

Sensitivity tests are used to assess the susceptibility of parasites to antimalarials. The currently available *in vitro* sensitivity assays can be divided into genotypic and phenotypic; the latter are grouped according to the method used to assess parasite growth – direct visual parasite count by microscopy, incorporation of radioisotopes or non-radioactive methods. The latter includes assays with a flow cytometer, fluorometric assays, and ELISA-based methods. [24,26-28]

All these approaches evaluate intrinsic sensitivity of malaria parasites by directly exposing them to drugs in culture plates and measuring their effect on the growth and development of the parasites; this allows direct and quantitative evaluation of actual resistance of the parasite to the drug.

In spite of the great amount of sensitivity assays for *P. falciparum*, there are still no guidelines for antimalarials sensitivity testing nor is there an official reference procedure; this makes it necessary to explore new methodologies feasible in any situation to draw inhibition profiles for existing antimalarials and to screen antimalarial candidates.

Recently, there has been an increasing number of new techniques, using the malarial pigment as the target material, being the magneto-optical detection one of them. The idea to take advantage of the unique magnetic properties of the malarial pigment and to use it as an alternative target in optical diagnosis has been proposed by several groups.

When suspended in a fluid, the long axes of the haemozoin crystals randomly orientate, while when applying a magnetic field to said fluid, the crystals orientate along the applied field direction. [30]

When the haemozoin-containing fluid is interrogated with polarized light it expresses preferred direction of optical absorption, producing an optical modulation signal directly proportional to crystal concentration – and this can be used to differentiate a haemozoin-containing sample from one which did not.

Taking this into consideration, Butykai *et al.* (2013) developed a new magneto-optical test (MOT). Magnetically-induced linear birefringence/dichroism and polarization-dependent light scattering are referred to as a whole as magnetically-induced linear dichroism (MLD), which is what the MO instrument measures.

In the used configuration, a laser beam passes through a polariser and probes the sample, which will be under the influence of a uniform magnetic field. [31]

To establish the usefulness of the MOT for field trials and its further application in a diagnostic setup, Orbán *et al.* (2014) conducted a study where the aim was to address issues such as the difference in haemozoin suspensions and true infected blood samples and how these behave in the MOT setup.

The authors evaluated the MOT performance using synchronized laboratory cultures of P. falciparum and investigated the limit of detection in samples with low levels of parasitaemia.

Preliminary results showed the MOT could provide an efficient tool to assess sensibility of *Plasmodium* parasites to novel or clinically relevant antimalarial drugs by detecting haemozoin as an indirect indicator of parasite development. However, further development and optimisation should be conducted in order to judge the value of this method. [32]

Methods

Culture maintenance and synchronization, Giemsa staining, and SYBR Green I staining were made as in Rebelo *et al.* (2013).

The used lysis solution consisted of 20 mM NaOH and 0.063% Triton in distilled water. Both reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Dihydroartemisinin (DHA), piperaquine (PQ), chloroquine (CQ), and pyrimethamine (PYR) were the tested drugs (see Table 1). DHA, CQ and PYR were purchased from Sigma-Aldrich (St. Louis, MO, USA), and PQ was kindly given by Sigma-tau (Pomezia, Italy). In order to have the correct final concentration in the mixture culture and drugs, drugs had to be prepared twice as concentrated, since they were going to be diluted by half. Intermediate and working solutions were all prepared in MCM.

Table 1 – Prepared solutions of the studied antimalarials.

Drug	Stock solution (solvent)	Intermediate solution (µM)	Working solutions (nM)
DHA	1.758 mM (DMSO)	10	0.24, 1, 4, 16, 64 (growth-curve assay); 8, 16, 32, 64, 128 (confirmation assays)
PQ	1 mM (Milli-Q water)	1	12, 36, 108, 324, 972
CQ	77.5 µM (distilled water)	1	12, 25, 50, 100, 200
PYR	1 mM (absolute ethanol)	10	25, 50, 100, 200, 400

DHA: dihydroartemisinin; PQ: piperaquine; CQ: chloroquine; PYR: pyrimethamine; DMSO: dimethyl sulfoxide.

Antimalarial drugs sensitivity testing with the Magneto-Optical Test (MOT)

The assays were divided into two sets: the first consisted in assessing the growth of the *P. falciparum* strains over 48 hours, in order to determine the best time-point for IC50 calculation. Negative controls were uninfected samples, and positive controls were drug-free samples. Infected samples were prepared from continuous synchronized cultures of either *P. falciparum* 3D7 or Dd2, of which parasitaemia and haematocrit were adjusted to 1% and 5%, respectively. The samples were prepared in 96-well plates in triplicate and each well contained a final volume of 160 µL.

The second set of assays was performed with the goal of assessing the robustness of the MO method and the reproducibility of its results. This consisted of repetition assays performed with DHA, PQ, and CQ with the 3D7 strain. Sample preparation followed the same procedure as before.

The MLD signal of all samples was measured (0-hour timepoint) and the plates were incubated at 37ºC. For the assays of DHA, PQ, and CQ, samples were measured again at 6, 8, 10, 14, 20, 24, 30, 36, and 48 hours of incubation; the PYR assay followed until 96 hours of incubation, with extra timepoints at 60, 72, 84, and 96 hours of incubation.

To analyse the samples with the MO instrument, each well content was lysed and transferred to sample holders, prepared specifically for the MOT; the MLD signal was measured, and converted into numerical values and observed in a computer software, also prepared specifically for the MO instrument.

In the second set of assays, sample measurement was only at the beginning of the assay and 24 hours after incubation, but followed the same procedure as before.

The resulting data were analysed with the software Microsoft Office Excel (in the case of the time-curves) and GraphPad Prism (for repetition assays), where the mean of the measurements, together with the standard deviation, was calculated and used as the value for each triplicate set. IC50 values were calculated through a nonlinear regression model with SigmaPlot - Systat Software (Chicago, IL, USA).

Variability tests

Several additional tests were carried, in order to test which steps of the previous procedure would add more variability to the resulting data. Samples were always prepared in triplicated and measured with the MO instrument at the beginning of every assay and again 24 hours later; subsequent data was analysed as before.

Synthetic and native haemozoin, previously prepares by others, were used for these variability tests. With these tests, the goal was to test whether lysing the samples would produce different results than non-lysed ones; whether the outcome of an assay would be different if samples contained PBS instead of MCM; and if RBC had a significant influence on the MLD signal of a sample, whether the samples was in PBS or in MCM.

To understand if the regularly used lysis solution had a detrimental influence on the outcome, a new lysis solution was prepared, which consisted of Triton 0.063 % in distilled water, without NaOH. A running unsynchronized culture of *P. falciparum* NF54 was used, without adjusting the parasitaemia.

Results

The MOT discerns between an uninfected sample and an infected one, even at the beginning of each assay, since the first always presents MLD values under 1, while the latter presents much higher MLD values.

For all the tested antimalarials, the MOT could assess drug effects, according to the different drug concentrations and, at the 24-hour timepoint, it was already possible to distinguish two groups of samples; one consisting of the two higher concentrations of drug and the other comprising the three lower concentrations (see Figure 1). This timepoint was hence chosen for further confirmation assays.

For the pyrimethamine assay, this is only visible at the 60-hour timepoint, as this is a slow-acting drug; for the assay with chloroquine with the Dd2 strain, all samples grew as a drug-free sample, expected, since this strain is chloroquine-resistant. Since these were not further tested, these data are not shown.

Figure 2 shows the obtained results from the confirmation assays of dihydroartemisinin (DHA), piperaquine (PQ), and chloroquine (CQ). There was some variability among replicates of the same drug, whether at the 0-hour timepoint – despite the standardised initial conditions – or at the end of the assays – meaning the inhibition effects were not the same in every replicate with the same drug, which reflects in the diverse IC50 (data not shown).

Nonetheless, inhibition effects are visible on all assays. Chloroquine was the most consistent drug in terms of inhibition effects and subsequent IC50, with the smaller IC50 range (25-41 nM), while PQ originated the most variable results, with IC50 ranging from 18 nM to 62 nM.

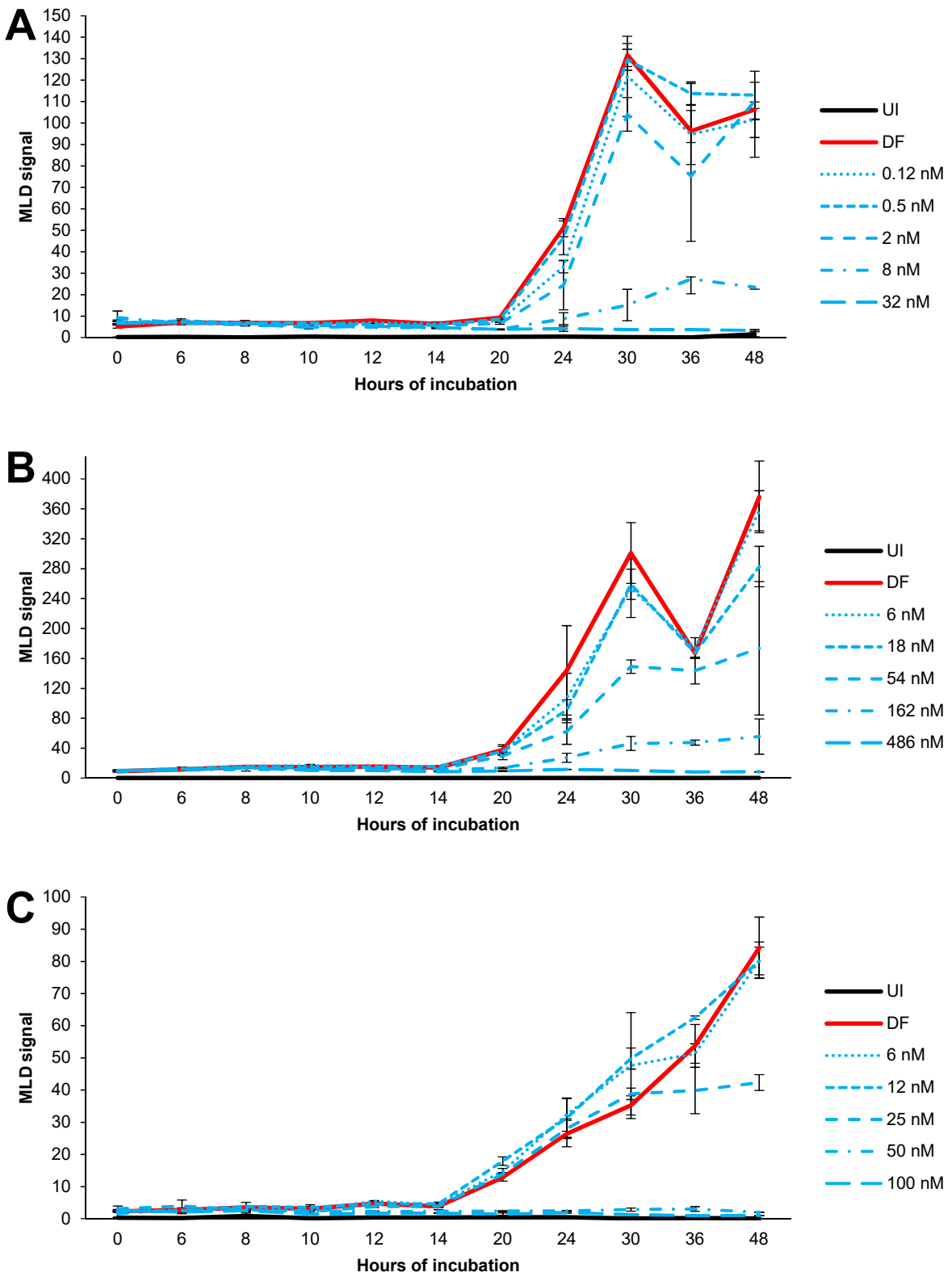

Figure 1 – Growth curve of *P. falciparum* 3D7 in the presence of several concentrations of dihydroartemisinin (A), piperaquine (B), and chloroquine (C). The means and standard deviations of triplicates are represented. The negative control was an uninfected (UI) sample and the positive control was a drug-free (DF) sample.

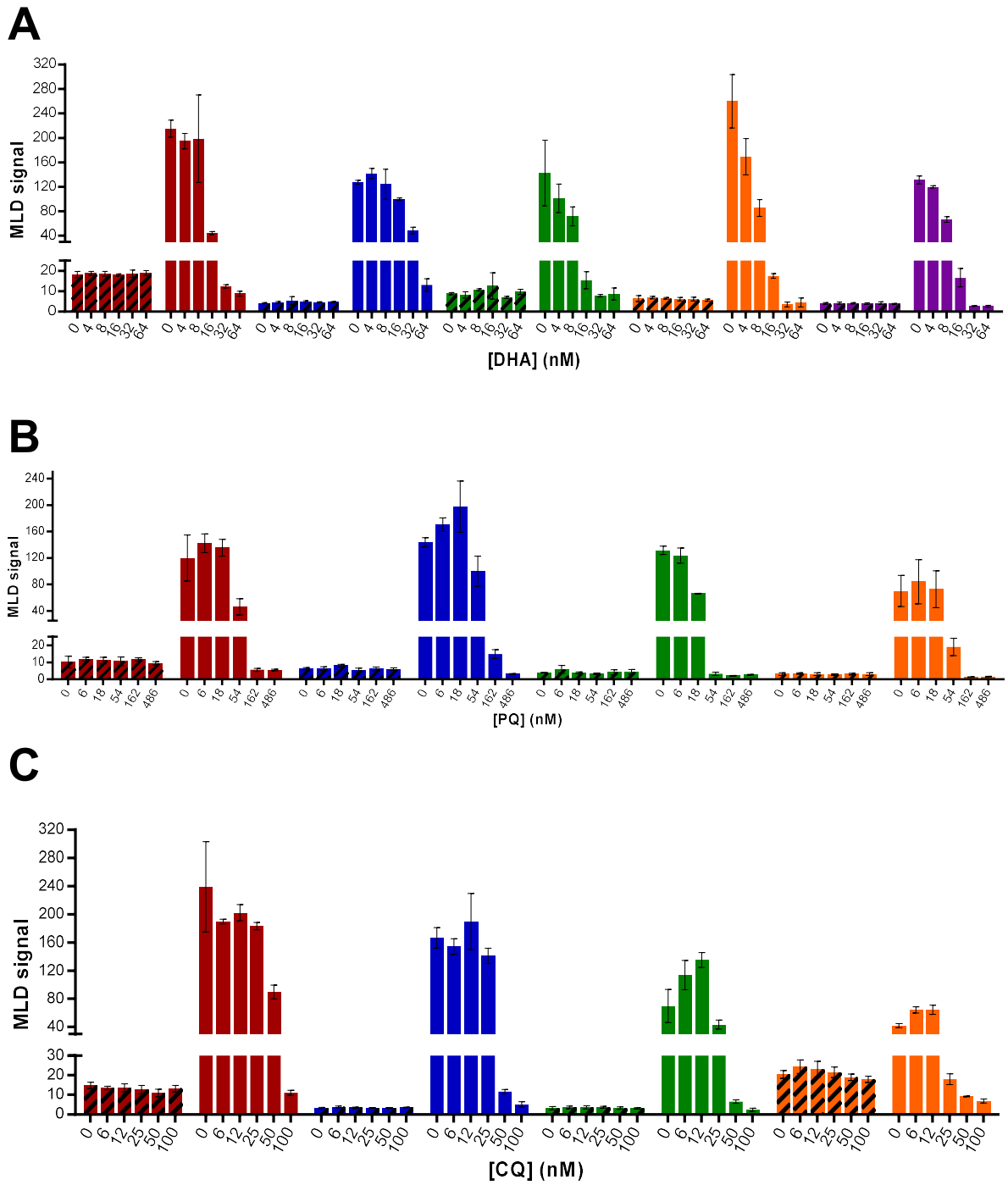

Figure 2 – Repetition assays of the sensibility of *P. falciparum* to (A) dihydroartemisinin (DHA), (B) piperaquine (PQ), and (C) chloroquine (CQ). The means and standard deviations of triplicates are represented. The first replicate is represented by the red bars, the second by the blue, the third by the green, the fourth by the orange, and the fifth and last (only in the case of DHA) by the purple bars. Striped bars represent the 0-hour timepoints of each assay, while solid bars represent the 24-hour timepoint.

Discussion

By performing the first drug assays with Plasmodium falciparum cultures and obtaining the respective growth curves, it was confirmed this method indeed detects maturation of the parasites. Since the assays start with parasites in the initial forms of the blood stage development (ring forms), at 24 hours of incubation, the parasites are schizonts and hence full with haemozoin. This haemozoin will be liberated into the culture well and merozoites will invade new red blood cells and produce more haemozoin until the next 24 hours of incubation.

Therefore, the exponential increase of signal – indirect indicator of the amount of haemozoin – until 48 hours of incubation was expected. Consequently, after 24 hours of incubation, it is already possible to see growth in all drugs, except for pyrimethamine, and to easily distinguish a grown sample from a non-grown sample. In fact, with piperaquine and chloroquine – which, being quinolones, interact with haemozoin production [9,33,36,37-39] –, growth is already seen at 20 hours of incubation.

For pyrimethamine, growth is observable at 14 hours and clear distinction from inhibited samples is possible at the 60-hour timepoint; for chloroquine with the P. falciparum Dd2 strain, since none of the drug concentrations influence parasite growth, growth is observed already at the 14-hour timepoint.

Since the purpose was to further evaluate this method as a sensitivity test for Plasmodium, the 24-hour timepoint was chosen for subsequent confirmation assays, since this is where the inhibition effects start to diverge.

This MO method promised to overcome some of the mentioned drawbacks for current sensitivity tests, while still being an effective tool in assessing inhibition effects of known antimalarials. Orbán and colleagues' (2014) preliminary data indicated it might be an efficient tool to assess the amount of hemozoin produced by the different parasite stages in synchronized cultures; therefore, it could eventually be used for testing the susceptibility of Plasmodium parasites to antimalarial drugs. [32]

With this study, it was possible to verify the MOT indeed detects inhibition effects of some of the most commonly used antimalarials nowadays. These effects were, for the most part, easily assessed and showed a correlation between drug concentrations and inhibitory effects. However, they did not occur the same way in all confirmation assays with the same drug; in other words, it seemed as the same concentration of a drug did not behave the same way in all replicate assays.

This variability was reflected on the diversity of IC50 obtained for the same antimalarials – for instance, the IC50 for DHA raged from 2 nM to 26. This variability could be due to the MOT or to the drug assay itself. The fact at the beginning of each replicate assay, all samples have somewhat the same signal, indicates the observed variability might me due to the whole process before the MOT measurements – since the difference is between 0-hour timepoint of replicates and not between samples of the same replicate.

Ring and early trophozoites are thought to convert about 3-5% and 15-20% of host haemoglobin, respectively, and schizonts about 50–70%; [20,22] there is hence no precise haemoglobin digestion and consequent haemozoin production rate, so these could vary along these ranges within the same population of parasites. This variation would implicate a wide variation on haemozoin amount in each well – which would mean triplicates of the same sample would behave differently. Despite the initial conditions being standardized, the initial MLD values were never the same for correspondent samples. Since at each timepoint a different microplate was measured – meaning there were not the same parasites being assessed at different timepoints –, the different proteolysis and subsequent haemozoin production rates would translate in such different results, where similar amounts of haemozoin should be produced, theoretically.

Additionally, the assays where the initial MLD values were higher were not necessarily those where the final MLD values were also the higher. Thus, this seems to be the most likely reason for most of the obtained variability. Therefore, reproducibility of the MOT does not seem questionable at this point. Nevertheless, being just a prototype, the instrument might also have introduced some variability in the assays' results.

Since this prototype consisted of several optical components screwed to a steady platform, these components got progressively loosened due to extensive use; this would turn the instrument increasingly unstable, which would obviously introduce variability while measuring the same sample. Even if the components were screwed back, this replacement was obviously not completely accurate and hence the photodetector could possibly detect differences in MLD signal.

The protocol of the sensitivity assays might also not be optimised to obtain the best results possible. Basco (2007) summarised several parameters which could influence the outcome of a sensitivity assay, some intrinsic to in vitro sensitivity assays. These range from the use of Albumax in the composition of MCM, going through the fact different solvents were used for different drugs, to the tendency of some drugs to bind to specific types of materials. [28]

Desjardins et al. (1979) recommended preparation of stock solutions in 70% ethanol and further dilutions in RPMI 1640 with HEPES, NaHCO3 and 10% human plasma/serum; [29] this would standardise this parameter and eliminate subsequent variability. However, each antimalarial has its own optimal solubility and requires an appropriate solvent; despite all working solutions being prepared in MCM, each stock solution was prepared with a different solvent.

Lell, Binh & Kremsner (2000) studied the effects of alcohol on malaria parasite growth; the authors concluded the presence of ethanol inhibited parasite growth by 20-30% during a 48-hour incubation. When fresh drug dilutions containing DMSO or ethanol – which was the case for DHA and PYR, respectively – the final concentrations of these solvents should hence not overcome 0.1%, in order to avoid toxic effects on the parasites which will obviously alter growth-effects. [34]

Most of the used materials in the drug assays were plastic and some drugs highly adsorb in plastic materials; since the final drug concentrations are in the nanomolar range, this could lead to extremely inconsistent results and therefore increased estimated IC50. According to Basco (2007), chloroquine is the only used drug not to bind to plastics at the assays' temperatures.

According to Wein et al. (2010), reliability of antimalarial sensitivity tests also depends on drug mechanisms of action. [35] This means a sensitivity test based on haemozoin detection might assess inhibition effects of haemozoin-affecting antimalarials, such as chloroquine, more reliably; this, together with the fact that chloroquine does not bind to plastics, could explain why the results obtained for this antimalarial were less variable than the other used drugs.

Chloroquine was, indeed, the antimalarial which produced the most stable results and, consequently, IC50. IC50 calculated from flow cytometry values and found in the literature were very similar to those obtained from MOT values, unlike what happened with other drugs.

Lastly, Orbán et al. (2014) believed freshly lysed blood samples would have an improved detection limit with the MOT, and the performance of the method was limited by a residual MLD signal due to some part of the lysed cell suspension – since the used samples were kept frozen and only thawed at the time of measurements.

In fact, the variability test performed to assess the influence of red blood cells in the signal of a sample (data not shown), showed samples with no red blood cells had a much lower signal than their counterparts with red blood cells – although they all contained the same amount of synthetic haemozoin –, indicating the presence of red blood cells disguises the true lower signal derived from the present haemozoin.

Overall, with this method, the researcher is able to assess inhibition effects of some common antimalarials on a Plasmodium falciparum strain, in a laboratory context, seemingly producing reproducible results.

The whole process of the sensitivity assays produced a high amount of waste, mainly plastic. Since each timepoint was assessed with a different microplate, and each sample was prepared in triplicate and then each triplicate transferred to a microtube, the amount of plastic waste produced was massive. This is obviously not feasible field-wise, since the amount of available material is not as much as in a laboratory in a developed country.

The process was also very time-consuming; it took a considerable amount of time between taking the plate from the incubator, transferring wells' content to the microtubes for sample lysing, transferring this to the sample holders, performing the measurements, and cleaning the materials. This would cause some samples to be in contact with the lysis solution for more time than others, until measurement. The different lysing times could also introduce a confounding factor to the end result, since the lysis solution seems to have a detrimental effect over time in the signal obtained – as it was observed in the variability tests.

Additionally, electrical power was used for the rotation of the magnet and batteries for the laser; this would not be feasible in malaria-endemic countries, where electric power is not available at all times. if this method was to be used in malaria-endemic countries in the future, this would also have to be optimised for field-use.

Overall, the assay process is not very practical, besides being laborious; nonetheless, the used protocol could be optimised in order to facilitate the process and subsequent measurement of a great number of samples. The amount of plastic material used should be decreased by cutting steps in the protocol and directly transfer the content of each well to the sample holders. These should also be developed to a more practical form, by automated filling with samples, instead of the manual filling by the handler.

Conclusion

There are currently some sensitivity assays available for P. falciparum; although some are suitable to inform the authorities of inhibition effects in the context of creating public health guidelines and antimalarial drug development, each has its inherent disadvantages. The radioisotopic assay with [3H]-hypoxanthine and the WHO microtest are the most widely accepted methods for sensitivity testing and subsequent IC50 determination.

Although the radioactive assay has become the global reference sensitivity test in developed countries, it cannot be the reference method for most malaria-endemic countries; thus, there is the need for standard non-radioactive sensitivity tests which are feasible even field-wise in malaria-endemic countries.

On the other hand, the WHO microtest is field-applicable and the required incubation period is of at least 24 hours. However, it is also labour-intensive and has the need for highly trained personnel, which still does not eliminate subjectivity issues; again, in malaria-endemic countries, highly trained personnel are scarce. Additionally, the WHO has recently discontinued the production of this sensitivity test – although it is still used.

Although molecular techniques are increasingly gaining recognition in the sensitivity tests field, and more

molecular markers for drug resistance are being discovered, in vitro sensitivity testing is still crucial for drug screening and for the study of drug resistance of currently used antimalarials.

This Magneto-Optical Test has thus great potential for the use in malaria parasites detection for sensitivity testing, since it can in fact differentiate a non-infected sample from an infected one, and among infected between different amounts of haemozoin; however, it is still far from being at its optimal form.

Therefore, this method is still not feasible field-wise; nevertheless, when the instrument is optimised into a more portable version and the process of drug assays is further developed into a more practical – and less waste-producing protocol –, this method has the potential to be the new reference sensitivity test, since it would be automated, fast, and inexpensive.

Aknowledgements

To the Department of Physics of the Budapest University of Technology and Economics and the MTA-BME Lendület Magneto-optical Spectroscopy Research Group, especially István Kézsmárki, for lending the MOT instrument, and Ágnes Orbán, for all the technical support throughout the project.

To Instituto de Medicina Molecular, especially to MRamirez Lab and the Flow Cytometry Unit, for all the help given, and materials and equipment provided.

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