Publishable Executive Summary

Worldwide activities to fight malaria have been massively extended during recent years. Nevertheless, the disease remains devastating on society and economy in developing countries. Our consortium focussed on the question whether a novel approach to attack the parasite from the outside rather than the intracellular compartment provides a better way to overcome rapid resistance development. Scientific background and objectives have not changed during the project.

By analysing genome data from *P. falciparum*, the coordinator of MalariaPorin has identified a single water/glycerol channel (aquaglyceroporin, PfAQP). PfAQP is the only member of the aquaporin family encoded in the *P. falciparum* genome. Immunolabeling showed its presence at the parasite/host interface. The PfAQP protein belongs to the major facilitator super family for nutrients and metabolites and is a bi-functional pore with high permeability for water and glycerol. The fact, that the aquaglyceroporin is a component of the reduced interface strongly suggests basic functions in the parasite biochemistry. The channel may play important roles in three essential cellular processes:

Protection against osmotic stress. During the process of transmission between the *Anopheles* mosquito and a human host changes in the osmotic environment are likely to occur. Later, in the host's blood stream, the parasite encounters drastic osmotic gradients during kidney passages where serum osmolarity can rise 4-fold compared to normal serum.

Access to the serum glycerol pool as a precursor for lipid synthesis. Malaria parasites grow rapidly. From a single parasite, which has invaded an erythrocyte in the merozoite stage, up to 32 new merozoites can be derived within a 48 h replication cycle. This calls for high rates of lipid synthesis due to the massive demand for plasma membrane extension. Using tracer studies, it has been shown that the parasite efficiently uses glycerol from the host serum for its glycerolipid backbone. The PfAQP channel may provide critical access to this pool.

Glycerol uptake and oxidation to cope with oxidative stress. An alternative glycerol source for lipid synthesis is from glucose metabolism (glycolysis). Glucose-to-glycerol conversion, however, shifts the $NADH/NAD^+$ balance towards the oxidised form, which may contribute to cell damaging oxidative stress in the parasite. Glycerol uptake through PfAQP for lipid synthesis and as a substrate for oxidation to replenish NADH may thus be beneficial for the parasite.

In fact, novel physiological data that we obtained in the course of MalariaPorin confirmed several of the above assumptions.

PfAQP complies with important requirements for being used as a drug target for chemotherapy due to the likely involvement in basic systems of the parasite and to unique functional and structural properties. To address the question of suitability we have assembled a highly interdisciplinary consortium:

Partner 1 (University of Kiel, Germany) is headed by Eric Beitz who also coordinates the project. One post-doc and two students are involved in the project. The team has long-standing expertise in the biochemical characterisation of aquaporins, incl. PfAQP, which has been identified by the team. The team provides assay systems for compound testing based on *Xenopus* oocytes and yeast.

Partner 2 (University of Göteborg, Sweden) is headed by Stefan Hohmann and involves three senior scientists plus PhD students. This group establishes yeast deletion strains for use in

glycerol permeability assays. Techniques are developed and applied for the production and purification of PfAQP in the 10 mg range. Crystallisation conditions for PfAQP are being obtained from these efforts.

Partner 3 (University of Edinburgh, United Kingdom) left the consortium due to a move of the principal investigator Sabine Flitsch to Manchester, see new partner 6.

Partner 4 (Max-Planck Institut für Biophysikalische Physik, Göttingen, Germany) is headed by Helmut Grubmüller and Bert de Groot. Three senior scientists, one post-doc and one PhD student are involved. The team has a long-term expertise in molecular dynamics simulations. The group was the first to simulate water passage through aquaglyceroporins and was also the first to address the question of proton exclusion in the aquaglyceroporin family. They provide simulations and modelling of the PfAQP aquaglyceroporin and design of potential inhibitors.

Partner 5 (Johns-Hopkins University, Baltimore, USA) is headed by Peter Agre and Nirbhay Kumar and involves a post-doctoral researcher. All contributed work is funded by own resources. The team has discovered the aquaporins and has developed major techniques for studying the function, structure, cell biology and physiology of aquaporins. The group has a strong background in haematology, physiology and parasitology. *Plasmodium* aquaporin gene knockouts are generated and physiologically analysed. The gene deletion technology will be transferred to a European researcher.

Partner 6 (University of Manchester, United Kingdom) headed by Sabine Flitsch, involves three post-doctoral researchers and is equipped with state-of-the-art techniques for parallel chemical synthesis. The group has in-depth know-how on the design of compound libraries and is situated in a research environment that focuses on the interaction of small molecules with proteins. This experience has been successfully employed before for the generation of blockers of AQP1 and AQP2.

Partner 7 (University of Tübingen, Germany) is headed by Jürgen Kun whose team has long-standing expertise in the in studying effects of drugs on *P. falciparum* parasites. The team provides assay systems for compound testing based on malaria parasites and determines PfAQP polymorphisms from *Plasmodium* field isolates and lab strains. The coordinator, Eric Beitz, split from the team to start an independent group at the university of Kiel, see partner 1.

The consortium has worked on an ambitious and innovative program that wants to answer the question whether PfAQP is a suitable drug target in plasmodia. To approach this question the following objectives were addressed:

Objective 1. *To assess the physiological role of PfAQP in cell and animal models.* A PbAQP knockout strain was generated and thoroughly analysed. A *P. falciparum* aquaglyceroporin *knockout* strain is at the clone selection stage. Comparison of ^{14}C labeled glycerol uptake in wild type and PbAQP knockout parasites confirms that PbAQP is the main pathway for glycerol

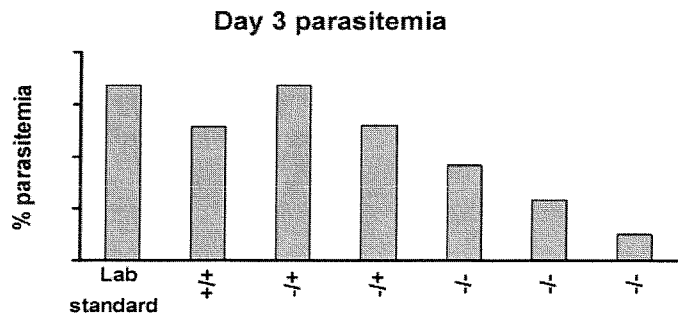

Fig. 1: Parasite growth in red blood cells that are homozygous (+/+), heterozygous (+/–) for the aquaglyeroporin or lack it completely (–/–).

uptake. PbAQP is expressed in many insect stages of *P. berghei* (ookinete, oocyst). However, the parasite development within the mosquito is not affected by the deletion of PbAQP. The PbAQP knockout sporozoites were found to be infectious to mice. *P. berghei* and *P. falciparum* survival was studied in blood that lacks the red cell aquaglyceroporin. The results indicate that red cell aquaglyceroporins contribute to *P. berghei* survival (Fig. 1).

Objective 2. *To establish test systems suitable for assessing the function and specificity of potential blockers of PfAQP.* The three assay systems based on *Xenopus laevis* oocytes, yeast, and *P. falciparum* parasites that have been established in the first funding period were optimized for clear read-out and higher throughput. A new yeast based system in 96-well format was set up (Fig. 2). Around 170 candidate compounds have already been tested in this assay. Indeed, we identified more than 10 compounds with an inhibitory effect on PfAQP permeability with IC_{50} values, in the high micro-molar range suggesting that the noted parasite growth inhibition is due to inhibition of an additional target. The yeast assay complements the *P. falciparum* culture based test system. Already 200 compounds have been evaluated in the *P. falciparum* test system, more than 50 have an antiproliferative effect on different strains of *P. falciparum*; the best compounds inhibit parasite growth in the single-digit nano-molar range at reasonable toxicity for mammalian cells. In addition to the workprogramme we established an assay for testing the effect of compounds on *Leishmania braziliensis* parasites and found inhibiting compounds in our library.

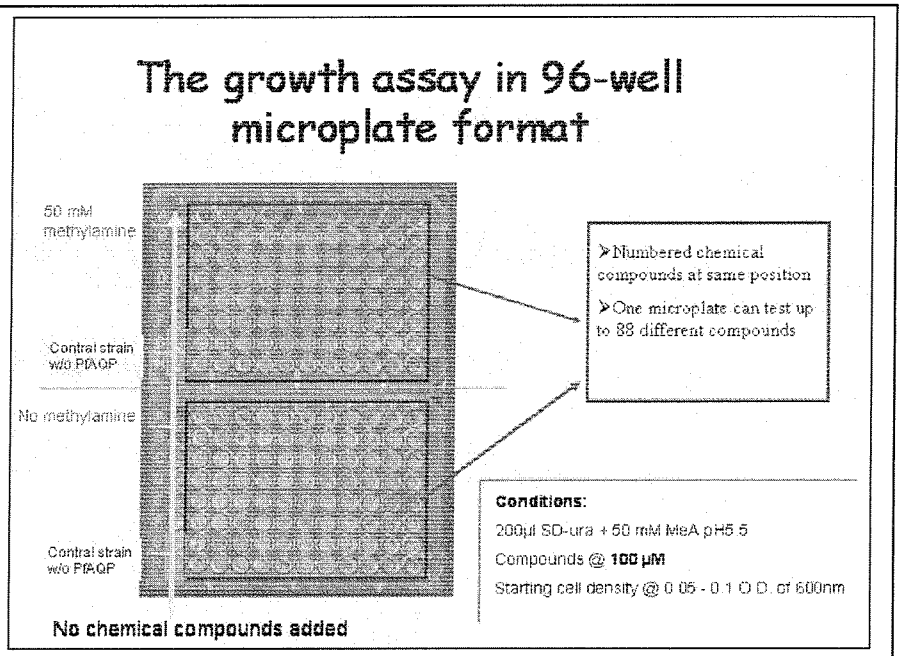

Fig. 2: Yeast based high-throughput screening for potential chemical inhibitors of PfAQP.

Objective 3. *To determine the occurrence and functional consequences of natural polymorphisms of the PfAQP gene.* This part of the project was basically finished in the first funding period. Hence, we did not follow-up on the results. In summary, 65 genomic DNA samples of *P. falciparum* were obtained from laboratory strains and from natural isolates from patients (Lambaréné, Gabon). Two rather conservative mutations were found, which due to their localisation do not suggest involvement in pore selection. The PfAQP gene shows surprisingly high conservation even in the somewhat variable connecting loops. This may render PfAQP less prone to resistance development in the future.

Objective 4. *To determine the 3D structure of PfAQP.* High-level PfAQP protein expression in *Pichia pastoris* yeast and protein purification have been successfully established.

Crystallisation yielded diffracting crystals in various shapes. However, the structural resolution remained above a 6 Å threshold. We are determined to elucidate the structure. Therefore, crystal optimisation is further being carried out after the end of the project funding time.

Objective 5. *To generate lead compounds suitable for development of a highly specific and effective blocker of PfAQP.* Using tetraethylammonium (TEA) as a lead compound, an extensive characterisation of the binding pocket of this known inhibitor was achieved in the structure of aquaporin-1 using atomistic molecular dynamics simulations (Fig. 3). Inhibitor binding was found to take place on the extracellular face of the channel, involving interactions with mainly loops C and E, and, surprisingly, loop A of the neighbouring monomer. The mode of action was confirmed experimentally by site-directed mutagenesis, in which designed mutants lost TEA sensitivity. The identification of key interactions allowed the specific design of two novel inhibitors that were recently confirmed in a cell-based assay as much more potent inhibitors than TEA.

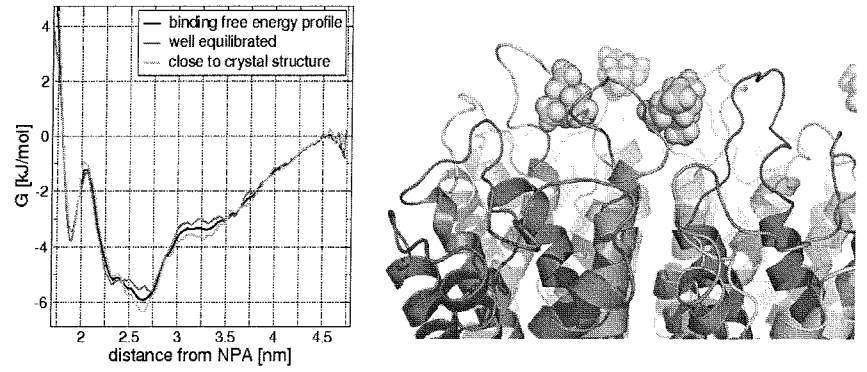

Fig. 3. TEA binding to aquaporin-1.

The major achievements can be summarised as that MalariaPorin

1. enhanced knowledge about the physiological role of aquaglyceroporins in plasmodia,
2. identified aquaporin protein structures that are involved in inhibitor binding,
3. optimised the throughput of assay systems for testing potential aquaporin blockers, and
4. created novel lead compounds for antimalarial chemotherapy.

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