The oligomeric protein interference assay method for validation of antimalarial targets

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SUMMARY AND FUTURE PERSPECTIVES
6.1. SUMMARY

Possessing a good repertoire of manipulative tools in assessing the role of a gene's product is a must in drug discovery. The antimalarial validation toolbox is currently filled with a number of genetic and proteomic-based tools that can report on a protein's biological activity. However, knock out/in and knock down systems are often highly complex or difficult to perform, time-consuming, too costly or are simply not effective for essential genes of the parasite. Moreover, small molecule inhibitor-based tools are frequently non-specific and do not always yield the desired efficacy partly owing to the complex nature of the parasite's life cycle. These and many other challenges found in the current toolset highlight the need for novel specific validation techniques. An alternative and highly specific biological activity validation tool could be represented by the oligomeric Protein Interference Assay (PIA). By disrupting oligomeric interfaces using mutant subunits of the target enzymes, we have clearly demonstrated a significant reduction in specific enzyme activity in vitro and in whole cell assays. Moreover, we demonstrated a phenotypic effect of the introduction of these mutant subunits within the parasite, validating the aspartate metabolism from \textit{P. falciparum} as a druggable pathway without recourse to complex genetic approaches.

In Chapter 1, the reader was introduced to the currently used validation methods in malaria. As disclosed in this chapter, despite the particular advantages of the genetic techniques, the overall unlikelihood of these methods to be applied to essential genes represent a significant drawback for their use as validation methods. In this scenario, the conditional and inducible validation tools are of great value. These methods indeed represent a better alternative for validation of essential genes/proteins, but neither of them is applicable to all classes of proteins or all species of \textit{Plasmodium} or possess full efficacy in disturbing/degrading the target. With the proteomic approaches, a significant advantage is the ability to assess targets with different functional isoforms with further post-translational variations. But non-specificity for their target of interest, rapid metabolism, poor cell membrane passage and subcellular location after successful entry are still significant barriers. In this scenario, we introduce the concept of the Protein Interference Assay, a highly specific method to control biological activity by disrupting oligomeric interfaces using mutant subunits of the target protein.

In Chapter 2, we describe the \textit{in vitro} application of PIA to two enzymes from the aspartate metabolism pathway of \textit{P. falciparum}: aspartate aminotransferase (\textit{Pf}AspAT) and malate dehydrogenase (\textit{Pf}MDH). Here we reported the use of the structural information of \textit{Pf}MDH to generate a mutant protein capable of disrupting the native tetrameric con-
formation of this enzyme and thus reducing its catalytic activity. Structural information of PfAspAT was also used to generate a double mutant capable of reducing its catalytic activity, but not disrupting its native dimeric structure. Both AspAT and MDH mutants were able to form a complex with their respective wild-type forms. We also demonstrated that these heterocomplexes are inactive, revealing the potential of these mutants for application of PIA using transfected parasites.

Chapter 3 follows up the work with AspAT and MDH. Parasites were transfected with the mutant forms of these enzymes and their expression was confirmed by qRT-PCR and western blot. Whole-cell assays revealed decreased specific activity of AspAT and MDH compared to control cultures. Most importantly, when cultivated in aspartate-limited media (to mimic biological conditions), parasites transfected with both mutants presented a significant growth defect compared to control or single transfected parasites. These results suggested that, although inhibition of AspAT or MDH alone is not sufficient to hamper parasites’ growth, future drug targets to treat malaria infection may be found within downstream components of the aspartate metabolism pathway. Moreover, these data provided initial evidence that distortion of oligomeric interfaces can be used to specifically influence protein behaviour in vivo.

In Chapter 4 we report the initial PIA development targeting the enzyme aspartate transcarbamoylase from Plasmodium falciparum (PfATC). In this chapter, we reported the structure of the wild-type enzyme and the use of the structural information in mutagenic studies. The crystallographic analysis revealed a trimeric structure organization with the active sites formed across the oligomeric interfaces. Each active site comprises the residues from two adjacent subunits in the trimer with a high degree of evolutionary conservation, allowing for the design of a double mutant that could potentially form a complex with wild-type subunits, similarly to our mutant AspAT. The reduced catalytic activity demonstrated for the double mutant established the basis for the PIA validation of PfATC using transfected parasites.

Chapter 5 was focused on the PIA analysis of PfATC. Here we reported the crystallographic structure of the double mutant that, combined with static light scattering experiments, confirmed that the mutations have no impact on the overall folding or oligomeric state of this enzyme. Transfection of parasites with the mutant gene caused a significant delay in growth compared to control culture. The double transfection with mutants ATC and AspAT caused a significantly stronger effect in proliferation, reinforcing the importance of aspartate for the parasite’s survivability and strengthening the potential of PIA as a powerful addition to the current antimalarial validation toolbox.
6.2. Future Perspectives

The results presented in this thesis provide the proof-of-concept for the validation of other oligomeric essential enzymes of Plasmodium parasites. It is important to remark that the PIA approach does not represent a replacement for other validation techniques applied to malaria. As other methods, PIA possesses limitations, the most noticeable being the inability to provide a complete knockout effect. Instead of being used as a single definitive method, PIA can be combined with other methods to provide a more complete understanding of the assessed target. PIA also represents a powerful method to validate metabolic pathways instead of single targets. When different components are assessed in parallel, like demonstrated for AspAT/MDH and ATC/AspAT, the negative effect in parasites’ growth is much more pronounced.

An important addition to the setup of PIA experiments could be represented by the use of bioinformatic tools. Molecular modelling could potentially suppress the need for crystallographic information for some targets. Furthermore, these tools could potentially be used in the development of a pipeline able to identify residues that, if mutated, would cause the desired effect.

Our report on the PIA approach is limited to three enzymes, mainly involved in two different pathways of P. falciparum. But the successful assessment of these targets represents a much broader potential for this technique. In principle, the two main requirements for the application of PIA to a specific target are structure information and oligomerization. The constant addition of crystallographic structures to the protein data bank added to the fact that more than 60% of these structures are reported in an oligomeric state different than monomeric support the hypothesis that the PIA approach can be applied not only to more metabolic pathway within P. falciparum but also extrapolated to other infectious diseases.