ANEXO 7

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3D structure and immunogenicity of *Plasmodium falciparum* sporozoite induced associated protein peptides as components of fully-protective anti-malarial vaccine

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ABSTRACT

SIAP-1 and SIAP-2 are proteins which are implicated in early events involving *Plasmodium falciparum* infection of the *Anopheles* mosquito vector and the human host. High affinity HeLa and HepG2 cell binding conserved peptides have been previously identified in these proteins, i.e. SIAP-1 34893 (^{421}KVQGLSYLLRRKNGTKHPVY^{440}) and SIAP-1 34899 (^{541}YVLNSKLLNSRSFDKFKWIQ^{560}) and SIAP-2 36879 (^{181}LLLYSTNSEDNLDISFGELQ^{200}). When amino acid sequences have been properly modified (replacements shown in bold) they have induced high antibody titres against sporozoites in *Aotus* monkeys (assessed by IFA) and in the corresponding recombinant proteins (determined by ELISA and Western blot). ^{1}H NMR studies of these conserved native and modified high activity binding peptides (HABPs) revealed that all had α-helical structures in different locations and lengths. Conserved and corresponding modified HABPs displayed different lengths between the residues fitting into MHCII molecule pockets 1–9 and different amino acid orientation based on their different HLA-DRβ1* binding motifs and binding registers, suggesting that such modifications were associated with making them immunogenic. The results suggested that these modified HAPBs could be potential targets for inclusion as components of a fully-effective, minimal sub-unit based, multi-epitope, and multistage anti-malarial vaccine.

1. Introduction

The *Plasmodium falciparum* apicomplexan parasite is one of the five causative agents of human malaria, being responsible for more than 200 million cases and 1 million deaths per year worldwide, mainly in children aged less than five [1]. Multiple control strategies to fight this deadly disease must be thus be developed, among them a fully-protective anti-malarial vaccine.

During an infected mosquito's blood meal during which sporozoites are injected into the vertebrate host's skin and, after multiple parasite protein receptor-ligand interactions with epithelial, endothelial, macrophage-derived and liver cells, sporozoites arrive at the hepatocytes (hepatic intracellular stage) [2,3]. All sporozoite invasion-mediating proteins are excellent targets for inclusion as components of a fully-effective anti-malarial vaccine as the first line of defence, prior to red blood cells becoming infected by merozoites as the second line of defence. The circumsporozoite protein (CSP), a surface protein, and the micronemal localised and membrane translocated thrombospondin-related anonymous protein (TRAP) are among the sporozoite-specific invasion-related proteins most studied to date [3,4].

Siau et al. [5] have recently analysed the sporozoite's transcriptoma, identifying a new set of molecules as promising vaccine candidates. They described two new sporozoite-invasion associated proteins involved in hepatocyte invasion SIAP-1 and SIAP-2, being up-regulated at 37 °C and having 113 kDa and 45 kDa molecular masses, respectively [5].

These proteins are encoded by a single-exon gene and the sporozoite-expressed products display a predicted signal peptide, indicating a probable translocation to the sporozoites's surface. A functional multi-stage role for SIAP-1 has been described recently involving the targeted disruption of the *siap-1* gene, having a marked effect on sporozoite migration to the *Anopheles* mosquito's salivary gland as well as on sporozoite infection of mammalian host cells [6]. Immunofluorescence assays (IFA) have shown that such micronemal proteins are also located on sporozoite surface, following a similar pattern to that observed for CSP [5]. Western-blot (WB) analysis has shown that SIAP-1 suffers temperature-dependent proteolytic cleavage into a 75-kDa molecule at

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37 °C [5], similar to apical membrane protein antigen-1 (AMA-1), CSP and TRAP processing.

A highly robust and sensitive methodology has been used for identifying minimal subunits (or peptides) from proteins involved in epithelial cell transversal (HeLa cells) and binding to hepatoma-derived HepG-2 cells to facilitate invasion of primary human hepatocytes isolated from human liver fragments [7]. Our group has previously identified twelve HepG-2 high-activity binding peptides (HABPs) and 17 HeLa HABPs in SIAP-1, eight of them binding to both cell types, while four HepG-2 HABPs and two HeLa HABPs have been identified in SIAP-2, only one of them binding to both cell lines. The immunological response induced in *Aotus* monkeys by some of these SIAP-1- and SIAP-2-derived HABPs and their respective modified analogues and their 3D structure has been determined by ^{1}H NMR spectroscopy. The presence of HLA-DRβ1* molecule binding motifs and registers led to searching for correlation between their immunological response and 3D structure to develop a logical and rational methodology for vaccine development and include them as components of a fully-protective, multi-antigen, multistage, minimal subunit-based, and chemically-synthesised *P. falciparum* anti-malarial vaccine.

2. Materials and methods

2.1. Synthetic peptides

Previously identified HABPs and their analogues (named according to our laboratory's serial numbering system) were selected from SIAP-1 and SIAP-2 binding profiles. Conserved SIAP-1 HABPs 34893 (^{421}K**V**QG**L**SY**L**LRRKNG**T**KHPVY^{440}) and 34899 (^{541}Y**V**L**N**SKL**L**NSR**S**FDKFKWIQ^{560}) and SIAP-2 36879 (^{181}L**L**LYST**NSE**DNLDISFGELQ^{200}) bound to both cell types. Please note that modified HABP numbers are written in bold and amino acids which were replaced in sequences are also shown in bold throughout this text. The aforementioned native HABPs, their respectively modified analogues **38162** (K**T**QG**H**SY**H**LRRKNG**V**KHPVY), **38166** (YVLNSKL**H**NSRSFDKFKWIQ) and **38156** (LL**H**YST**ISQ**DNLDISFGELQ).

Peptides used to immunise BALB/c mice to determine these proteins' immunogenicity were recognised using specific B-cell epitope BcePred and BCPREDS prediction servers [7]. Non-HABP conserved peptide 37873 (^{488}VSKELKEKWIQIGQWYKELPL^{507}, present in recombinant fragment II) was used for SIAP-1.

Mixture A consisting of N-terminus non-HABP conserved peptides 37877 (^{61}SPSEVPMLIDDEEEYEILEG^{80}) and 37878 (^{101}TMIDLD NIITEISKLKKKKL^{120}) and mixture B containing C-terminus non-HABP conserved peptides 37879 (^{281}LYNLKDSSDNQEEYYELLAG^{300}) and 37880 (^{341}VSKKEELFFYFISLLKNNF^{360}) were used for SIAP-2, all four sequences being present in the SIAP-2 recombinant fragment.

Non-HABPs, native HABPs, their modified analogues and corresponding polymers were synthesised by the multiple-solid-phase system using the tert-butoxycarbonyl (t-Boc) strategy [8,9]. Monomers were purified by RP-HPLC and their purity assessed by analytical RP-HPLC and mass spectrometry (MALDI-TOF). Peptide polymers were produced for immunisation purposes, as previously described [10–12].

2.2. Recombinant protein production

The gene sequence encoding putative proteins in the *P. falciparum* 3D7 strain was used for designing primers to produce a recombinant fragment from SIAP-1 (PlasmoDB accession number: PFD0425w) and SIAP-2 (PlasmoDB accession number: PF08_0005). The rSIAP-1 fragment included HABPs 34893 and 34899 from amino acids 323–699 (Fig. 1A) where forward primer was (5′-ATGCATGCTTT TGAATATTCTAA-3′) and reverse primer (5′-ATATAAATAAAAATGG AAGGA-3′); *P. falciparum* 3D7 strain genomic DNA was used for amplifying the fragment. SIAP-2 primers were (forward 5′-ATGT ACTTATCTTCCGAAAACC-3′ and reverse: 5′- TTTTTTAGATATCATA GCTTC-3′); the ensuing sequence covered amino acids 22–398 and *P. falciparum* FCB2 strain genomic DNA was used as template for amplification.

PCR were carried out with the Platinum Pfx DNA polymerase enzyme (Invitrogen, California, USA). PCR products were purified using a Wizard PCR preps kit (Promega, Wisconsin, USA) and cloned in pEXP5-CT/TOPO vector (Invitrogen, California, USA). Recombinant plasmids were purified using an UltraClean mini plasmid prep purification kit (MO BIO Laboratory, Solana Beach, California) and the integrity of five clones per target protein, obtained from five independent PCRs each, was corroborated in an ABI PRISM 310 automatic genetic analyser (PE Applied Biosystems, California, USA). Sequencing results from the FCB2 and 3D7 strains were compared to the reference strain by nucleotide and amino acid alignments using ClustalW [13]. The pEXP5-CT/TOPO vector containing the *P. falciparum* SIAP-1 or SIAP-2 genes added a six-histidine tag to each protein's C-terminus to facilitate its immune-detection through anti-histidine monoclonal antibodies and later purification by solid-phase affinity chromatography using a Ni^{2+}–NTA agarose column (Qiagen, CA), according to the manufacturer's recommendations. Fractions where each pure protein was observed were pooled and thoroughly dialysed against 1× PBS, pH 7.5.

2.3. Animals and immunisation

The animal experiments involved using spleen-intact *Aotus* monkeys captured in the Amazon jungle with the permission of the Colombian government's environmental authority (CORPOAMAZONIA) and kept at our primate field station in Leticia (Colombia) where they were permanently monitored by CORPOAMAZONIA officials [14].

Groups of 5–9 monkeys proving IFA-negative for sporozoites and merozoites were subcutaneously inoculated on day zero (0) with 250 μg polymerised peptide homogenised with Freund's Complete Adjuvant for the first dose and Freund's Incomplete Adjuvant for the second (day 20) and third (day 40) doses. Blood was drawn for immunological analysis on day 1 before (P_0) the first immunisation and 20 days after the second (II_{20}) and third (III_{20}) immunisations.

2.4. Immunofluorescent antibodies tests (IFA)

Slides containing air dried *P. falciparum* sporozoites (3D7 strain) purchased from Sanaria Inc (Rockville, Maryland USA) were used for double-labelling experiments; they were blocked for 10 min with 1% skimmed milk and incubated for 30 min with appropriate monkey sera dilutions (starting at 1:40) [14]. Reactivity for *Aotus* anti-CSP modified peptide 24258 (4383) [12] diluted 1:500 was detected using $F(ab')_2$ fragments from a 1:200 dilution of an affinity purified goat anti-*Aotus* IgG:FITC (fluorescein isothiocyanate) conjugate (showing green by fluorescent). The second antibody from an *Aotus* immunised with SIAP-1 or 2 modified peptides was used at 1:50 dilution and further detected by purified goat anti *Aotus* IgG $F(ab)_2$ fragment RITC (rhodamine isothiocyanate) conjugate at 1:200 dilution (showing red by fluorescence microscopy). Slides were further stained with DAPI (4′,6-diamidino-2-phenylindole) which binds to parasite nuclear DNA (showing a bright blue) [15]. Pre-immune sera from all monkeys were used as negative controls.

2.5. Western blot analysis

SIAP-1 and SIAP-2 recombinant fragments were separated in SDS–PAGE for WB using 12% acrylamide gels (w/v) and transferred

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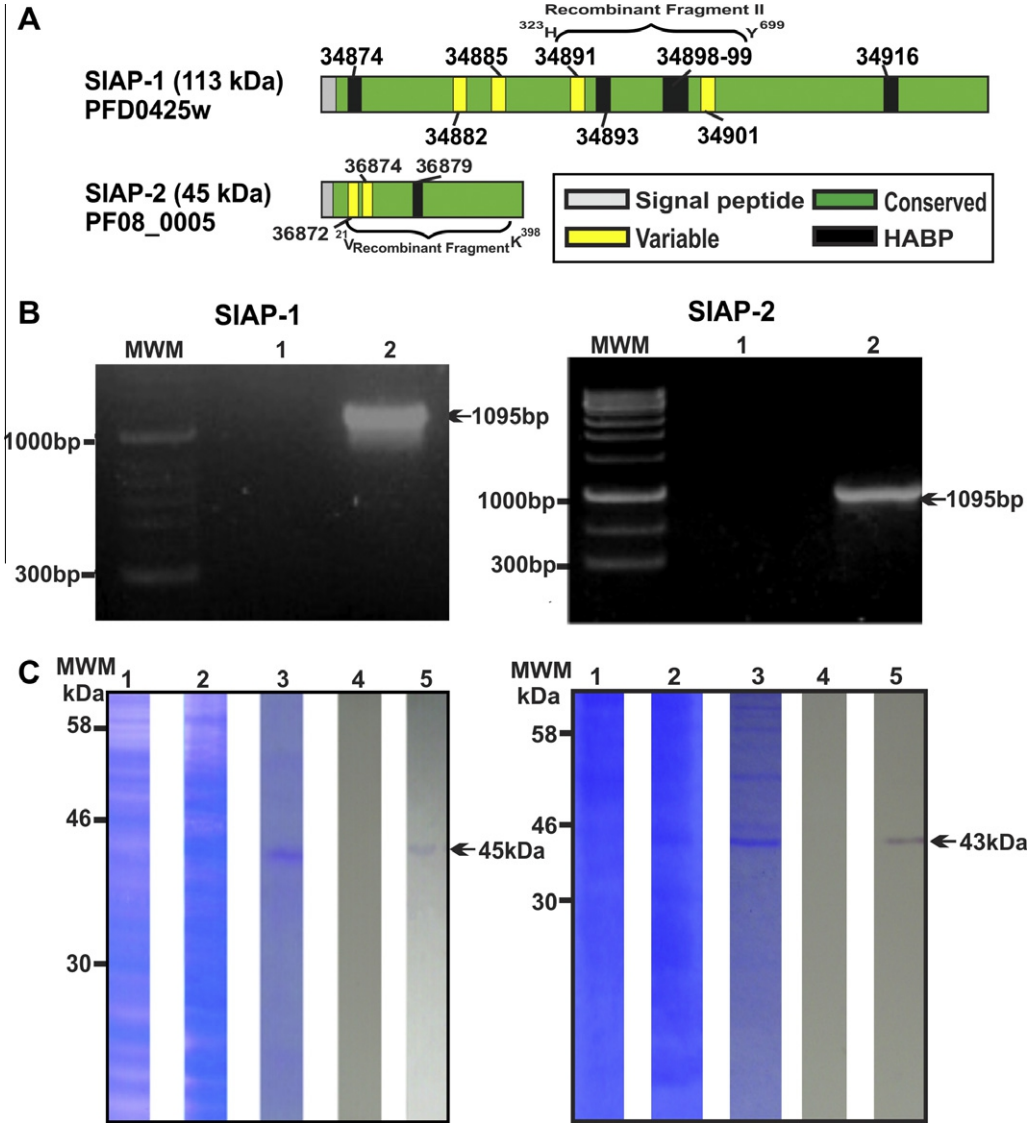

Fig. 1. (A) Schematic representation of SIAP-1 and SIAP-2 molecules and localisation of recombinant fragments and HABPs common to HeLa and HepG-2 cells. Each molecule's molecular mass and accession codes are shown. Bar length represents approximate molecular weight. A colour code is included in Fig. 1. (B) PCR amplification of a *P. falciparum* SIAP-1 fragment and the whole SIAP-2 encoding gene in the 3D7 and FCB-2 strains, respectively. MWM: molecular weight marker. Lane (1), negative control. Lane (2), SIAP-1 (left) and SIAP-2 (right) amplified from genomic DNA. (C) SDS–PAGE and Western-blot of *P. falciparum* SIAP-1 (left) and SIAP-2 (right) purified proteins. Lane (1), non-induced bacterial lysate. Lane (2), L-arabinose-induced bacterial lysate. Lane (3), SDS–PAGE of purified proteins. Lane (4), Western-blot of non-induced lysate, and Lane (5), Western-blot of the purified protein using anti-polyhistidine monoclonal antibody. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

to nitrocellulose membranes, cut into strips and treated as previously described [12]. Each strip was individually incubated with serum from each monkey diluted 1:100 in blocking solution for 1 h, washed several times with TBS-T and then incubated with alkaline phosphatase (AP) coupled goat anti *Aotus* IgG F(ab)$_2$ fragment, conjugated at 1:1000 dilution for 1 h before developing the reaction with NTB/BCIP [12].

2.6. Enzyme-linked immunosorbent assay (ELISA)

This procedure has been clearly described by our group [12], where the recombinant fragments were used at 5 μg/ml and *Aotus* sera was diluted 1:100.

2.7. NMR spectroscopy

NMR samples were prepared by dissolving 6–10 mg monomer peptide in 500 μl trifluoroethanol (TFE-d$_3$/H$_2$O 30:70). All NMR spectra were run on Bruker DRX-500 spectrometer and processed on a computer equipped with TOPSPIN 1.3 software (Bruker). Spin systems were assigned by double-quantum-filtered-correlation (DFQ-COSY) [16] and total correlation spectroscopy (TOCSY) [17] using MLEV-17 pulse sequences and Nuclear Overhauser effect spectroscopy (NOESY) [18] ^{1}H–^{1}H 2D experiments. Standard spectrum procedure was used for sequential assignment.

Temperature coefficients for predicting hydrogen bonds ($-\Delta\partial$HN/$\Delta T < 5$) were determined by TOCSY experiments using a 30 K temperature range (285–315 K). All experiments were done at 295 K (except temperature-dependant ones).

2.8. Structure calculation

Distance constraints were extracted from NOESY spectra to obtain structural models at 295 K. All NOE intensities are converted into distance range (strong, medium, weak) and Insight II package was used for structure calculations (Accelrys, Inc., Software, USA),

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run on Silicon Graphics. Distance geometry (DGII) software was used to build a set of 50-structure conformers; these were then refined by using simulated annealing protocol (Discover software).

3. Results and discussion

3.1. Reasons for a multi-epitope, multistage, minimal subunit-based vaccine development strategy

A fully-protective vaccine against the *P. falciparum* parasite, the most lethal form of malaria, requires a multi-epitope, multi-stage strategy for blocking or destroying the multiple pathways used by the parasite to infect (~200 million) and kill (~1 million) susceptible humans worldwide, per year. As the sporozoite and merozoite proteins' repertoire involved in invasion is so large, recombinant, DNA, viral or microbial vector-targeted approaches using complete proteins for fully-effective vaccines including both parasite stages seems to be an impossible challenge.

Therefore the minimal subunited based multi-epitope, multi-stage, chemically synthesised vaccine approach thus appears to be the most logical and rational methodology for performing this phenomenal task. Due to the *P. falciparum* parasite's tremendous genetic variability (~200,000 strains or variants) to escape host immune pressure, an immunological approach seems practically an insurmountable problem since microbe-induced immunity is strain-specific. It was thus decided to tackle vaccine development from a functional point of view, recognising the conserved amino acid sequences of proteins or HABPs involved in binding to and invasion of host cells. However, these HABPs turned out to be immunologically silent since they were not antigenic, or immunogenic, or protection-inducers [19].

To solve this problem, and after years of thorough studies working with conserved HABP-derived merozoite proteins (the second line of defence) it was found that specific modifications had to be made to critical binding residues [20] to allow them to perfectly fit into the Major Histocompatibility Class II molecule-peptide-T-Cell Receptor (MHCII-peptide-TCR) complex to render them highly immunogenic and protection-inducers against experimental challenge with a highly infectious *P. falciparum* strain (FVO) in the Aotus monkey (an animal model having an immune system almost identical to that of humans) [21].

A similar approach is now being used by our group to try to block the sporozoite journey to the liver, impeding its gliding motility and traversing activity through different cell types to

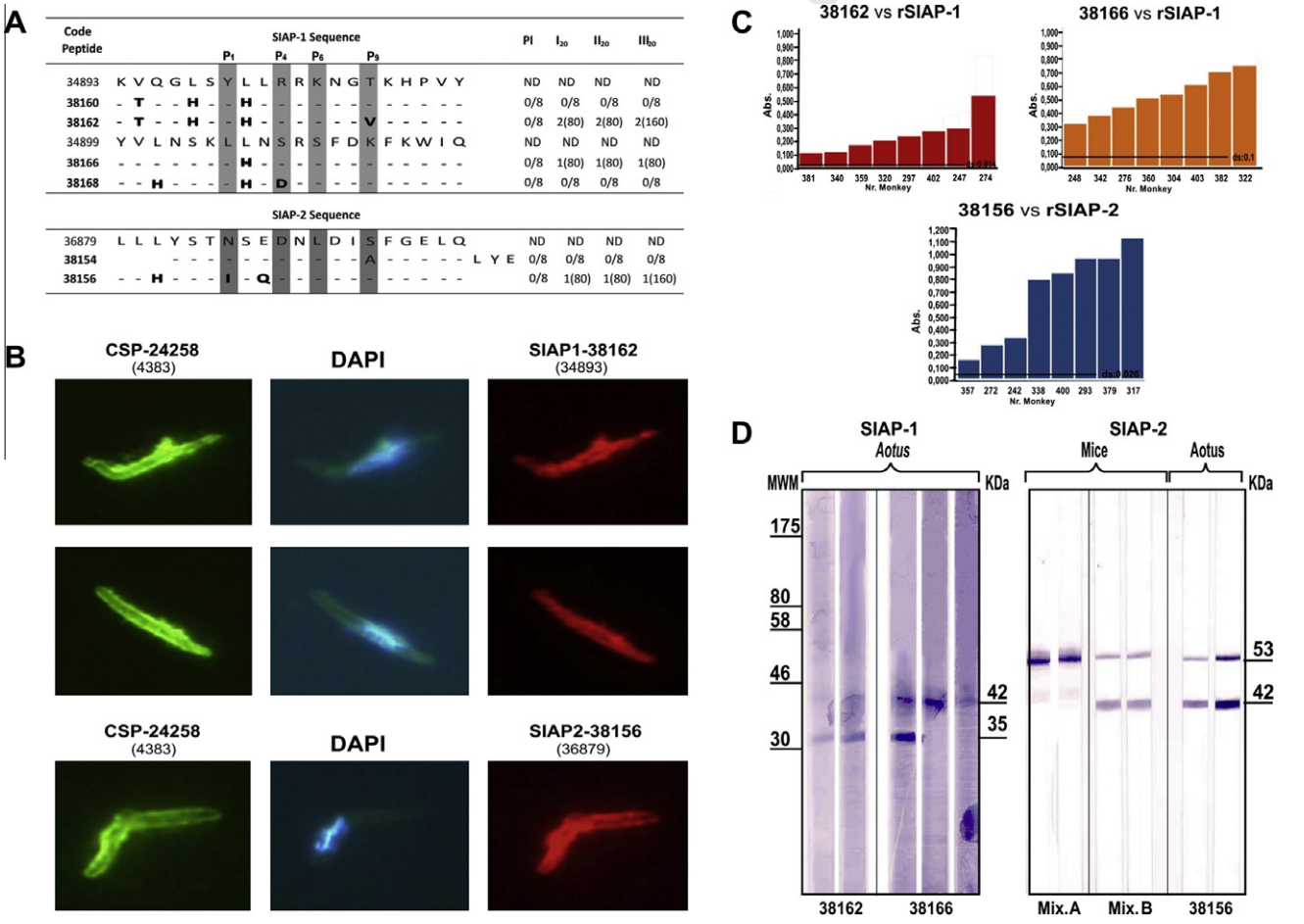

Fig. 2. (A) Antibody titres (reciprocal in parenthesis) as assessed by IFA against sporozoites, determined prior to immunisation (PI) 20 days after the first (I_{20}), second (II_{20}) and third (III_{20}) doses induced by native and modified HABPs (in bold) with their corresponding amino acid sequences. Shadowed are residues fitting HLA-DRβ1* binding registers corresponding to pockets (P1, P4, P6, P9). (B) Sporozoite immunofluorescence pattern and localisation recognised by anti-CSP **24258** (4383) reference sera, reacting with membrane structures (green fluorescence) while anti-SIAP-1 **38162** (34893) and anti-SIAP-2 **38156** (36879) reacted with small intracytoplasmatic (probably micronemes) and membrane structures (red fluorescence). DAPI localises DNA as bright blue. (C) Recognition of rSIAP-1 and rSIAP-2 for individual *Aotus* sera immunised with correspondingly modified HABPs (in bold), as assessed by ELISA. (D) Western blot analysis of mice and *Aotus* sera diluted 1:100 reacting with respective recombinant fragments and their molecular weights. *Aotus* sera reacted with 42 kDa and 35 kDa molecules in SIAP-1. Sera from mice immunised with mixture A peptides localised in the N-terminal reacted with a 53 kDa recombinant fragment in SIAP-2 while mouse sera from mice immunised with mixture B peptides located in the C-terminal reacted with 53 kDa and 42 kDa molecules, identical to *Aotus* sera reactivity when immunised with **38156** (36879). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

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finally infect the hepatic cells. Ongoing work has thus focused on CSP [12], SPECT [22], TRAP [15], STARP [23], some liver stage proteins like LSA-1 [24] and SALSA [24] to establish this first line of defence.

Now that a large number of highly immunogenic and protection-inducing merozoite-derived modified HABPs (the second line of defence) have been identified [25], SIAP-1 and SIAP-2-derived conserved HABPs appear to be excellent targets for inclusion as components of a first line of defence (against sporozoites) in the development of a fully-effective anti-malarial vaccine [26].

3.2. SIAP-1 and SIAP-2 recombinant fragments

A 1131 bp fragment obtained from amplifying the *P. falciparum* 3D7 strain and which encodes about half of SIAP-1 (rSIAP-1-RII), as well as the region encoding the whole SIAP-2, obtained from the FCB2 strain, were cloned and expressed in *Escherichia coli* BL21Al. The SIAP-1 sequence did not have any nucleotide changes with respect to the 3D7 sequence reported in PlasmoDB, while the SIAP-2 sequence had 3 nucleotide changes corresponding to non-synonymous substitutions located in positions 283 bp, 740 bp and 895 bp, shifting H95Y, S247I and A299P, respectively.

These proteins' recombinant expression was assessed by WB with anti-polyhistidine monoclonal antibody, resulting in a single ~45 kDa band for SIAP-1 and ~53 kDa (data not shown) and ~43 kDa bands for SIAP-2, thereby agreeing with the expected molecular weight (Fig. 1C). BCA quantification gave 150 μg/mL for SIAP-1 and 80 μg/mL for SIAP-2 production.

3.3. Immunological studies

Previously established rules and principles [20,26] regarding merozoite proteins mean that very few modified HABPs have to be synthesised nowadays to obtain highly immunogenic peptides. SIAP-1-derived 38162 (34893) induced high recognition against the rSIAP-1-RII fragment in five out of eight monkeys immunised against sporozoites, as assessed by ELISA (Fig. 2C) and in 2 out of 8 monkeys (1:160 titres, determined by IFA in Fig. 2A), small intracellular structures being recognised inside sporozoites (red fluorescent dots in Fig. 2B) while reference anti-CSP antibody 24258

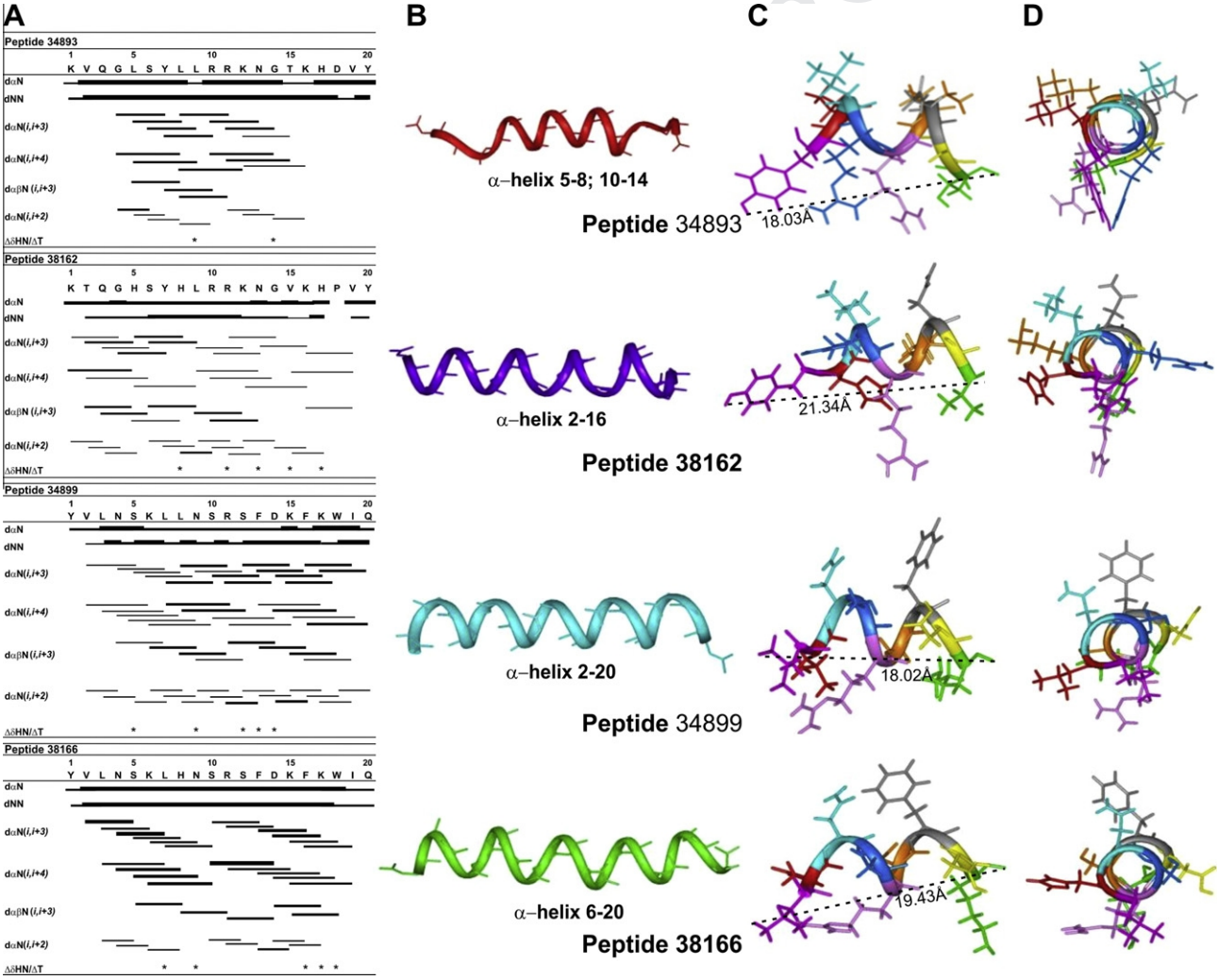

Fig. 3. (A) Native and modified HABP connectivity (NOE intensity is represented by line thickness). Amide protons having low coefficients used for structure calculations are shown by (*). (B) Ribbon representation of native and modified HABP 3D structure as determined by ^{1}H NMR displaying α-helical localisations. (C) Lateral view of native and modified HABP 3D structure. Residues assigned according to corresponding HLA-DRβ1* molecule binding motifs and binding registers recognised by immunogenic **38162** (34893) corresponded to HLA-DRβ1*0802 and **38166** (34899) bound to HLA-DRβ1*0302. The colours represent residues fitting into the MHC-II-p-TCR complex: fuchsia in pocket 1, red in P2, turquoise in P3, dark-blue in P4, pink in P5, orange in P6, grey in P7, yellow in P8 and green in P9. The difference in distance was measured in Å. (D) Front view of native and modified HABPs. Note the difference in residue orientation between native and modified HABPs which was most clearly observed between 34893 and **38162**, this being the highest immunogenic so modified. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

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(4383) reacted with membrane structures (green fluorescence in Fig. 2B) [26]. These 38162-induced antibodies were also seen to react with rSIAP-1 RII fragment (where 34893 HABP is located) by WB, recognising a ~42 kDa band and another at ~35 kDa which was probably a purification cleavage fragment (Fig. 2D, right panel). Something similar occurred with 38166 (34899), being immunogenic for six out of eight immunised monkeys by ELISA (Fig. 2C) and a smaller number of monkeys by IFA (Fig. 2B) since only one *Aotus* developed a 1:80 antibody titre against sporozoites (Fig. 2A); four of them recognised the same fragments by WB.

Using the same assays, SIAP-2-derived 38156 (36879) was highly immunogenic for 5 out of 8 monkeys (by ELISA in Fig. 2C) and a 1:160 antibody titre was induced in 1 of them (by IFA in Fig. 2A), reacting with intracellular and membrane structures (red fluorescence in Fig. 2B, lower panel). WB revealed that this monkey's serum recognised the recombinant SIAP-2 protein (42 kDa molecular mass) and a higher molecular weight precursor (53 kDa) (Fig. 2D, left panel).

This *Aotus*' serum reactivity was similar to that observed with mice sera immunised with non-HABP conserved amino acid sequences from SIAP-2 (produced to determine this protein's immunogenicity) located in this protein's N-terminal (mixture A) which reacted exclusively with the high molecular weight precursor (53 kDa) while sera from mice immunised with peptides located in the C-terminal (mixture B) reacted with both 53 kDa and 42 kDa molecules (Fig. 2D left panel). It has been suggested that SIAP-2's greater molecular weight may be due to post-translational modification [5] and one could speculate that these modifications are located at the N-terminal region since mice anti-mixture A sera exclusively recognised the high molecular weight precursor (53 kDa) and anti-mixture B (C-terminal) reacted with both 53 kDa and 42 kDa molecules.

3.4. Structural analysis

Sequential- and medium-range NOE connectivity from NOESY spectra analysis of 34893, 34899, **38162** and **38166** showed $d_{NN}(i,i+1)$, $d_{\alpha\beta}(i,i+3)$, $d_{\alpha N}(i,i+3)$, $d_{\alpha N}(i,i+4)$ connectivity and low amide proton temperature coefficients (Fig. 3A), indicating the presence α-helical structures in all peptides. Peptide 34893 had helical regions from residues L5 to L8 and R10 to G14 while **38162** displayed an α-helix between residues T2 to K16, 34899 between residues V2 to Q20 and **38166** from L6 to Q20 (Fig. 3A and B). These results were consistent with structure calculation (Supplemental Table 1).

SIAP-2 36879 HABP and its corresponding **38156** were not soluble at the required concentrations for ^{1}H NMR studies, so their 3D structure could not be determined.

3.5. Native and modified HABPs 3D structure

The following was found when searching for an association between native and modified HABPs' immunogenicity and 3D structure aimed at choosing the best modified HABP which was able to induce high antibody titres as assessed by three techniques. When highly immunogenic **38162** (modified in H5L, H8L and V15T) was compared to 34893, based on predicted HLA-DRβ1*0802 binding motifs and binding registers, the distance between residues fitting into the most distant MHCII pockets 1 (Y7, fuchsia) and pocket 9 (T15, green) was 3.31 Å longer in **38162** (Fig. 3C) and residues fitting into pocket 1, (Y7, fuchsia), pocket 4 (R10, dark blue) and pocket 6 (K12, orange) had a weird orientation in 34893 compared to **38162** amino acids (Fig. 3D), thereby not following the canonical downward orientation these residues should display to enable their fit into MHCII molecule pockets. By the same token, residues pointing upwards to make contact with the TCR (R9, turquoise and N13, grey) had completely different side orientation in 34893 and **38162**.

Something quite similar occurred with 34899 and **38166**, the latter displaying characteristic HLA-DRβ1*0302 binding motifs and binding registers where **38166** was 1.41 Å longer than native 34899 between residues fitting into this molecule's pockets 1–9, also having differences in lateral chain orientation, mainly in K15 (green) fitting into pocket 9 (Fig. 3C and D).

Immunogenicity studies of highly relevant SIAP-1 and SIAP-2 in *Aotus* monkeys regarding conserved HABPs successful binding to HeLa and HepG-2 cells which are involved in sporozoite cell traversal and invasion of hepatic cells (**38162** genetically controlled by HLA-DRβ1*0802 and **38166** by HLA-DRβ1*0302) have suggested that both modified HABPs are excellent candidates for inclusion in a desperately needed multi-epitope, multi-stage, minimal subunit-based chemically-synthesised anti-malarial vaccine

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.039.

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ANEXO 8

Patarroyo ME, **Alba MP**, Curtidor H, Vanegas M, Almonacid H, Patarroyo MA. Using the PfEMP1 head structure binding motif to deal a blow at severe malaria. PLoS One. 2014. 9:e88420.

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Using the *Pf*EMP1 Head Structure Binding Motif to Deal a Blow at Severe Malaria

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Abstract

Plasmodium falciparum (*Pf*) malaria causes 200 million cases worldwide, 8 million being severe and complicated leading to ~1 million deaths and ~100,000 abortions annually. *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) has been implicated in cytoadherence and infected erythrocyte rosette formation, associated with cerebral malaria; chondroitin sulphate-A attachment and infected erythrocyte sequestration related to pregnancy-associated malaria and other severe forms of disease. An endothelial cell high activity binding peptide is described in several of this ~300 kDa hypervariable protein's domains displaying a conserved motif (GACxPxRRxxLC); it established H-bonds with other binding peptides to mediate red blood cell group A and chondroitin sulphate attachment. This motif (when properly modified) induced *Pf*EMP1-specific strain-transcending, fully-protective immunity for the first time in experimental challenge in *Aotus* monkeys, opening the way forward for a long sought-after vaccine against severe malaria.

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Introduction

Malaria-infected children's sera originally recognised *Pf*EMP1 in infected erythrocyte (IE) agglutination tests [1], as a highly polymorphic very large (~300 kDa molecular weight); protein encoded by >60 variable, genes (*Pf var*). *Pf*EMP1 has an extracellular ectodomain consisting of 2 to 9 highly variable in amino acid sequence, length and composition domains; constituted by an N-terminal segment (NTS), a Duffy-binding-like (DBL) 1α domain and a cysteine interdomain region (CIDR) α1 (forming the head structure) and DBL2X, C2, DBL3X, DBL4ε, DBL5ε, DBL6ε and DBL7ε domains followed by a transmembrane region (TM), and an intracytoplasmic acidic terminal segment (ATS), inserted into IE membrane [2–4].

*Pf*EMP1 can be classified into 5 groups (A–E) based on the nucleotide sequence similarity of the upstream promoter sequence (UPS) [5], having 6 major DBL domain classes (α, β, γ, δ, ε and X). Each DBL domain consist of hypervariable and conserved regions and contains 3 subdomains (S1, S2 and S3) having 10 semi-conserved homology blocks (HB 1–10 consisting of 7 to 21 residues) conserved in all domain classes, most frequently localised in subdomains S1 (HB4), S2 (HB3, HB5) and S3 (HB2, HB1) [5,6].

*Pf*EMP1 can also be grouped according to 23 domain cassettes (DC), the most frequent ones DC1 to 3, spanning the entire protein while the others include 2–4 domains [6].

The DBLα1 domain, binds blood group A and forms rosettes by adhering to uninfected erythrocytes (UE) [7] being associated with cerebral malaria (CM) [8]. DBL3X and DBL6ε bind to chondroitin sulphate proteoglycans (CSPG) whilst DBL2X, DBL3X, DBL5ε and DBL6ε bind to chondroitin sulphate-A (CSA) [9,10], leading to IE sequestration in the placenta, thereby inducing pregnancy-associated malaria (PAM) and abortions, mainly in primigravidas.

A robust, highly specific, sensitive functional methodology has been thoroughly described for tailor-made vaccine development aimed at *Pf*EMP1 (*ipso facto* severe malaria), recognising variable and conserved HABPs (cHABPs) in relevant invasion molecules by working with ~15 to 20 mer-long peptides [11]. cHABPs are immunologically silent since they do not induce immune responses; however, when their critical binding residues have been properly modified [12–14] they become highly immunogenic and protection-inducing modified HABPs (mHABPs).

Materials and Methods

Ethics Statement

The present study was approved by the Fundación Instituto de Inmunología's animal ethics committee. The capture of *Aotus* monkeys (International Union for Conservation of Nature and Natural Resources (IUCN) status: least concern), the pertinent maintenance, immunization challenge and research procedures have been authorized by the official Colombian environmental authority in the Amazonian region (CORPOAMAZONIA, resolutions 0066/Sep/2006, 0028/May/2010, 0632/Jun/2010 and 0042/Jan/2011 and previous authorizations beginning in 1982).

The US Committee on the Care and Use of Laboratory Animals' guidelines were followed for all animal handling procedures, in turn complying with Colombian regulations for

ANEXO 9

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The high immunogenicity induced by modified sporozoites' malarial peptides depends on their phi (ϕ) and psi (ψ) angles

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ABSTRACT

The importance of CSP- and STARP-derived ϕ and ψ dihedral angles in mHABP structure was analysed by ^{1}H NMR in the search for molecules which can be included as components of a first-line-of-defence *Plasmodium falciparum* sporozoite multi-epitope vaccine against the most lethal form of human malaria. Most of the aforementioned dihedral angles were left-hand-like polyproline type II ($PPII_L$) structures whilst others had right-hand-like α-helix (α_R), thus allowing mHABPS to fit better into MHCII molecules and thereby form an appropriate pMHCII complex and also establish the H-bonds which stabilise such complex and by this means induce an appropriate immune response. This information has great implications for vaccine development, malaria being one of them.

1. Introduction

Developing a totally effective and definitive vaccine against the main parasite causing human malaria (*Plasmodium falciparum*, producing ~200 million cases and 1.2 million deaths annually) [1] needs highly immunogenic components in its first line-of-defence, such as molecules from the parasite's sporozoite (the parasite form which invades liver cells after being inoculated during an infected *Anopheles* mosquito's bite) [2].

However, obtaining enough amounts of sporozoites for biological, biochemical, functional and immunological studies from the mosquito's salivary glands where they are localised is not an easy task but rather a very difficult one. It is equally impossible to culture sporozoites *in vitro* [2,3] and a lack of *Anopheles* mosquito strains which have been adapted for infecting *Aotus* monkeys further hampers developing a totally effective vaccine against this stage and thus against this deadly disease.

Our institute has thus opted for defining the principles or rules for developing second-line-of-defence vaccines by working with the merozoite, the parasite's infective form which invades the red blood cells (RBCs). This is easily cultured and can be obtained in large amounts from infected blood *in vivo* or *in vitro* [4] for biological, biochemical and immunological studies. Such rules can then be applied to developing a totally effective vaccine against the sporozoite stage.

Our institute has also taken advantage of having access to the *Aotus* monkey which is an appropriate experimental model for studying merozoites; it has a ~90–100% identical immunological system to that of humans [5]. These monkeys can be easily infected by intravenous route and such monkeys' blood can be monitored daily regarding the development of the disease (or parasitaemia) by simple methods such as Giemsa staining or fluorescence (Acridine Orange) or molecular biology (PCR).

Plasmodium falciparum genome encodes ~5600 proteins, ~50 of which have been found to be involved in merozoite invasion of RBC in elegant proteome studies [6] and it has been calculated that a similar number of sporozoite proteins is involved in invasion of hepatocytes [7]. Our group has identified conserved amino acid sequences having high specific binding capacity to both RBC and hepatocytes which are involved in the invasion of such cells, called conserved **h**igh **a**ctivity **b**inding **p**eptides (cHABPs). Their critical residues have been identified, as well as fundamental residues establishing H-bonds with other cHABPs or with receptor molecules [8] for designing modified HABPs (mHABPs) according to thoroughly-described previously established principles and rules [9–11] and thus converting such immunologically silent cHABPs into highly immunogenic, protection-inducing mHABPs.

Based on such principles and rules, our group has identified cHABPs from ~20 sporozoite proteins [12,13] which have been recognised to date as being involved in sporozoite traverse of endothelial and Kuppfer cells to reach and invade hepatocytes, the circumsporozoite protein (CSP) [14] and the sporozoite threonine- and asparagine-rich protein (STARP).

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ANEXO 10

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Functional, Immunological and Three-Dimensional Analysis of Chemically Synthesised Sporozoite Peptides as Components of a Fully-Effective Antimalarial Vaccine

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Abstract: Our ongoing search for a fully-effective vaccine against the *Plasmodium falciparum* parasite (causing the most lethal form of human malaria) has been focused on identifying and characterising proteins' amino acid sequences (**h**igh **a**ctivity **b**inding **p**eptides or HABPs) involved in parasite invasion of red blood cells (RBC) by the merozoite and hepatocytes by the sporozoite. Many such merozoite HABPs have been recognised and molecularly and structurally characterised; however, native HABPs are immunologically silent since they do not induce any immune response or protection against *P. falciparum* malaria infection and they have to be structurally modified to allow them to fit perfectly into immune system molecules.

A deeply structural analysis of these conserved merozoite HABPs and their modified analogues has led to rules or principles becoming recognised for constructing a logical and rational methodology for a minimal subunit-based, multi-epitope, multi-stage, chemically-synthesised vaccine. The same in-depth analysis of the most relevant sporozoite proteins involved in sporozoite cell-traversal and hepatocyte invasion as well as the hepatic stage is shown here.

Specifically modifying these HABPs has resulted in a new set of potential pre-erythrocyte targets which are able to induce high, long-lasting antibody titres in *Aotus* monkeys, against their corresponding recombinant proteins and the complete parasite native molecules.

This review shows how these rules may be applied against the first stage of parasite invasion (i.e. the sporozoite) to mount the first line of defence against the malarial parasite, which may indeed be the most effective one. Our results strongly support including some of these modified sporozoite HABPs in combination with the previously-described modified merozoite HABPs for obtaining the aforementioned fully-protective, multiepitope, multi-stage, minimal subunit-based, chemically-synthesized, antimalarial vaccine.

Keywords: Malaria, *Plasmodium*, sporozoite, vaccine, peptide, synthesis, analogue, structure, HLA, *Aotus*.

INTRODUCTION

Apicomplexan parasites from the *Plasmodium* genus are some of the most successful intracellular pathogens infecting both vertebrate and invertebrate hosts; they do so during their development stages by means of three specialised invasive forms: the sporozoite, the merozoite and the ookinete [1, 2]. Their success as pathogens is reflected in the close to 3.2 billion people constantly at risk of developing the most lethal form of human malaria, that caused by the *Plasmodium falciparum* parasite; this disease afflicts around 300 million people each year, killing more than 3 million of them [3, 4]. The severity of the global situation regarding malaria has fostered thousands of studies aimed at developing new and more effective control mechanisms for tackling this scourge. Most of these efforts have been based on the chemical, physical or biological elimination of anopheline mosquito vectors, producing more effective antimalarial drugs and treatment and using different approaches for developing antimalarial vaccines [5-8].

However, identifying new and more potent vaccine candidates and immunoprophylactic strategies becomes an impossible task without a deep knowledge of the molecular mechanisms involved in parasite survival and host-pathogen interactions. The *Plasmodium* parasite uses exquisitely complex host-pathogen interaction mechanisms to invade specific target cells in its human and invertebrate hosts during each life-cycle stage. Infection begins in humans when an infected female *Anopheles* mosquito injects around 100 to 1,000 **sporozoites** into the human host during a blood meal (this is the parasite's larva-like form shown in Fig. **1**). Some injected sporozoites travel through the bloodstream until reaching the liver (whilst others cannot find their way to the blood) where they recognise and invade the host's liver cells or hepatocytes (named the pre-erythrocyte stage in this review), thereby giving rise to thousands (~30,000) of **merozoites** (pear-like parasite forms) per infected hepatocyte. Merozoites are then released into the bloodstream to specifically invade and infect erythrocytes or **r**ed **b**lood **c**ells (RBCs), thus marking the beginning of the intra-erythrocyte asexual development cycle or stage. Merozoites are released from infected RBCs (iRBC) and the subsequent invasion and infection of new RBCs causes the clinical symptoms associated with malaria and can eventually lead to a human host's death.

Hepatocyte and RBC invasion by their corresponding invading forms (the sporozoite and merozoite, respectively) is mediated by a large number of molecules fulfilling a variety of different tasks related to host cell binding, recognition and invasion. Such invasion-associated proteins are therefore considered attractive candidates for developing a fully-effective, antimalarial vaccine capable of conferring protection against *P. falciparum*'s different life-cycle stages. How some of these proteins may be used as vaccine candidates can be found in two recent reviews by our institute [9, 10] and several reviews by other authors [11-14].

The mechanisms underlying **merozoit**e invasion of RBCs have been the target of numerous functional, structural and immunological studies thus leading to unravelling a wide repertoire of invasion mechanisms and potential antimalarial vaccine targets [11, 15, 16]. It is thus fitting that the same deep and rigorous analysis should be conducted for the **sporozoite**, the first stage during *Plasmodium* infection of humans and thus the first level needing to be blocked by an effective vaccine.

This review summarises the systematic efforts made in identifying (at atom level) the rules defining the immunogenic potential of pre-erythrocyte (sporozoite) malarial parasite proteins. Its second objective, the *raison d'être* of this manuscript, is to describe our research achievements leading to the production of a new generation of minimal, subunit-based, multi-antigenic, multi-stage, chemically-synthesised, fully-effective vaccines for the health and well-being of humankind (malaria being one of them).

THE PLASMODIUM SPOROZOITE LIFE-CYCLE

Two main phases can be distinguished in the parasite's life-cycle during the sporozoite stage; the first phase is associated with

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