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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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The modifications made to sporozoite cHABPs have led to developing mHABPs inducing high antibody titres when inoculated into *Aotus* monkeys, as determined by immunofluorescence antibody test (IFA), using sporozoites from infected mosquitoes salivary glands or by Western blot (WB) or ELISA test using their respective recombinant proteins.

Ascertaining these cHABP and mHABP 3D structure by ^{1}H NMR has led to showing that such modifications provide a better fit into the trimolecular complex formed by molecules from the major histocompatibility complex class II-peptide-T-cell receptor (MHCII-p-TCR) [15].

The accompanying paper by our group has shown that merozoite-derived mHABPs which were highly immunogenic and induced protection against experimental challenge in *Aotus* monkeys had a specific 3D structure which was associated with left-handed-polyproline type II ($PPII_L$) and/or left-handed α-helix (α_L) physical–chemical characteristics [16], with defined ϕ and ψ torsion angles ensuring a perfect fit into the MHCII-p-TCR complex, to induce a highly immunogenic, protection-inducing response [15].

This manuscript has thus been aimed at describing the development and analysis of CSP-derived [17,18] and STARP-derived mHABPs [19,20] to include them as first-line-of-defence components (the sporozoite) in a totally effective and definitive multi-epitope, multistage minimal subunit-based, chemically-synthesised [9,12] antimalarial vaccine.

2. Materials and methods

Peptide synthesis, *Aotus* immunizations, mHABPs' 3D structure determined by ^{1}H NMR and superimposition studies onto HLA-DRβ1* molecules have been previously reported and have been summarised in a previous accompanying paper [21]. Sporozoites for IFA studies were purchased from Sanaria Inc. (Bethesda. USA). WB analysis was performed with STARP, CSP-N-terminal construct 2 and CSP-C-terminal recombinant proteins which were kindly provided by Professors Pierre Druille (Institute Pasteur, France), Mauricio Calvo Calle (Boston University) and Manuel Alfonso Patarroyo (FIDIC), respectively.

3. Results and discussion

3.1. Immunological studies

It has been thoroughly demonstrated [9] that cHABPs must be specifically modified to render them highly immunogenic and protection-inducing mHABPs against intravenous experimental challenge with a highly virulent *Aotus*-adapted *P. falciparum* strain, thereby opening the way forward for vaccine development (i.e., malaria).

Unfortunately, the Santa Lucia strain (the only *P. falciparum* strain adapted to *Anopheles* mosquitoes for transmitting malarial infection via sporozoites to *Aotus* monkeys via direct mosquito bites) gives very weird and irreproducible results, leaving immunogenicity (as assessed by different methods) as the only way to determine a sporozoite protein-induced humoral immune response.

The most relevant protein in sporozoite invasion (CSP) has two non-antigenic, non-immunogenic cHABPs (4383 and 4388 according to our institute's serial numbering system) [17]; they have become highly immunogenic when they have been properly modified (mHABP are indicated in bold numbers from this point onwards whilst native cHABPs are not shown in bold but in parenthesis) [18]. These were **25608** (4383) and **32958** (4388) which induced very high antibody titres as assess by IFA (Fig. 1B) [18] and reacted with sporozoite membrane, as determined by double immunofluorescence (**25608** shown in red in Fig. 1C and **32958** in Fig. 1D).

STARP, another very important molecule in sporozoite invasion, contained highly relevant cHABP 20546 [19] which, when properly modified as **24320,** became highly immunogenic in *Aotus* monkeys as assessed by IFA titres (Fig. 1B) and Western blot (WB) [20]. **24320** mHABP reacted with sporozoite membrane and small intra-cytoplasmatic structures (Fig. 1C and D green), probably corresponding to the micronemes where it is deposited before translocation to the membrane. STARP ranked second in importance in a prospective study using protein microarrays carried out in Mali regarding Ab response to *P. falciparum* before and after the malaria season in such hyper-endemic area [22]; the importance of identifying this mHABP as a component in a fully-protective multistage, multi-epitope antimalarial vaccine can thus be seen.

The fact that some regions of these highly immunogenic mHABPs (also protection-inducing against merozoites) could adopt configurations different to the canonical $PPII_L$, such as the α_L region in **10014.35** or α_R in **24320.18**, has suggested that some other transitional structures could fit into the MHCII PBR to be presented to the TCR to induce an appropriate immune response as long as they could form a stable MHCII-p-TCR complex [22].

These *Aotus*' sera also reacted with their corresponding recombinant proteins or their fragments in WB in such a way that anti-**25608** sera recognised 36 kDa MW CSP N-terminal construct 2 where only the last 5 residues were present [18], **32958** reacted with 10 kDa CSP C-terminal fragment (including residues 283–379 where 4383 cHABP was present) and anti-**24320** reacted with the complete 68 kDa recombinant STARP molecule [20] (Fig. 1E).

Amino-acid replacements in these cHABPs clearly induced modifications in their peptides' 3D structure, changing 4383 random structure into a mHABP having a type II-β-turn in **25608**. By the same token, 4388 random structure became changed in **32958** into a mHABP having a type Iβ-turn [18] and the α-helix from S9 to L16 in 20546 became displaced to K3 to D10 in **24320** [20].

The aforementioned modifications involved some other biological implications associated with changes in their immunological behaviour, such as binding to HLA-DRβ1* molecules (Fig. 1B); i.e., CSP 4383 did not bind to any of the HLA-DRβ1* purified molecules studied here, but **25608** had high binding capacity (58%) to HLA-DRβ1*0401, also displaying the characteristic binding motifs and binding registers for this molecule. Something similar occurred with cHABP 4388 which did not bind to any HLA-DRβ1* purified molecule but **32958** carrying both HLA-DRβ1*0101 and HLA-DRβ1*0401 binding motifs and registers, simultaneously bound to them with high capacity (65% and 52%, respectively) [18]. The latter was chosen for superposition studies; STARP cHABP 20546 was highly promiscuous regarding its binding to HLA-DRβ1* purified molecules, binding to practically all of them, (Fig. 1B) but mHABP **24320** displaying the binding motifs and registers characteristic of HLA-DRβ1*0301 and HLA-DRβ1*0101 binding to both (even though weakly so to the latter) [20]. High HLA-DRβ1*0101 binding capacity was assumed for technical reasons.

3.2. Structural characteristics

Previous papers with highly immunogenic, protection-inducing, merozoite protein-derived mHABPs [9] and sporozoite protein-derived mHABPs [12,18,20,23] have shown that the distance between the furthest atoms capable of fitting into HLA-DRβ1* pockets 1 to 9 was 27.50 Å for **25608.37**, 27.10 Å for **32958.2** and 21.35 Å for **24320.18** conformers (Fig. 2A, D, G), this being a perfect distance to fit into MHCII molecules' most distant and relevant pockets.

Since steric restriction has been recognised as the major organisational force in proteins, and the amide bond being planar, each

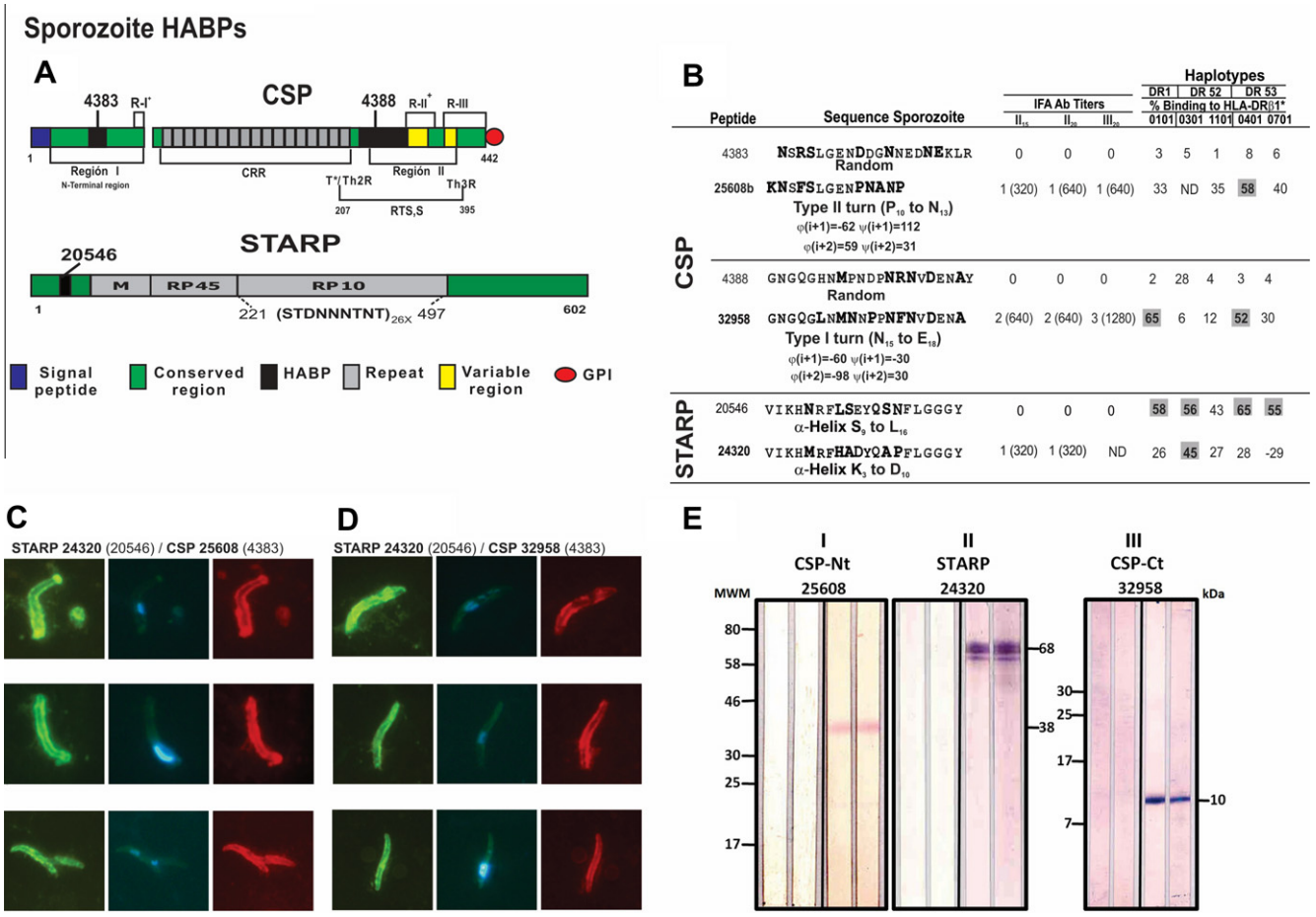

Fig. 1. (A) CSP and STARP molecule schematic representation according to the colour code below, showing cHABP localisation in vertical black bars; the bar length corresponds to approximate molecular weight. (B) CSP and STARP with their corresponding amino acid sequences and structural features elucidated by ^{1}H NMR; antibody titres as assessed by IFA against cHABP- and mHABP-induced sporozoites (in bold). Their capacity to bind to HLA-DRβ1* purified allele molecules where >50% binding (shadowed) was considered positive. (C) and (D) Double immunofluorescence (IFA) assays for sporozoite protein patterns and localisation, determined with high antibody titre sera produced in *Aotus* monkeys immunised with mHABPs. *Aotus* antibodies against STARP-modified HABP **24320** (20546) used at 1:40 dilution, detected with fluorescein isothiocyanate (FITC)-labelled goat purified IgG directed against *Aotus* IgG (1:100 dilution) displaying a green membrane fluorescence and small intracytoplasmatic dots inside the sporozoite. *Aotus* anti-CSP mHABP **25608** (4383) and anti-**32958** (4388) reactivity (serum dilution 1:100) detected by purified goat IgG anti-*Aotus* IgG conjugate with rhodamine isothyocynate (RITC), diluted 1:10, showing red membrane fluorescence. For reference, the localisation of the sporozoite's nucleus detected by 4,6 diamino-2phenylindole (DAPI) (bright blue). Sporozoites were purchased from Sanaria Inc: Bethesda USA. (E) Western blot analysis showing *Aotus* monkey reactivity. In panel I, sera from monkeys immunised with **25608** (4383), reacting with recombinant CSP construt 2 having 35 kDa MW; in panel II **24320** (20546) immunised monkeys' sera reactivity with rSTARP (68 kDa MW) and panel III anti **32958** (4388) monkey sera reacting with rCSP C-terminal (10 kDa MW).

peptide has only two degrees of freedom: ϕ and ψ; this is limited by atom clashes which "disallow" their minimal energy configurations. The two dihedral angles in question were thus obtained from ^{1}H NMR information choosing these mHABPs' lowest energy conformer (numbered according to our institute's serial number, followed by a dot and its corresponding conformer number) to identify their role in immunogenicity and protection (as shown in the accompanying paper) induced by these mHABPs in our endeavour to ensure a logical and rational methodology for fully-protective, definitive vaccine development.

Two contiguous left-handed polyproline II-like helices ($PPII_L$) were clearly identified in the CSP-derived **25608.37** conformer (Fig. 2B, highlighted in green), ϕ angles ranging from −91.8° to −46.5° (−93.5 ± 25° canonical range) and +149.4° to +85.1° for the ψ angles (+135 ± 20° canonical range) [24–28]. Such angles formed part of a sequence also displaying a $PPII_L$-like structure three residues upstream, thereby confirming, together with the other two mHABPs, the elegant work by Jardetzky et al., [29] that the predominant structures bindings to class II molecules display $PPII_L$-like structural characteristics.

Modifying the HLA-DRβ1*0401 molecule according to the amino-acid sequence differences found in the *Aotus* [15] in HLA-DRβ1*0422 led to 12 H-bonds (<4.0 Å distance) spontaneous formation when the **25608.37** conformer was superimposed onto HLA-DRβ1*0422 (Fig. 2C and Table 1) without any further manipulation, keeping in mind that these two molecules' 3D structures were determined by two different methodologies (^{1}H NMR for mHABPs and X-ray crystallography for HLA-DRβ1* molecules). The foregoing striking and outstanding finding confirmed that mHABPs must be properly modified to render them highly immunogenic, thereby fitting perfectly well into MHCII molecules, as elegantly shown by other groups and that such interaction largely depends on mHABPs structural conformation as dictated by ϕ and ψ dihedral angle rotations.

25608.37 formed five 9–11-member ring bidentate H-bonds with Sα53, Nβ82, Qα9, Nα62 and Kβ71 and established a single H-bond between Nα69 and Wβ61 (Fig. 2C and Table 1), anchoring it very stably to HLA-DRβ1*0422 to induce a very high immune response, as assessed by different immunological methods. It is worth mentioning that, although HLA-DRβ1*0422 occurs very frequently in the *Aotus* population (~20%), it is relatively rare in humans (<5%).

The **32958.2** conformer also displayed two sequential $PPII_L$ structures (Fig. 2E, highlighted in green) involving residues p2N

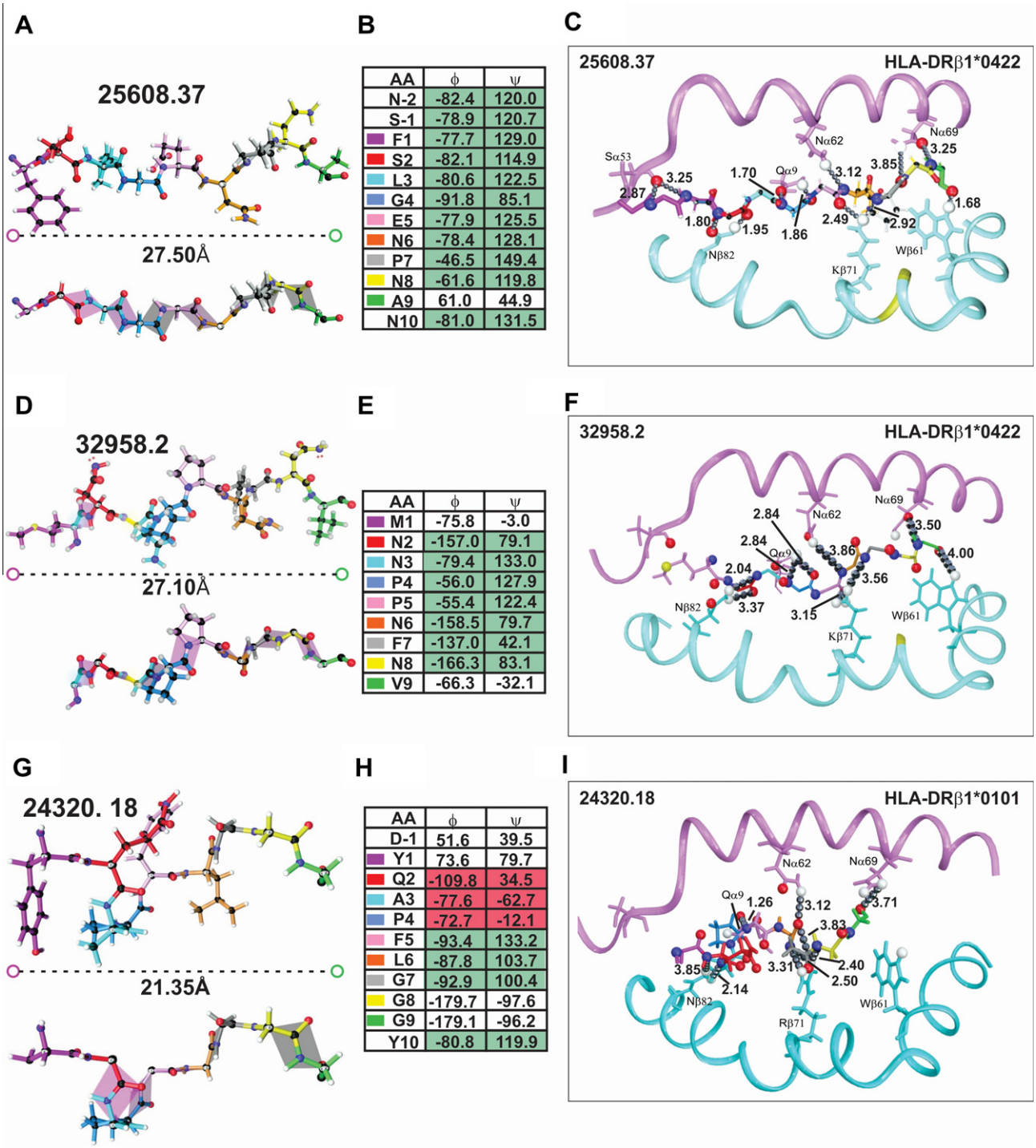

Fig. 2. Left panel: Lowest energy conformer 3D structure determined by ^{1}H NMR, identified by our serial number followed by dot and corresponding conformer number (A) CSP **25608.**37 (4383), (D) CSP **32958**.2 (4388) and (G) STARP **24320.**18 (25608). Central panel: B, E, H dihedral angles ϕ and ψ for the corresponding conformer. Right panel: C, F, I superimposition of mHABPs determined by ^{1}H NMR on HLA-DRβ1* molecules and their inter-atomic distances between peptide backbone and HLA-DR lateral chain atoms. (C) **25608**.37 and (F) **32958**.2 on HLA-DRβ1*0422 and (I) **24320**.18 on HLA-DRβ1*0101.

to p5P within the first $PPII_L$ segment, containing four residues, and p6N to p8N within the second one containing three residues, both being characteristic of $PPII_L$-helices [24]; their ϕ and ψ dihedral angles very closely followed the characteristics for the $PPII_L$ structures described in the accompanying article.

Eight H-bonds and one van der Waals (vdW) interaction were established between **32958**.2 backbone atoms and HLA-DRβ1*0422 lateral chains' atoms when **32958**.2 was superimposed onto the same HLA-DRβ1*0422 3D structure (Fig. 2F and Table 1).

Three bidentate 9–11-member ring H-bonds [28] were spontaneously formed between Nβ82 with p2N, Qα9 with p4P and Kβ71 with p5P and p7F while Nα62, Nα69 and Wβ61 formed individual H-bonds with p5P, p9V and p9V, respectively (Fig. 2F and Table 1); this led to a very stable pMHCII complex involving **32958.2** being formed, partly explaining this mHABP's very high immunogenicity.

The situation with STARP-derived **24320.18** conformer was slightly different as this mHABP was shorter than the other two, (21.35 Å), confirming that the inter-atom distance between the

Table 1
Atoms involved in H-bond formation between mHABPs and their corresponding HLA-DRβ1* molecule lateral chains. Distances shown for these H-bonds in Å are represented by silver dots.

A.			B.			C.		
	P1 P9 KNSFSLGENPNANP			P1 P9 GNGQGLNMNNPPNFNVDENA			P1 P9 VIKHMRFHADYQAPFLGGGY	
HLA-DRb1*0422	**25608.37**	Distance Å	HLA-DRb1*0422	**32958.2**	Distance Å	HLA-DRβ1*0101	**24320.18**	Distance Å
Sα53:O	HN:S-1	3.81	Nβ82:Hδ21	N:N2	2.04	Nβ82:Hδ21	N:A3	2.14
Sα53:O	HN:F1	3.67	Nβ82:Hδ22	O:N2	3.37	Nβ82:Hδ22	N:Q2	3.85
Nβ82:Hδ21	O:S2	1.95	Qα9:Hε22	N:P4	2.84	Qα9:Hε22	N:P5	1.26
Nβ82:Oδ1	HN:S2	1.8	Qα9:Hε22	O:P4	2.84	Nα62:Hδ22	O:L6	3.12
Qα9:Oε1	HN:G4	1.7	Nα62:Hδ22	N:P5	3.86	Nβ69:Hδ21	O:G9	3.71
Qα9:Hε22	O:G4	1.86	Nα69:Oδ1	HN:V9	3.1	Rβ71:HH21	O:L6	3.83
Nα62:Hδ21	O:P7	3.85	Kβ71:HH11	N:F7	3.56	Rβ71:HH21	N:G8	2.4
Nα62:Hδ22	N:N6	3.12	Kβ71:HH13	O:P5	3.15	Rβ71:HH21	N:G7	2.5
Nα69:Oδ1	HN:A9	2.44	Wβ61:Hε1	O:V9	4	Rβ71:HH12	N:G7	3.31
Kβ71:HH11	N:P7	2.92						
Kβ71:HH12	O:E5	2.49						
Wβ61:Hε1	O:A9	1.68						

furthest atoms fitting into p1 and p9 was 23.5 ± 2.5 Å, as we have thoroughly shown for merozoite-derived mHABPs [9].

The dihedral angles in this mHABP adopted a particular conformation involving a typical $PPII_L$ from p5F to p7G (−93.4° to −87.8° in ϕ and +133.2° to +100.4° in ψ) [27] (Fig. 2H, highlighted in green); however, as in some merozoite highly-immunogenic, protection-inducing mHABPs reported in the accompanying paper, there was a deviation from this rule with other structures different to $PPII_L$.

24320.18 mHABP conformer had a right-handed-like α-helix (α_R) region having ϕ −109.8° to −72.7° to and ψ −62.7° to +34.5° (Fig. 2H, highlighted in pink), such angles corresponded to this helical structure spanning p2Q to p4P, suggesting that some other structures besides the canonical $PPII_L$ structure [29] could be implicated in the binding to HLA-DRβ1* molecules to form an appropriate pMHCII complex and thereby induce an appropriate immune response. Hypothetically, such non-canonical structures could have high segmental atomic mobility in some areas (as occurs with Ab reacting regions in a protein) [30] thereby partly explaining their promiscuity in binding to HLA-DRβ1* molecules as another mechanism to escape immune pressure.

Nine H-bonds were spontaneously formed when **24320.18** was superimposed onto HLA-DRβ1*0101 3D structure without any modifications having been made, as explained before (Fig. 2I and Table 1). One was a bidentate 9-member ring H-bond between Nβ82 lateral chain atoms and p2Q and p3A and a very complex chain of H-bonds between Rβ71 and p5L and consecutive glycines p7G and p8G; the others consisted of three individual H-bonds established between Qα9 with p5P, Nα62 with p6L and Nα69 with p9G, showing that other structures than $PPII_L$ could also activate a high immune response, as shown for this mHABP.

In support of such molecule segmental atomic mobility we could cite Porter and Rosen's argument [31] for a new folding pathway of proteins where consecutive intermediates could successfully maintain an unbroken series of intra-molecular H-bonds. $PPII_L$ is frequently found in conditions following an H-bond low energy pathway (preserving intermediates) traversing a progressive continuum from β-turns to 3_{10}, to α_R-helices (α) through a bridge region defined by an area having $\phi = -80$ and $\psi = 30$, in such a way that the proposed low energy pathway would follow a sequence of events regarding their ϕ and ψ angles: $PPII_L$ (−60; 150) ↔ inverse γ-turn (−75; 80), hybrid turn (h) (−90; 35), bridge turn (b) (−90; 0.0), α-helices (α) (60; −40). This would facilitate switching handedness from left to right, without breaking any H-bonds and thus partly explaining $PPII_L$ transition into an α_R-helix, as occurred in **24320.18** mHABP; such situation was much more observable in ^{1}H NMR structures as occurred with our HABPs which are in solution than in rigid crystal structures as determined by X-ray crystallography.

This, and the accompanying paper, have clearly demonstrated that these principles or rules can be universally applied based on the following: (a) they deal with mHABPs derived from different proteins, performing different biological functions, (b) they are derived from different *P. falciparum* stages (sporozoites and merozoites) infecting different cell types and (c) when properly modified, they bind to different HLA-DRβ1* alleles corresponding to different haplotypes covering most of the genetic traits controlling specific humoral immune responses: HLA-DRβ1*0101 represents the HLA DR1 haplotype (including HLA-DRβ1*0101, 0104, 1001, etc.), HLA-DRβ1*0301 represents HLA DR52 (including HLA-DRβ1*03, 08, 11, 12, 13, 14) alleles and HLA-DRβ1*04 represents HLA DR53 (including HLA-DRβ1*04, 07, 09). The data presented here could thus be applied to any vaccine.

Physical–chemical rules determined by ϕ and ψ dihedral angles could thus be applied to a logical and rational methodology for a definitive minimal subunit-based, multi-epitope, multistage, chemically-synthesised, fully-protective vaccine, an antimalarial vaccine being one of them.

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