Vaccine 28 (2010) 4989–4996

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

3D structure determination of STARP peptides implicated in *P. falciparum* invasion of hepatic cells

Adriana Bermúdez[a,b], Martha Patricia Alba[a,b], Magnolia Vanegas[a,b], Manuel Elkin Patarroyo[a,c,*]

[a] *Fundación Instituto de Inmunología de Colombia (FIDIC), Colombia*
[b] *Universidad del Rosario, Colombia*
[c] *Universidad Nacional de Colombia, Colombia*

ARTICLE INFO

Article history:
Received 11 March 2010
Received in revised form 17 April 2010
Accepted 7 May 2010
Available online 22 May 2010

Keywords:
Structure
1H NMR
Malaria vaccine
Sporozoite

ABSTRACT

To block the different stages of *Plasmodium falciparum* invasion into human hepatocytes and red blood cells, we have focused on those proteins belonging to the pre-erythrocytic stage. One of these proteins is Sporozoite Threonine and Asparagine Rich Protein (STARP), which is a ligand used by *P. falciparum* parasites to bind Hepatic cells (HepG2). Previous studies on this protein identified two conserved peptides binding with high activity to HepG2 cells (namely 20546 and 20570) with corresponding critical hepatic-cell binding residues and determined an important role for these two peptides in the invasion process. This study shows the results of immunization trials in *Aotus* monkeys with native STARP peptides and analogues modified in critical hepatic-cell binding residues. The results show that native peptides are not immunogenic but can induce high-antibody titers when their critical residues are replaced by other with similar volume and mass but different polarity. Nuclear Magnetic Resonance (^{1}H NMR) studies revealed that native peptides (non-immunogenic) displayed shorter α-helical regions compared to their highly immunogenic modified analogues. Binding assays with HLA-DRβ1* molecules showed that 20546 modified peptides inducing high-antibody titers (**24972**, **24320** and **24486**) bound to HLA-DRβ1*0301 molecules, while the 20570 modified analogue (**24322**) bound to HLA-DRβ1*0101. The results support including these high-immunogenic STARP-derived modified peptides as pre-erythrocytic candidates to be included in the design of a synthetic antimalarial vaccine.

1. Introduction

The Sporozoite Threonine and Asparagine Rich Protein (STARP) antigen was cloned by Fidoch et al. using *Plasmodium falciparum* malaria laboratory strains and field isolates from a wide range of endemic regions [1]. The STARP antigen has been found to be present in other *Plasmodium* species and its gene is highly conserved in *P. falciparum* strains [1,2].

Immunoflourescence and immunoelectromicroscopy assays carried out using immune sera targeting the protein's central and C-terminal region have shown that STARP is expressed on the surface of the sporozoite forms that invade hepatic cells, suggesting a role during parasite's entry to the hepatic cell and infection [1].

The transcription of the 2.0 kb STARP gene has been demonstrated by reverse PCR and Northern blot hybridization. This gene encodes a highly conserved 604-residues-long protein of about 78 kDa containing a considerable amount of asparagine and threonine residues, for which the protein receives its name [1]. STARP contains 3 central repeat regions: 1) a mosaic (M) region between residues T85 to I134 containing various degenerated small repeats, 2) Rp45, located between residues N135 and T229 which contains two 45-amino-acid-long identical tandem repeats, and 3) Rp10, constituted by 26 tandem repeat units of 10 amino acids spanning from residue N223 to N489. The central region shows limited size variations, whereas the non-repetitive N and C termini have no length variations and show low degree of polymorphism. A highly hydrophobic region is located toward the protein's C-terminal end [1–3] (Fig. 1A).

In the search for optimal vaccine candidate proteins expressed by sporozoite stages of the *P. falciparum* parasite and capable of potentiating the immune responses induced by previously reported sporozoite antigens [4,5], we have targeted our research toward the study of the STARP antigen. Twelve peptides binding with high ability to HepG2 cells were identified in STARP by Lopez et al. by performing receptor–ligand binding competition assays [6]. Three of these high-ability binding peptides (HABPs) were successively situated inside the non-repetitive N-terminal region, one of which overlapped the mosaic (M) region; whereas six HABPs were located

* Corresponding author at: Fundación Instituto de Inmunología de Colombia (FIDIC), Carrera 50, No. 26-00, Bogotá, Colombia. Tel.: +57 14815219; fax: +57 1 4815269.
E-mail address: mepatarr@mail.com (M.E. Patarroyo).

0264-410X/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2010.05.025

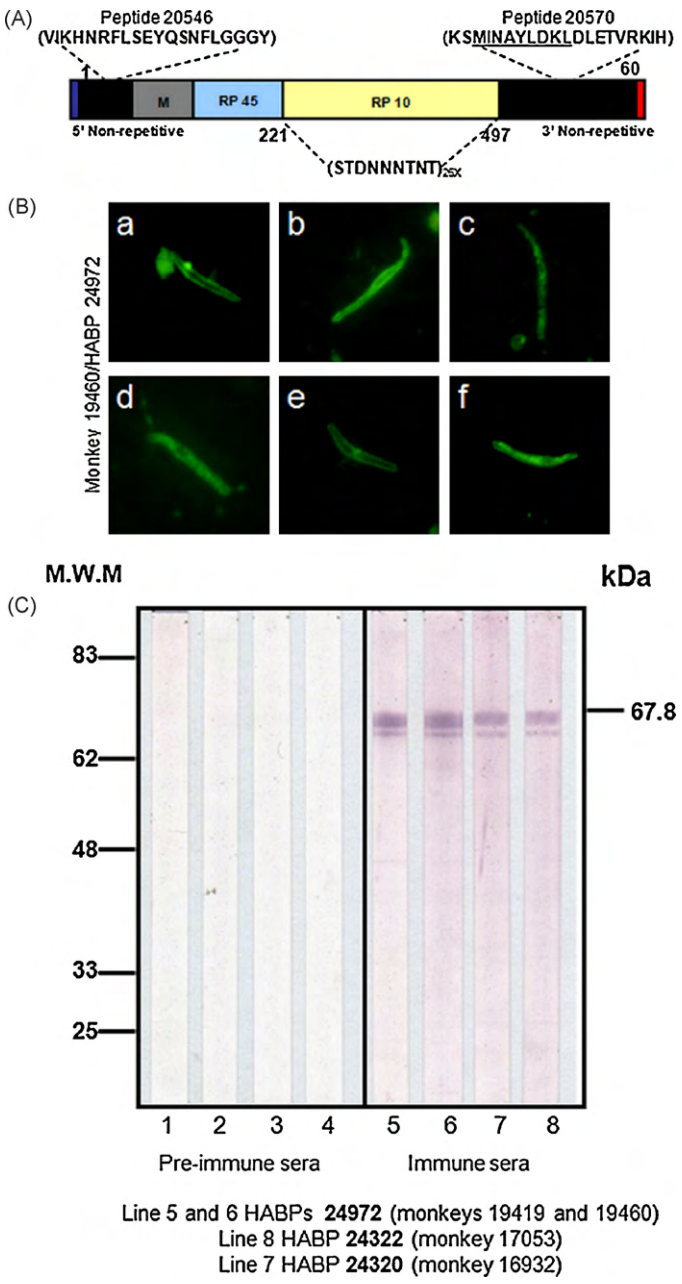

Fig. 1. (A) Schematic representation of the *Plasmodium falciparum* STARP antigen's structure showing the localization of native HABPs 20546 and 20570. (B) Sporozoite immunofluorescence patterns obtained using sera from monkey 19460 immunized with **24972** (20546) which shows recognition of membrane and granular membrane structures. (C) Western blot analysis of sera from *Aotus* monkeys immunized with native and modified STARP HABPs showing the recognition of *P. falciparum* STARP recombinant protein with a 67.8 kDa molecular weight.

in the Rp10 region of the central domain and three HABPs were found in the non-repetitive C-terminal end, one of which overlapped the Rp10 region.

For the development of the present study, we selected those STARP conserved HABPs located outside the repetitive regions M, Rp45 and Rp10 because it has been shown that repetitive sequences are highly antigenic and highly immunogenic but non-protection inducers. Therefore we choose those conserved HABPs located inside the non-repetitive N and C terminal regions: HABP 20546 (^{41}VIKHNRFLSEYQSNFLGGGY^{60}) localized inside the 5′ non-repetitive region and HABP 20570 (^{521}KSMINAYLDKLDLETVRKIH^{540}) located in the 3′ non-repetitive region. Besides binding with high capacity to HepG2, HABP 20570 contained a previously described CTL-inducing epitope (sequence underlined above and in Fig. 1A) associated to the HLA-A2.2 genetic characteristic. Both peptides presented saturable bindings with dissociation constants ranging between 18 and 219 nM and HABP 20570 cross-linked to two hepatocyte membrane proteins of about 38 and 44 kDa [6].

But it has been thoroughly shown that conserved HABPs are non-antigenic, non-immunogenic, non-protective inducers and that to render them into highly immunogenic and protective inducer peptides critical binding residues (identified by glycine analogue scanning) have to be properly modified [7–9].

We have consistently shown that such modifications are associated with the appropriate fit of these modified HABPs inside the macromolecular complex formed by Class II Major Histocompatibility molecules (MHC II) and T cell receptors (TCR), necessary to induce an effective immune response [10–13]. Based on previously published data, some residues were replaced trying to maintain their volume and mass but changing their polarity [13–15] so that they could properly fit into a particular HLA–DRβ1* molecule to be presented to the TCR. Therefore non-immunogenic natives peptides 20546 (VIKH**N**RF**LS**EYQ**SN**FLGGGY) and 20570 (KSMINAYLDK**LD**L**E**TVRKIH) were render into immunogenic-inducing peptides by replacing the critical HepG2 binding residues (in bold and underlined above) according to principles previously described [13–15]. Immunogenicity studies were performed with the above mentioned natives peptides and their corresponding modified analogues **24972**, **24320** and **24486** (modified from 20546); **24322** (modified from 20570) seeking for a correlation between their ^{1}H NMR 3D structures and their immunological activity.

The role played by STARP in the *P. falciparum* exo-erythrocytic cycle inside the human host is still unclear; however, all the aforementioned evidence supports the idea that STARP is involved in sporozoite invasion of hepatocytes and also that STARP HABPs (when being property modified) may be good candidates to be included in the design of a minimal subunit-based, multi-epitopic, multi-stage, chemically synthesized vaccine against *P. falciparum* malaria, so urgently needed.

2. Materials and methods

2.1. Peptide chemical synthesis

Native peptides 20546, 20570 and its corresponding **24972**, **24320**, **24486**, and **24322** modified analogues (shown in bold throughout the paper) were synthesized by the standard solid-phase peptide synthesis methodology [16], purified by reverse-phase HPLC and analyzed by MALDI-TOF mass spectrometry to determine their molecular masses (Autoflex Bruker Daltonics). A glycine-cysteine (GC) tag was added to the peptide's C and N termini during synthesis to allow polymerization following oxidation. The so obtained polymerized peptides were used to immunize *Aotus* monkeys.

2.2. Animals and immunization trials

Aotus monkeys were kept in stainless-steel cages at FIDIC's primate station in Leticia, Amazonas, Colombia, and maintained in strict accordance with the NIH and the Colombian Ministry of Health (Law 84/1989) guidelines for animal care, under the weekly supervision of CORPOAMAZONIA officials and a primatologist. All procedures were approved and supervised by FIDIC's Ethics Committee in Health Research (Resolution No. 008430 of 1993, Colombian Ministry of Health) and FIDIC's Primate

Station Ethics Committee. Once studies had concluded and monkeys were in excellent health conditions, they were released back into the jungle close to the places where they had been captured.

All monkey serum samples were first tested for the presence of antibodies against air-dried fixed *P. falciparum* sporozoites and infected red blood cells (iRBCs) at the schizont stag (1:20 dilution). Monkeys testing positive were returned to the jungle without further manipulation whereas groups of 5 to 8 *Aotus* IFA-negative monkeys were immunized with 125 μg of peptide same as described in previous works [7–15].

2.3. Indirect immunofluorescence assays (IFA)

Slides containing air-dried *P. falciparum* sporozoites (3D7 strain) kindly provided by Dr. Patricia de la Vega (formerly of the Department of Microbiology, University of Maryland School of Medicine; Baltimore, USA) were used for IFA assays. The slides were blocked and processed as described elsewhere [7–15].

2.4. Western blot analysis

A total of 125 μg of *P. falciparum* recombinant STARP (kindly provided by Dr. Pierre Druilhe from the Pasteur Institute, Paris) were separated by discontinuous SDS-PAGE using 12% acrylamide gels (*w*/*v*) and transferred to nitrocellulose membranes. Nitrocellulose membrane strips were individually incubated with each monkey sera diluted 1:100 in blocking solution, washed several times and then incubated with alkaline phosphatase-conjugated goat anti-*Aotus* IgG at a 1:1000 dilution and developed with NBT/BCI.

2.5. Purifying HLA-DR molecules

Purified human molecules were obtained from DR1, WT100BIS (DRβ1*0101), DR3, COX (DRβ1*0301), DR4, BSM (DRβ1*0401), DR7 EKR (DRβ1*0701) and DR11 BM21 (DRβ1*1101) homozygous EBV-B cell lysates by affinity chromatography using anti-HLA-DR L-243 monoclonal antibodies cross-linked to protein A-Sepharose CL-4B (Amersham Pharmacia Biotech AB) as affinity support.

2.6. Peptide-binding competition assays

The ability of unlabeled peptides to compete with biotinylated indicator peptides for purified HLA-DR molecules was assessed in peptide-binding competition assays, as previously described elsewhere [17]. The biotinylated-labeled hemagglutinin (HA) peptide residues 306–318 (PKYVKQNTLKLAT) was used as control peptide for DRβ1*0101 and DRβ1*0401; *Mycobacterium tuberculosis* (MT) 65-kDa Y3–13 peptide (YKTIAFDEEARR) for DRβ1*0301, and tetanus toxin (TT) 830–843 (QYIKANSKFIGITE) for DRβ1*0701 and DRβ1*1101. Relative binding affinities were determined in competition assays, where a peptide inhibiting binding of indicator peptide to the HLA molecule being tested by more than 45% was considered good competitor.

2.7. Circular dichroism (CD) analysis

The CD spectra of the peptides were measured in 50 mM phosphate buffer, pH 7.0, and TFE–water (30:70, *v*/*v*) in a JASCO J810 spectropolarimeter using a 1-mm pathlength cuvette. CD data were expressed as mean residue ellipticity (deg cm^2 $dmol^{-1}$) [18].

2.8. NMR analysis and structure calculations

Ten milligrams of pure peptide were dissolved in 600 μl TFE–water (30/70, *v*/*v*) for NMR experiments. NMR spectra were recorded on a Bruker DRX-600 spectrometer at 295 K. Spectra were assigned according to double-quantum filter correlation spectroscopy (DQF-COSY) [19], total correlation spectroscopy (TOCSY)[20] and nuclear overhauser enhancement spectroscopy (NOESY) experiments [21]. 2D NMR data were processed using TOPSPIN software. NOESY spectra recorded at different temperatures (285–315 K) were used to obtain amide temperature coefficients for predicting hydrogen bonds ($-\Delta\delta H^N/\Delta T$ ppm/K). Distance Geometry (DGII) software was used for gathering a family of 50 structures. These structures were refined by using simulated annealing protocol (DISCOVER software). NOE intensities were calculated and classified into strong (1.8–2.5 Å), medium (2.5–3.5 Å) and weak (3.5–5.0 Å) range interactions (for more details see [22]). Only structures having reasonable geometry and minimum angle and distance violations were selected.

3. Results

3.1. Peptide characterization

Molecular mass determinations of HPLC-purified STARP HABPs and their corresponding modified analogues assessed by MALDI-TOF spectrometry showed a single signal for all peptides which corresponded to their expected molecular masses. The polymers used for immunization had molecular weights in the 8 kDa to 24 kDa range, as assessed by size exclusion chromatography (SEC), suggesting a variable but consistent degree of polymerization.

3.2. Immunogenicity studies

While immunization of *Aotus* monkeys with native 20546 induced no detectable antibodies as assessed by IFA and Western blot, its analogues **24972** and **24320** proved once again that specific modifications had to be performed on native HABP in order to render them into highly immunogenic modified peptides (as assessed by IFA test and Western blot), since antibody titers ranging between 1:320 and 1:1280 were induced 20 days after the first immunization and remained detectable after the 2nd immunization (as determined by IFA and Western blot). Modifications leading to obtaining peptide **24486** induced no antibody responses in *Aotus* monkeys being immunized with this modified peptide, proving once more the specificity and selectivity of the changes needed to render peptides into strong immunogens (Table 1).

The other native HABP (20570) was not immunogenic but its modified analogue **24322** induced high-antibody titers against the sporozoite, as assessed by IFA (1:320 to 1:640) In essence, the studies of immunization trials in *Aotus* monkeys confirmed that conserved HABPs are non-immunogenic (Table 1) unless they were specifically modified according to rules previously described with merozoite's conserved HABPs [23].

Monkey antisera induced by immunization with modified STARP peptide **24972** showed a strong reactivity against membrane (Fig. 1B), cytosol and perinuclear antigens in of air-dried sporozoites by IFA (see Fig. 1B), and a similar immunofluorescence pattern was observed with the antibodies induced by the other modified STARP peptides assessed in this study (data not shown). Such reactivity pattern agrees with the different localizations of microneme organelles in sporozoite forms, inside which STARP is deposited.

Fig. 1C shows the Western blot analysis of sera obtained from *Aotus* monkeys immunized with modified **24972** (lanes 5 and 6) and **24320** (lane 7), both of which were derived from 20546, and **24322** derived from HABP 20570 (lane 8). All three modified peptides show a clear recognition of the 67.8-kDa recombinant STARP protein kindly provided by Prof. Pierre Druilhe).

Table 1
Amino acid sequences of the STARP conserved and modified HABPs (numbered according to our Institute's serial system) used for immunizing *Aotus* monkeys and whose 3D structures were determined by ^{1}H NMR.

Polymerized peptide	Peptide sequence																			PI	I_{20}	II_{10}	II_{15}	II_{20}
STARP							**P_1**			**P_4**		**P_6**			**P_9**									
20546	V	I	K	H	N	R	F	L	S	E	Y	Q	S	N	F	L	G	G	G	Y	0/5	0/5	0/5	0/5	0/5
24972	-	-	-	-	**M**	-	-	H	**V**	D	-	-	**A**	**I**	-	-	-	-	-		0/8	3(1280)	2(640)	ND	3(1280)
24320	-	-	-	-	**M**	-	-	H	**A**	D	-	-	**A**	**P**	-	-	-	-	-	-	0/8	1(640)	1(320)	1(320)	1(320)
24486	-	-	-	-	**N**	-	-	H	**V**	D	-	-	**A**	**P**	-	-	-	-	-	-	0/8	0/8	0/8	0/8	0/8
20570	K	S	M	I	N	A	Y	L	D	K	L	D	L	E	T	V	R	K	I	H	0/5	0/5	0/5	0/5	0/5
24322	-	-	-	-	-	-	-	-	-	-	H	P	-	M	-	-	-	-	-	-	0/8	3(640)	1(320)	1(320)	1(320)

Sequences are aligned according to their binding motifs and HLA-DRβ1 molecule's reading registers to Pockets 1, 4, 6 and 9 (shadowed residues). Antibody titers induced by each peptide in *Aotus* monkeys are shown to the left. PI, I_{20}, II_{10}, II_{15} and II_{20} correspond to the days when monkeys were bled and antibody titers were determined (shown in brackets).

3.3. Interaction with purified HLA-DRβ1* molecules

Binding assays to HLA-DRβ1* isolated molecules showed that native STARP 20546 binds promiscuously and with high capacity to HLA-DRβ1*0101, HLA-DRβ1*0301, HLA-DRβ1*0401 and HLA-DRβ1*0701 molecules a phenomenon very often observed when working with native, non immunogenic HAPBs. However, when modifications were performed to render this peptide into a long-lasting antibody-titer inducer (Table 1), modified HABP **24972** (analogue to 20546) bound with high activity to HLA-DRβ1*0301, (Table 2) displaying the classical binding motifs and binding registers characteristic for this molecule [24]: F7 fitting into Pocket 1, D10 into Pocket 4, Q12 into Pocket 6 and F15 into Pocket 9, suggesting that probably this modified HABP was binding preferentially to HLA-DRβ1*0301-like *Aotus* molecules to induce production of high long-lasting antibody titers in these monkeys. This genetic trait is present in ∼27% of a group of 100 *Aotus* monkeys genotyped by molecular biology methods [25], and in another large of these monkeys recently genotyped (Suárez C. et al. unpublished results), a proportion similar to that of those of monkeys giving a positive immune response (∼37%) when immunized with this modified peptide. The same phenomenon occurred with modified HABP **24320**, which bound to HLA-DRβ1*0301 with a lower binding capacity, inducing also lower antibody titers. Meanwhile, the modified HABP **24486** (analogue to 20546) bound simultaneously with high capacity to HLA-DRβ1*0401 and HLA-DRβ1*0701, both genetic markers showing a combined genetic frequency of ∼40% in the same population of random heterozygous wild Amazonian *Aotus* monkeys from Colombia. This modified HABP did not induce any antibody titers at any point of the trial, as assessed by IFA and Western blot.

The other native STARP-derived HABP (20570) bound with high capacity to HLA-DRβ1*0301 and did not induce any antibody titers, whereas modified analogue **24322** bound to HLA-DRβ1*0101 as well as to HLA-DRβ1*0301. However, binding to HLA-DRβ1*0101 has been uncommon in our assays with different HABPs but since the classical binding register displayed by this peptide is more in agreement with binding to HLA-DRβ1*0101, where Y7 fits into pocket 1, K10 into pocket 4, P12 into pocket 6 and T15 into Pocket 9; therefore it was assigned an HLA-DRβ1*0101 binding capacity. This genetic trait it present in ∼10% of the genotyped *Aotus* population [25] a similar proportion to the proportion of monkeys giving a positive immune response (∼12.5%) when immunized with this **24322** modified HABP.

3.4. Structural analysis

NOESY spectra obtained for peptides 20546 and its modified analogues **24972**, **24320**, **24486**, and for peptide 20570 and its modified analogue **24322** showed sequential, short and medium range $d_{NN}(i,i+1)$ $d_{\alpha\beta}(i,i+3)$, $d_{\alpha N}(i,i+3)$, $d_{\alpha N}(i,i+4)$ NOE connectivities, low amide proton chemical shift temperature coefficients for some of the amino acids, which altogether suggest the presence of an α-helix structure (Fig. 2A). These results were consistent with

Table 2
Structural features of native conserved HABPs from the *P. falciparum* STARP and their corresponding analogues determined by ^{1}H NMR and their associated binding capacity to HLA-DRβ1* molecules.

Protein	Peptide	#	Helical structure	NOEs used	Distance (Å)	Rmsd Å	Maximum NOE violations Å	Haplotypes				
								DR1	DR52		DR53	
								% Binding			HLA-DRβ1*	
								alleles				
								0101	0301	1101	0401	0701
STARP	20546	20	S9-L16	210	20.69	0.21	0.25	**58**	**56**	43	**65**	**55**
	24972	25–19	M5-V9 and I14-G19	212	21.85	0.36	0.30	11	**76**	ND	22	14
	24320	41	K3-D10	193	–	0.21	0.22	26	**45**	27	28	−29
	24486	25	K3-Q12	202	19.25	0.23	0.30	10	**47**	−103	**90**	**52**
	20570	24	I4-T15	184	21.71	0.34	0.35	−1	**72**	−26	14	−110
	24322	24–29	M3-K10 and P12-H20	180	23.10	0.28	0.20	**48**	**64**	0	19	−90

(#) Number of superimposed structures chosen from an initial set of 50 low-energy conformers. Distance (in Å) between the HABP residues theoretically fitting into HLA-DRβ1* Pockets 1 and 9; Rmsd: root mean square deviation of the superimposition. Peptides binding to MHC Class II molecules with ≥45% activity are shown in bold.

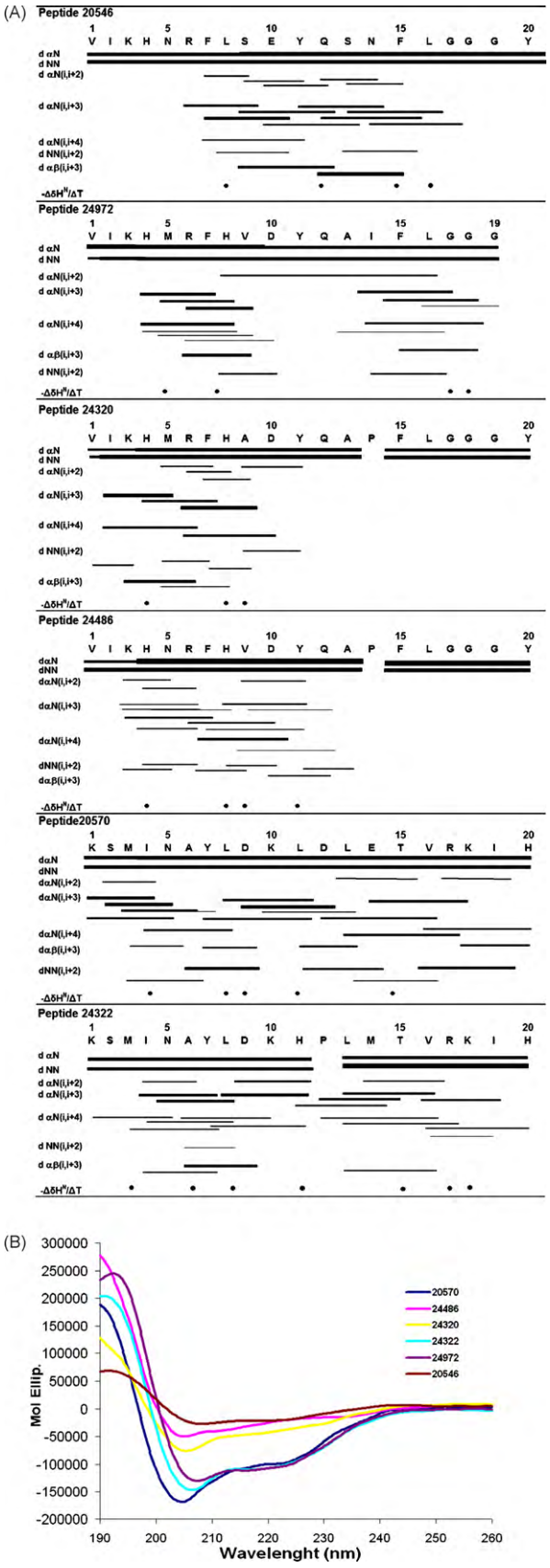

Fig. 2. (A) The most representative sequential medium range NOE connectivities used for determining the structure of native peptide 20546 and its modified analogues **24972**, **24320**, **24486**, and the structure of native 20570 and its modified analogue **24322**. Amide protons having temperature coefficients smaller than 4.0 are indicated by ●. Approximated NOE intensities are indicated by the thickness of the horizontal black bars. (B) Circular dichroism spectra of *P. falciparum* STARP peptides acquired in aqueous solution. Molar ellipticity (deg cm^2 dmol^{-1}) was plotted as a function of the wavelength (nm).

deconvolution analyses using CONTINLL, SELCON and CDSSTR programs [26,27], predicting a 50–95% content of α-helical features in the secondary structures of these peptides.

An average of 26 low energy conformers having no distance violations larger than 0.35 Å or ω angles greater than 1.4° were chosen out of the initial set of 50 structures calculated for peptide 20546 and its modified analogues **24972**, **24320**; **24486**, and for peptide 20570 and its modified analogue **24322**. Average root mean square deviations (RMSD), maximum NOE violations and the number of low energy conformers are shown in Table 2. RMSD values were obtained by superimposing backbone structures between amino acids S9–L16 in peptide 20546, M5–V9 and I14–G19 in peptide **24972**, K3–D10 in peptide **24320**, K3–Q12 in peptide **24486**, I4–T15 in peptide 20570, M3–K10 and P12–H20 in peptide **24322**. The DSSP program [28] assigned a clear α helical structure to all of these peptides (Table 2).

4. Discussion

Over the last 20 years, we have focused our efforts towards blocking red blood cell (RBC) invasion of merozoites with aim of obtaining a fully effective antimalarial vaccine. Such endeavor has comprised synthesizing and testing the RBC binding ability of thousands of peptides, as well as identificating their critical binding residues [29,30].

However, a large number of immunization studies with *P. falciparum* merozoite-derived peptides [31] demonstrated that conserved HABPs are neither antigenic, nor immunogenic or protection-inducing, a problem named by us as immunological silence. To solve this problem, critical RBC binding residues identified by glycine analogue scanning were replaced by others having the same mass and volume but opposite polarity [13] and tested in *Aotus* monkeys [32]. It was by following this strategy that we gathered a pool of potential candidate HABPs to be included in the design of a multi-epitopic, minimal subunit-based antimalarial vaccine [14,15,33].

Now that we have almost completed the functional the characterization of a large number of invasion-relevant merozoite proteins, identifying their HABPs with their critical binding residues we are now focusing our studies in proteins involved in invasion of hepatocytes by using the same strategy, seeking to identify molecules capable of blocking sporozoite's invasion of hepatic cells as the first line of defense against malaria infection and turn such non-immunogenic conserved HABPs into highly immunogenic molecules. Amongst such sporozoite proteins involved in invasion of hepatocytes is STARP, based on whose sequence we synthesized native peptides 20546 (**24972**, **24320** and **24486**) and 20570 (**24322**), together with their corresponding modified HABPs (shown above in brackets and in bold throughout this manuscript to distinguish them from the native peptides from which they were derived) and then proceeded to characterize these peptides regarding their binding ability and immunological and structural properties.

In different Nuclear Magnetic Resonance (NMR) studies performed on a large number of HABPs in 30:70 TFE–H_2O (*v*/*v*) it has been demonstrated that the tridimensional conformations of HABPs are identical to the ones displayed in the complete microbial protein when superimposing their 3D structures with the 3D structures of the protein segments where such HABPs were identified, independently of whether such structures were determined by X-ray crystallography or NMR, as indicated by the RMSD average

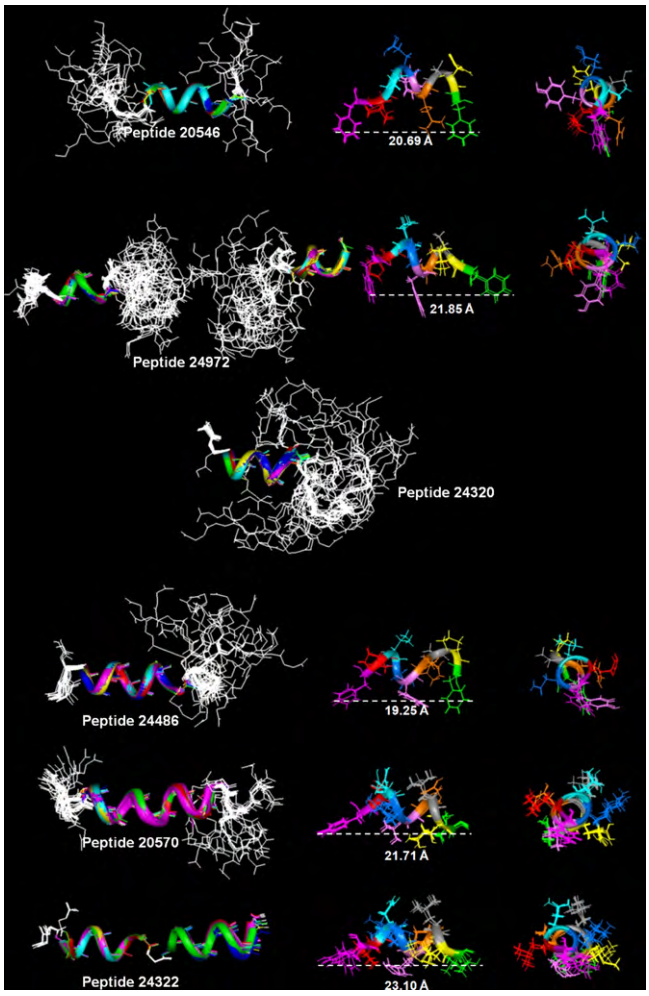

Fig. 3. Three-dimensional models of *P. falciparum* STARP peptides obtained by superimposing several of the structures obtained from an initial set of 50 structures calculated for each peptide. Left-hand and central panels show only the backbone of the molecule where well defined α helices are represented as colored ribbons. Right-hand panel: lateral chains of residues fitting into the HLA-DRβ1* molecules' pockets. Color code: fuchsia: **Pocket 1**, red: P2, turquoise: P3, blue: **Pocket 4**, pink: P5, orange: **Pocket 6**, gray: P7, yellow: P8 and green: **Pocket 9**. Pockets were assigned according to the characteristic peptide motifs and reading registers of each HLA-DRβ1* purified molecule to which these peptides bound. Peptides 20546, **24972**, **24320** and **24486** bound to HLA-DRβ1* 0301, 20570 whereas peptide **24322** bound to HLA-DRβ1* 0101.

values of 1.1 obtained in such peptide/protein superimpositions. These studies have reported α helical, β turn and random structural conformations [34] and not only α helical, therefore showing that such assay conditions help to stabilize rather than induce structural conformations [35].

In general, structural comparison between native HABPs and their modified analogue peptides showed that all modified STARP analogues presented α-helical conformations, which were different from the α-helical segments displayed by their corresponding native STARP HABPs. For instance, seventeen residues were involved in the α helical region formation of the highly immunogenic modified STARP analogue **24322** whereas only 12 amino acids displayed this structural feature in native STARP 20570, indicating that there is an extra α helix in the modified peptide.

Fig. 3 shows the ^{1}H NMR structures of native non-immunogenic peptide 20546 and its modified analogues: the antibody titer-inducing modified peptide **24972** and the modified non-immunogenic peptide**24486**. In each structure, binding motifs and binding register to HLA-DRβ1* molecules are indicated based on the results of binding assays with purified HLA-DRβ1* molecules (Table 2). The residue orientation of the highly immunogenic modified HABP **24972** can be clearly seen in the front view of these molecules (Fig. 3, right hand panel), showing how the Y11 residue (pink) in pocket 5 is downwardly orientated toward the MHC in modified HABPs **24972** and **24486** but does not have the same orientation in native HABP 20546; this same orientation is evidenced in the lateral view of the structures (Fig. 3, central panel).

Moreover, when the structure of the **24972** modified peptide is compared against the structure of the native 20546 peptide (Fig. 3, central panel), the most evident difference is observed in the distance existing between residues fitting into pocket 1 and pocket 9. Such distance is more than 1.3 Å longer in the high-antibody-titer inducing HABP **24972** (21.85 Å), which bound with high capacity to the HLA-DRβ1*0301 molecule, displaying the characteristic binding motifs and binding register for this allele: F7 in Pocket 1, D10 in Pocket 4, Q12 in Pocket 6 and F15 in Pocket 9, which is in complete agreement with the binding motifs and binding registers reported by Marsh et al. [24] for this allele. In spite to the fact that both native 20546 and highly immunogenic modified peptides **24792** and **24320** display the same binding motifs and binding registers to HLA-DRβ1*0301 molecules, reflected in the high binding capacity of all these peptides to this purified Class II molecule, striking differences are observed in HLA-DRβ1*0301 and the putative TCR contacting residues of these peptides. In Fig. 3 it can be clearly seen that in native 20546 HABP, residues fitting into Pockets 4 (E10, dark blue) and 6 (Q12, brown) are awkwardly oriented while in **24972** D10 and Q12, corresponding to residues fitting into Pockets 4 and 6 respectively of this molecule are horizontally oriented to properly fit into the canonical structure of HLA-DRβ1* 0301.

There are also differences in orientation of the lateral chain residues occupying pocket 3 (light blue) and 7 (gray), which are upwardly oriented in opposite direction to the MHC and therefore probably more available to TCR inspection, whereas the same orientation is not observed in native peptide 20546. These data suggest that peptide **24972** displays a different and probably a more appropriate structural conformation to properly fit into the HLA-DRβ1* 0301–**24972**–TCR complex and that modifications performed in residues N5/M, L8/H, S9/V, E10/D, S13/A, and N14/I altered the orientation of these residues and that such modifications shifted the immunological properties of conserved HABP 20546, rendering it into a immunogenic and high-antibody-titer inducing peptide.

A comparative analysis between structured regions of native HABPs and their modified analogues, correlated with their immunological activity, shows that native non-immunogenic HABPs contained different three-dimensional structural conformation when compared with their high-antibody-titer inducing modified analogues. Such was the case of HABP 20546 displaying an α-helix between residues S9–L16, in which modifications done to its critical residues resulted in appearance of two α-helical regions (M5–V9 and I14–G19) in its modified **24972**. In STARP modified peptides **24320** and **24486**, the N14P substitution resulted in a conformational change given that P, being an α helix breaker residue, disrupts the folding of the α helix. Even though proline is located 3 or 1 residues outside the structured region but anyhow inside the sequence of these peptides fitting into this Class II peptide binding region, it produces a conformational change by blocking the continuity of the α helix. This conformation shift might contribute to the appropriate fitting of modified **24972** inside the TCR–Peptide–MHCII complex and the improved immunogenicity shown in *Aotus* monkeys, since no strong conformational changes were evidenced.

The same behavior was evidenced with native HABP 20570 (containing an α helix between residues I4–T15) when compared to

its modified high-antibody-titer inducing **24322** analogue (which displayed 2 α-helical regions between M3–K10 and P12–H20). This analogue bound with high affinity to purified HLA-DRβ1*0101 molecules displaying the classical binding motifs and binding registers for this molecule: Y7 in Pocket 1, K10 in Pocket 4, P12 in Pocket 6 and T15 in Pocket 9. The data suggest that when native 20570 is modified in its critical hepatocyte binding residues (L11/H in Pocket 5, D12/P in Pocket 6 and E14/M in Pocket 8), the resulting modified peptide (HABP **24322**) fits into the HLA-DRβ1*0101 molecule, rendering it capable of inducing long-lasting high-antibody titers in *Aotus* monkeys.

The results also show that native 20546 binds promiscuously to different HLA-DRβ1* molecules, whereas its modified highly immunogenic **24972** and **24320** bind preferentially and with high capacity to HLA-DRβ1*0301 molecules while its modified **24486** (non-immunogenic) binds with high capacity to another Class II molecule, HLA-DRβ1*0401. These modified peptides' immunogenic properties were tested in *Aotus* monkeys, which have proven to be an ideal experimental model due to the high similarity existing between their Class II MHC molecules and their human counterparts [25,36]. Both modified peptides (**24972** and **24320**) induced high-antibody titers, whereas the modified **24486** did not induce antibody production in *Aotus* monkeys. Their 3D structure molecular models were thus determined based on ^{1}H NMR spectral parameters and showed that all peptides presented α-helical conformations in different regions of their structure. Native peptides presented less structured regions and different localization of their α helixes when compared with their modified high-antibody-titer inducing analogues, highlighting the important role played by the peptide's structural conformation in the induction of an appropriate immune response.

Therefore, in order for a minimal subunit-based pre-erythrocytic vaccine to be effective, only conserved HABPs of liver stage proteins must be also selected. We suggest that our modified **24972** and **24322** peptides derived from conserved STARP HABPs 20546 and 20570 could be some of these epitopes given that HABP **24322** contains an amino acid sequence known to induce a CTL-associated response in individuals bearing the HLA-A2.2 genetic characteristic [37]. Furthermore, this modified analogue **24322** was capable of inducing long-lasting high anti-sporozoite antibody titers in *Aotus* monkeys, as shown by the data reported in this study.

Therefore, the results of this study show an association between the NMR structures of native and modified STARP HABPs and the antibody responses induced by these peptides in *Aotus* monkeys. The data support the inclusion of modified STARP **24972** and **24320** HABPs, together with the LSA-1 modified **24322**, as liver-stage components of a multi-antigenic, multi-stage, minimal subunit-based, chemically synthesized antimalarial vaccine.

Funding

This research has been supported by the Asociación para la Investigación Solidaria SADAR (Pamplona, Spain 2009) and the Agencia Española de Cooperación Internacional para el Desarrollo AECID (Madrid, Spain 2009). The funders had no role in the study design, data collection and analysis, decision to publish, or the preparation of the manuscript.

Acknowledgements

We would like to thank Nora Martinez for her collaborations during the translation of this manuscript. Aurora Cortes, Jamer Gomez, Vicente Amaya and Oswaldo Escobar for their assistance and collaboration during the conduction of these studies.

References

[1] Fidock DA, Bottius E, Brahimi K, Moelans II, Aikawa M, Konings RN, et al. Cloning and characterization of a novel *Plasmodium falciparum* sporozoite surface antigen, STARP. Mol Biochem Parasitol 1994;64(April (2)):219–32.
[2] Fidock DA, Sallenave-Sales S, Sherwood JA, Gachihi GS, Ferreira-da-Cruz MF, Thomas AW, et al. Conservation of the *Plasmodium falciparum* sporozoite surface protein gene, STARP, in field isolates and distinct species of Plasmodium. Mol Biochem Parasitol 1994;67(October (2)):255–67.
[3] Pasquetto V, Fidock DA, Gras H, Badell E, Eling W, Ballou WR, et al. *Plasmodium falciparum* sporozoite invasion is inhibited by naturally acquired or experimentally induced polyclonal antibodies to the STARP antigen. Eur J Immunol 1997;27(October (10)):2502–13.
[4] Bermudez A, Vanegas M, Patarroyo ME. Structural and immunological analysis of circumsporozoite protein peptides: a further step in the identification of potential components of a minimal subunit-based, chemically synthesised antimalarial vaccine. Vaccine 2008;26(December (52)):6908–18.
[5] Patarroyo ME, Cifuentes G, Rodriguez R. Structural characterisation of sporozoite components for a multistage, multi-epitope, anti-malarial vaccine. Int J Biochem Cell Biol 2008;40(3):543–57.
[6] Lopez R, Garcia J, Puentes A, Curtidor H, Ocampo M, Vera R, et al. Identification of specific Hep G2 cell binding regions in *Plasmodium falciparum* sporozoite-threonine-asparagine-rich protein (STARP). Vaccine 2003;21(June (19–20)):2404–11.
[7] Espejo F, Cubillos M, Salazar LM, Guzman F, Urquiza M, Ocampo M, et al. Structure, immunogenicity, and protectivity relationship for the 1585 malarial peptide and its substitution analogues. Angew Chem Int Ed Engl 2001;40(December (24)):4654–7.
[8] Purmova J, Salazar LM, Espejo F, Torres MH, Cubillos M, Torres E, et al. NMR structure of *Plasmodium falciparum* malaria peptide correlates with protective immunity. Biochim Biophys Acta 2002;1571(May (1)):27–33.
[9] Salazar LM, Alba MP, Torres MH, Pinto M, Cortes X, Torres L, et al. Protection against experimental malaria associated with AMA-1 peptide analogue structures. FEBS Lett 2002;527(September (1–3)):95–100.
[10] Bermudez A, Cifuentes G, Guzman F, Salazar LM, Patarroyo ME. Immunogenicity and protectivity of *Plasmodium falciparum* EBA-175 peptide and its analog is associated with alpha-helical region shortening and displacement. Biol Chem 2003;384(October–November (10–11)):1443–50.
[11] Cifuentes G, Patarroyo ME, Urquiza M, Ramirez LE, Reyes C, Rodriguez R. Distorting malaria peptide backbone structure to enable fitting into MHC class II molecules renders modified peptides immunogenic and protective. J Med Chem 2003;46(May (11)):2250–3.
[12] Cubillos M, Alba MP, Bermudez A, Trujillo M, Patarroyo ME. *Plasmodium falciparum* SERA protein peptide analogues having short helical regions induce protection against malaria. Biochimie 2003;85(July (7)):651–7.
[13] Cifuentes G, Bermudez A, Rodriguez R, Patarroyo MA, Patarroyo ME. Shifting the polarity of some critical residues in malarial peptides' binding to host cells is a key factor in breaking conserved antigens' code of silence. Med Chem 2008;4(May (3)):278–92.
[14] Cifuentes G, Espejo F, Vargas LE, Parra C, Vanegas M, Patarroyo ME. Orientating peptide residues and increasing the distance between pockets to enable fitting into MHC-TCR complex determine protection against malaria. Biochemistry 2004;43(June (21)):6545–53.
[15] Alba MP, Salazar LM, Purmova J, Vanegas M, Rodriguez R, Patarroyo ME. Induction and displacement of an helix in the 6725 SERA peptide analogue confers protection against *P. falciparum* malaria. Vaccine 2004;22(March (9–10)):1281–9.
[16] Houghten RA. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. Proc Natl Acad Sci USA 1985;82(August (15)):5131–5.
[17] Frayser M, Sato AK, Xu L, Stern LJ. Empty and peptide-loaded class II major histocompatibility complex proteins produced by expression in Escherichia coli and folding in vitro. Protein Expr Purif 1999;15(February (1)):105–14.
[18] Chen YH, Yang JT, Martinez HM. Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. Biochemistry 1972;11(October (22)):4120–31.
[19] Rance M, Sorensen OW, Bodenhausen G, Wagner G, Ernst RR, Wuthrich K. Improved spectral resolution in cosy 1H NMR spectra of proteins via double quantum filtering. Biochem Biophys Res Commun 1983;117(December (2)):479–85.
[20] Bax A, Davis DG. MLEV-17 based two dimensional homonuclear magnetization transfer spectroscopy. J Magn Reson 1985:65.
[21] Jenner J, Meier BH, Backman P, Ernst RR. Investigation of enhance processes by two-dimensional NMR spectroscopy. J Chem Phys 1979;71:4546–5427.
[22] Bermudez A, Alba P, Espejo F, Vargas LE, Parra C, Rodriguez R, et al. Fitting modified HRP-I peptide analogue 3D structure into HLA-DR molecules induces protection against *Plasmodium falciparum* malaria. Int J Biochem Cell Biol 2005;37(February (2)):336–49.
[23] Patarroyo ME, Patarroyo MA. Emerging rules for subunit-based, multiantigenic, multistage chemically synthesized vaccines. Acc Chem Res 2008;41(March (3)):377–86.
[24] Marsh SG, Parham P, Barber LD. The HLA fact book; 2000.
[25] Suarez CF, Patarroyo ME, Trujillo E, Estupinan M, Baquero JE, Parra C, et al. Owl monkey MHC-DRB exon 2 reveals high similarity with several HLA-DRB lineages. Immunogenetics 2006;58(July (7)):542–58.

[26] Sreerama N, Venyaminov SY, Woody RW. Estimation of the number of alpha-helical and beta-strand segments in proteins using circular dichroism spectroscopy. Protein Sci 1999;8(February (2)):370–80.
[27] Sreerama N, Woody RW. Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. Anal Biochem 2000;287(December (2)):252–60.
[28] Kabsch W, Sander C. Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. Biopolymers 1983;22(December (12)):2577–637.
[29] Rodriguez LE, Urquiza M, Ocampo M, Suarez J, Curtidor H, Guzman F, et al. *Plasmodium falciparum* EBA-175 kDa protein peptides which bind to human red blood cells. Parasitology 2000;120(March (Pt 3)):225–35.
[30] Urquiza M, Rodriguez LE, Suarez JE, Guzman F, Ocampo M, Curtidor H, et al. Identification of *Plasmodium falciparum* MSP-1 peptides able to bind to human red blood cells. Parasite Immunol 1996;18(October (10)):515–26.
[31] Patarroyo ME, Cifuentes G, Bermudez A, Patarroyo MA. Strategies for developing multi-epitope, subunit-based, chemically synthesized anti-malarial vaccines. J Cell Mol Med 2008;12(October (5B)):1915–35.
[32] Rodriguez R, Moreno A, Guzman F, Calvo M, Patarroyo ME. Studies in owl monkeys leading to the development of a synthetic vaccine against the asexual blood stages of *Plasmodium falciparum*. Am J Trop Med Hyg 1990;43(October (4)):339–54.
[33] Espejo F, Bermudez A, Torres E, Urquiza M, Rodriguez R, Lopez Y, et al. Shortening and modifying the 1513 MSP-1 peptide's alpha-helical region induces protection against malaria. Biochem Biophys Res Commun 2004;315(March (2)):418–27.
[34] Rodriguez LE, Curtidor H, Urquiza M, Cifuentes G, Reyes C, Patarroyo ME. Intimate molecular interactions of *P. falciparum* merozoite proteins involved in invasion of red blood cells and their implications for vaccine design. Chem Rev 2008;108(September (9)):3656–705.
[35] Patarroyo ME, Cifuentes G, Martinez NL, Patarroyo MA. Atomic fidelity of subunit-based chemically-synthesized antimalarial vaccine components. Prog Biophys Mol Biol 2010;102(January (1)):38–44.
[36] Nino-Vasquez JJ, Vogel D, Rodriguez R, Moreno A, Patarroyo ME, Pluschke G, et al. Sequence and diversity of DRB genes of Aotus nancymaae, a primate model for human malaria parasites. Immunogenetics 2000;51(March (3)):219–30.
[37] BenMohamed L, Thomas A, Druilhe P. Long-term multiepitopic cytotoxic-T-lymphocyte responses induced in chimpanzees by combinations of *Plasmodium falciparum* liver-stage peptides and lipopeptides. Infect Immun 2004;72(August (8)):4376–84.