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Chapter 15

The Next Vaccine Generation Against Malaria: Structurally Modulated *Plasmodium* Antigens

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Additional information is available at the end of the chapter

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Abstract

Challenges for obtaining more effective malaria vaccines depend on precise selection of antigenic motifs and understanding the complexity of *Plasmodium* spp. life cycle. Naturally expressed antigens are characterized for being weak immunogenic when tested as vaccine components, thus these have to be strategically modified to render them immunogenic. A molecular clue in this pursuit is provided by the chemical peptide-bond processing by peptidases, which follows a multistep pathway including ephemeral high energy molecular complexes known as transition states. Thus, we have proposed non-natural peptide-bond isosteres as transition states mimetics, and therefore, stabilizing these high-energy states with site-directed designed immuno-mimetics have demonstrated being a rational approach for stimulating antibody populations harboring multiple functional capacities. Therefore, peptide-bond substitutes constitute a coherent pathway towards obtaining selected immuno-active compounds from specific plasmodial molecular objectives. Chemical strategies for synthesizing peptido-mimetics and antimalarial selected trials lead us to assess a number of peptide-bond substitutes for obtaining immuno-active and structurally defined molecules. *Plasmodium* antigens expressed on merozoite, sporozoite and gametocyte stages have been selected as targets and subsequently modified based on the presence of either a high-binding motif or a potential HLA-reading frame. This new family of immuno-mimetics is and efficient neutralizing antibody inducers when tested in *in vitro* and *in vivo* experiments, thus representing a new generation of malaria vaccine components.

Keywords: malaria, synthetic vaccine, non-natural elements, immuno-mimetic, functional antibody

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1. Introduction

Malaria is spreading from old *Plasmodium* colonized territories to other zones of the earth where this lethal disease did not exist before covering about 45% of the planet. Over decades an important number of public health trials for malaria eradication and control have been conducted with a limited success. Malaria is caused by *Plasmodium* spp. to a susceptible human being and is transmitted by the *Anopheles* female mosquito bite and is responsible of 660,000 deaths and around 219 million new cases annually (a range of 154–289 million) especially in children younger than 5 years of age and pregnant women inhabitants of endemic and high transmission areas [1–3].

A number of difficulties inherent to many causes among the pathogen resistance to antimalarials, poor coverage of public health programs and natural human host genetic restrictions make the finding of an effective malaria vaccine be an urgent need.

Malaria vaccine development has constituted a big challenge for researchers, up-to-date about 236 vaccine candidate prototypes are being tested. Approaches based on strategies such as immunization with irradiated sporozoites of *Plasmodium* spp., as well as DNA-based immunogens besides recombinantly expressed antigens such as RTS,S formulated in different vehicles and strong adjuvant systems and prototype delivery systems such as virosomes besides to the most promising strategy constituted by synthetic peptides representing subunit multistage immunogens formulated in human allowed adjuvants represent the main attempts for immuno-prophylaxis [2].

Plasmodium spp. express a number of antigens, more than 100 in its different life cycle stages and most of them have been regarded as vaccine targets such as the so-named merozoite surface antigens 1–10 (MSP1–MSP10), erythrocyte binding antigens EBA-140 and EBA-175, the ring-infected erythrocyte surface antigen (RESA-155), the apical membrane antigen AMA-1 among many others from the merozoite stage including a number of organelle's proteins. Besides the circumsporozoite surface protein (CSP), sporozoite threonine and asparagine-rich protein (STARP), the sporozoite and liver stage antigen (SALSA), the liver stage antigen (LSA) are some representative antigens of the sporozoite stage and the classical Pfs48/45, GSA and Pbs25/28 from gametocytes have been regarded as the targets for transmission blocking vaccines [4].

Natural immunity to malaria is related to hemoglobin structure, some disorders such as thalassemia confer resistance to *Plasmodium falciparum* while Duffy negative RBC constitutes the mechanisms associated to malaria resistance to *Plasmodium vivax*. Immune response against a natural malaria infection is nonspecific and has a weak effect on protection. Innate immunity mediated by NK and non-related auto antibody B-cells as well as the INF-gamma response to infected red blood cells act as a primary line of defense against *Plasmodium* infection. In the adaptative immunity step $CD4^{+}$, dendritic cells, macrophages, gamma delta T cells and NKT cells are able to detect the parasite and participate in the immune defense. Besides natural immunity to this disease, an effective malaria vaccine can be proposed for a strong stimulation of antibody B-cells. However, at is has been demonstrated that most *Plasmodium* native-derived

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sequences are proven to be poorly immunogenic and non-protection inducers against malaria. In order to understand this problem, Patarroyo have established a strategy based on selection of non-polymorphic regions of selected antigens and a subsequent rational mutation of those residues belonging to red blood cells and hepatic cells binding motifs allow modified active antigens [4], however, most clinical trials worldwide which have been performed with the above mentioned malaria vaccine candidates have failed to achieve significant protection levels, evidencing intrinsic difficulties for developing a potent fully protective vaccine formulation. Perhaps pathogens including *Plasmodium* spp. have evolved complex mechanisms to recognize, block and destroy natural-presented antigens as vaccines as well as others related to antigen structure modulation.

Our group has introduced in this pursuit some non-natural elements to be incorporated into synthetic antigens with the aim of governing both antigen presentation as well as specific B-cells for functional neutralizing antibody stimulation. Some of these non-natural elements included capture sequences for stimulating antigen degradation and others for the peptide-bond structure modulation. Peptide-bond isosteres included reversal configuration thereof, urea motifs and reduced amide as peptide-bond surrogates all constituting a novel immunogen family herein named as immuno-mimetics.

Once strategically incorporated into selected antigens, produced peptide-bond surrogates overcome non-desirable properties of native non-modified antigens such as cytotoxicity and hemolytic profiles, besides prolonging these new molecules half-life and a remarkably strong immuno-stimulating activity that can be associated to the newly introduced freedom degrees to the 3D structure of immuno-mimetics.

Also, we have consolidated the female BALB/c animal model for malaria vaccine candidate testing based on controlled challenging performed to immunized animals with two rodent malaria strains, being those *Plasmodium berghei ANKA* and *Plasmodium yoelii*-17XL. Additionally, passive transferring antibodies into infected animals have proven to be efficient for malaria disease control and parasite clearance.

2. Global health statistics, economical and environment determinants

In 2015, time for fulfillment of the millennium development goals (MDGs) was getting closer to the end, and a consequent protocol comprising 17 sustainable development goals (SDGs) constitute the next step. In its annual report, World Health Organization (WHO) analyzed 15 years of advances of those proposed MDG and evaluated the next challenges for the coming years.

As reported in world health statistics 2015 issued by the WHO [1], undernutrition was the main cause of mortality in an assessed 45% of all deaths of children under 5 years of age. In the 1990–2013 period, the estimate of underweight children in third-world countries decreased from 28 to 17%, and a sustainable decreasing rate to 16% was expected for the end of 2015. In spite of those proposed efforts for achievement of MDG, these numbers are not still sufficient

to the goals. The proportion of underweight children declined globally from 25% in 1990 to 15% in 2013.

Worryingly, poverty is strongly associated with public health especially to problems related to high transmission of infectious diseases. As observed in **Figure 1A**, among the main causes of deaths among children under 5 years (in neonatal and post-neonatal ages), between 2000 and 2013 are responsibility of infectious diseases, pneumonia, malaria, HIV/AIDS, measles, diarrhea and sepsis are the main reasons of mortality accounting 13 and 35%, respectively. Malaria represents 7% of children mortality mainly in the post-neonatal period between 1 and 59 months of age.

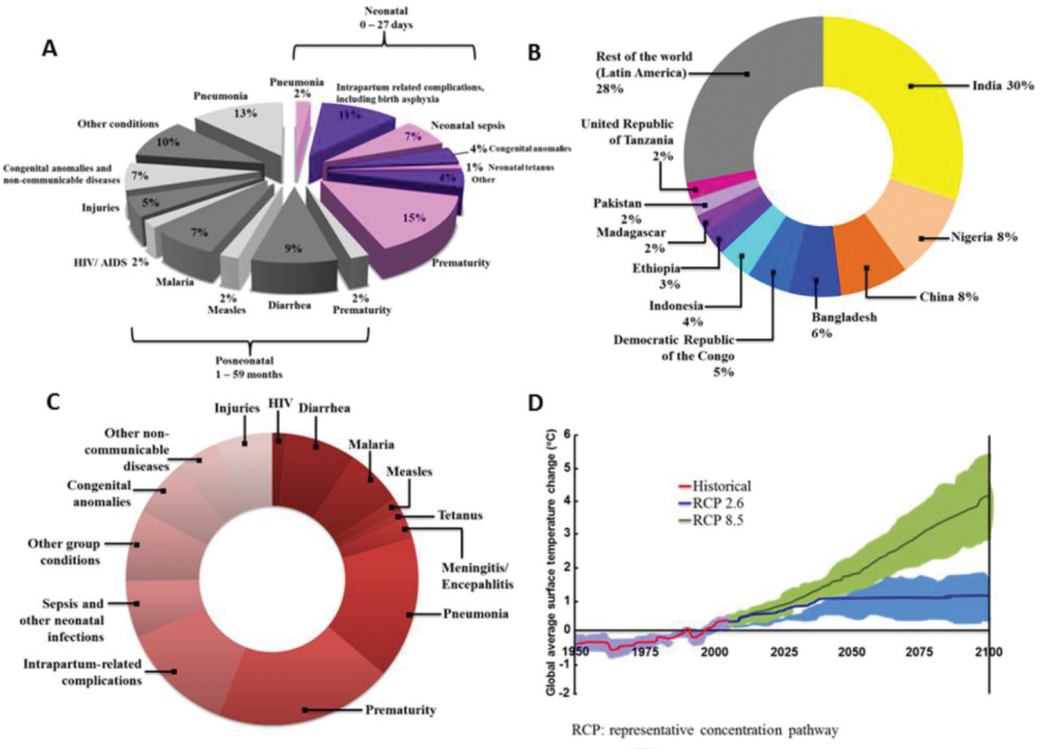

Figure 1. Health statistics and world climate. (**A**) Causes of deaths among children under 5 years of age. **(B)** Top 10 countries with largest share of the global extreme poverty. **(C)** Main causes of child mortality due to transmissible diseases. 5.9 million children under age five died in 2015, nearly 16,000 every day. **(D)** Climate changes and greenhouse effect. Global average surface temperature changes under two scenarios for considering the global greenhouse gas emissions between years 1950 and 2100. RCP for representative concentration pathway. This figure has been adapted from information provided by WHO [1–3].

In the last year, 836 million of the world population lived on less than US$1.25 daily in comparison with 1.9 billion in 1990. In those the so-named poor countries, 14% of the people lived on less than US$1.25 daily in the same year, regarding the 47% in 1990. Getting closer to an amount of US$2 daily has been difficult at higher poverty levels.

The most inhabited counties of the world such as People's Republic of China and India have been crucial for world reduction in poverty (indeed India remains the earth's country having most extreme poverty; **Figure 1B**) such reduction can be associated to growth of central

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economic sectors and labors. Other factors such as income transferring, remittances and evolving new demographic profiles have had a lesser impact. However, those efforts have not been enough since one of each seven people in poor countries live on less than US$1.25 daily. In the sub-Saharan countries, more than 40% people are living in extreme poverty in 2015. In the countries having middle-incomes, the 73% of the Earth's poverty is found [3].

Figure 1B displays the top 10 countries with largest share of the global extreme poor, accordingly with WHO classifications, these countries are inhabited by people living on less than US $1.25 per day. Therefore, poverty levels show India 30%, Latin America 28%, China 8% and dramatically 20% represents African countries (Nigeria, Democratic Republic of the Congo, Ethiopia, United Republic of Tanzania, among others). Child mortality was 5.9 million children under age five which died in 2015, nearly 16,000 per day, mainly caused by infectious diseases whose distribution can be observed in **Figure 1C**. Main causes of child death are due to measles, malaria, diarrhea, HIV/AIDS, meningitis/encephalitis, tetanus and sepsis and other neonatal infections besides prematurity among other causes [1–3].

The sustainable development goals (SDGs) also contain ambitious targets for child mortality, with SDG 3.2 seeking to end preventable deaths of newborns and children under five. Those have included local aims for reducing the under-five mortality rates (U5MR) around to 25 deaths per 1000 live births as well as the neonatal mortality rate (NMR) to lower than 12 per 1000 live births, in comparison with a world's U5MR rate of 43 per 1000 live births in the last year, representing 5.9 million deaths of children under 5 years and a NMR rate of 19 per 1000 live births, representing 2.7 million deaths in the first month of life. Main causes of newborn mortality during the last year were prematurity, birth-related complications and neonatal sepsis, while those post-neonatal causes of death were associated to pneumonia, diarrhea, injuries and malaria. Specifically, the so-called Target 4.2 in the document, which encourage for assuring that most children have access to good quality development, heath assistance and care, and basic education join to reducing child mortality while improving a better living quality for childhood in most poor countries [3].

On the other hand, **Figure 1D** displays climate changes and greenhouse effect on earth for a period between 1950 projected to the wear 2100. As described, global average surface temperature change is estimated under two scenarios for turning around global greenhouse gas emissions [1].

The global climate warming is a reality. The average data for the Earth's surface temperature showed a 0.85°C increasing (0.65–1.06) for the 1880–2012 period. Data show that the Earth's north hemisphere had the warmest period from 1983 to 2013, being the highest regarding the last 1400 years. Without any doubt, most causes for this fact can be associated to human activities. Mathematical and predictive algorithms for global warming-cooling allow establishing precise predictions on climate changes over long-time periods, these have included factors such as volcanic activity and gas emissions to the atmosphere. The Intergovernmental Panel on Climate Change's (IPCC) for temperature changing prediction have considered a number of factors and possibilities for future greenhouse gas emissions, which have been termed as representative concentration pathways (RCP). This ranges from RPC 2.6 which considers that global greenhouse gas emissions will reach a highest value between 2010 and

2020, then it significantly decrease after 2020, to RCP 8.5, in which greenhouse gas emissions will continue to increase during the present century. Middle-range positions consider that RCP 4.5 and 6.0 would reach the highest emission values in 2040 and 2080 in consequence [1].

World's predictive temperature changes for 2015–2016 period regarding those recorded between 1986 and 2005 are estimated to vary between 0.3 and 0.7°C. Similarly, increasing temperature ranges for the 2081–2100 period regarding the recorded changes between 1986 and 2005 has been estimated to be 0.3–1.7°C (RCP 2.6) to 2.6–4.8°C (RCP 8.5) (**Figure 1D**). In consequence, the Arctic region's warming rate will increase faster than the world's mean, and that for the land's rate will be higher than the mean for the ocean. Assessed RPCs led to estimate that sea level will growth from 0.26 to 0.82 m by the final of the current age. Earth's surface warming and climate variations will have a deep impact on human living, health and welfare, since obtaining drinking water and the possibility of cultivating the necessary quantities of agricultural products and all resources required for the future world's larger population will be compromised.

3. Malaria: a devastating disease

Malaria is a global disease responsibly of high levels of morbidity and mortality especially in developing countries whose inhabitant populations suffer the consequences of the disease besides the economic impact on these populations. At the beginning of the new millennium, a global strategy for controlling malaria by establishing a global founding for fighting three high impact diseases, i.e., AIDS, tuberculosis and malaria have been proposed by the World Health Organization (WHO) [5]. The 2015 world malaria report from WHO account data from 79 countries affected by this disease reflecting a slight improvement in controlling the disease impact but the problem still remains for a solution. In 2013, diagnosis tests were expanded to most malaria affecting countries and huge steps towards vector control were also conducted. In 2013, the use of insecticides impregnated mosquito nets were promoted and so the amount of populations protected against malaria were increased, thus mortality due to malaria was reduced to 47% between the years 2000 and 2013. However, endemic areas are still far away from reaching a total coverage for malaria control and available founding is each time decreased for managing this important problem. An estimated 278 million people in Africa live in households without a single insecticide mosquito net and 15 million pregnant women have no access to a preventive treatment for malaria. In addition, other diseases affecting these populations alter the development of related campaigns is the case of Ebola whose recent outbreak have conducted to a decreasing in health assistance in those affected zones.

In the last five years, it is estimated that 584,000 deaths due to malaria have occurred (367,000–755,000) of which 78% were children under 5 years of age and 90% came from Africa; today, there are an estimated 3.2 billion people at risk of contracting the disease since are living in areas influenced by the disease, of which 1.2 billion are at high risk (more than 1 into 1000 possibility of acquiring malaria in the year); in the Region of the Americas, it is presumed that the risk is 120 million people in 21 countries in the region [5].

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Eradication efforts by public health preventive measures are not sufficiently effective for many reasons, among which are the socioeconomic, demographic and technical policies, emerging resistance to insecticides by the vector and to antimalarial drugs by the parasite [6]. In 2010, vector resistance had been reported in 49 countries around the world of which 39 reported resistance above two or more pyrethroid insecticides. In 2013, this report increased to 82 countries reporting insecticide resistance [3], therefore, to develop an effective vaccine against the disease becomes an urgent need.

By 1967, major efforts were made to find an effective vaccine against human malaria, in one of the most important related studies of the time, 59% protection was achieved after an intravenous challenge of a malaria murine model after being vaccinated with 75,000 live attenuated irradiated sporozoites [7].

Currently among vaccine candidates that are in more advanced clinical trials are the RTS,S and *Pf*SPZ which incorporates the use of non-replicative attenuated sporozoites through controlled radiation [8], which has obtained a dose dependent protection in humans being necessary the application of 1.35×10^5 attenuated sporozoites in five doses [9]. It should be noted that the duration of protective antibodies has not been fully established, thus obtaining a vaccine is not a reality.

As can be observed in **Figure 2**, global malaria spreading accounts for more than 80 countries that are affected by malaria infection (purple background in the map). Besides, insecticide susceptibility status for malaria vectors (*Anopheles* female mosquitoes) demonstrates a resistance increasing to most insecticides concomitant with areas of high transmission of malaria.

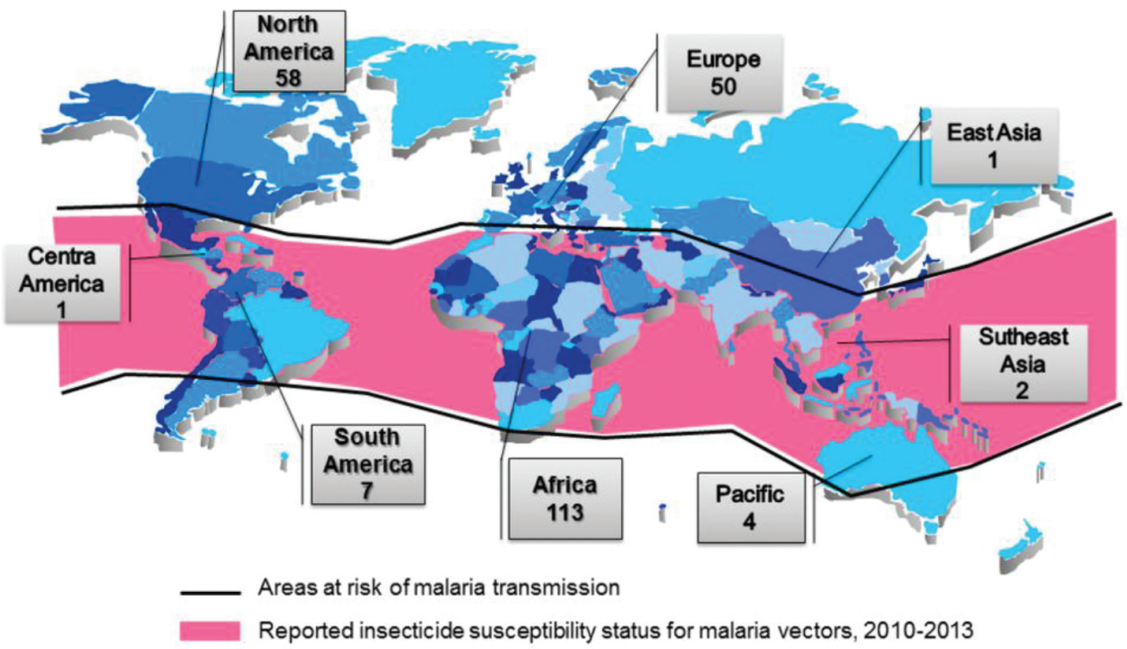

Figure 2. Areas of risk of malaria transmission and ongoing malaria vaccine candidate trials.

4. *Plasmodium* spp. life cycle

A deep knowledge and understanding of the *Plasmodium* parasite life cycle would be a key step towards antigen discovery, and it will establish the molecular basis for a proper immunogen designing to be further tested as a vaccine candidate. The *Plasmodium* spp. belong to the phylum Apicomplexa being the causative agent of malaria, whose clinical and pathological manifestations are associated with asexual erythrocytic stage of the parasite [10].

There are five species of *Plasmodium* causing human malaria, the most lethal disease is caused by *P. falciparum* and followed by *P. vivax*, and less prevalent are *Plasmodium malariae* and *Plasmodium ovale* [11] in 2011 *Plasmodium knowlesi* was included in this list. In Colombia for the year 2014, 356 clinical cases of uncomplicated malaria were reported, 20,074 cases of malaria by *P. vivax*, 19,789 by *P. falciparum*, 17 cases by *P. malariae* and 561 cases of mixed malaria according to data presented by the National Institute of Health (INS) in its weekly report SIVIGILA [12].

Plasmodium parasites have a complex life cycle involving interactions of invertebrates (vector) and vertebrates (mammalian host), besides presenting various stages in intracellular and extracellular environments (**Figure 3**) [13].

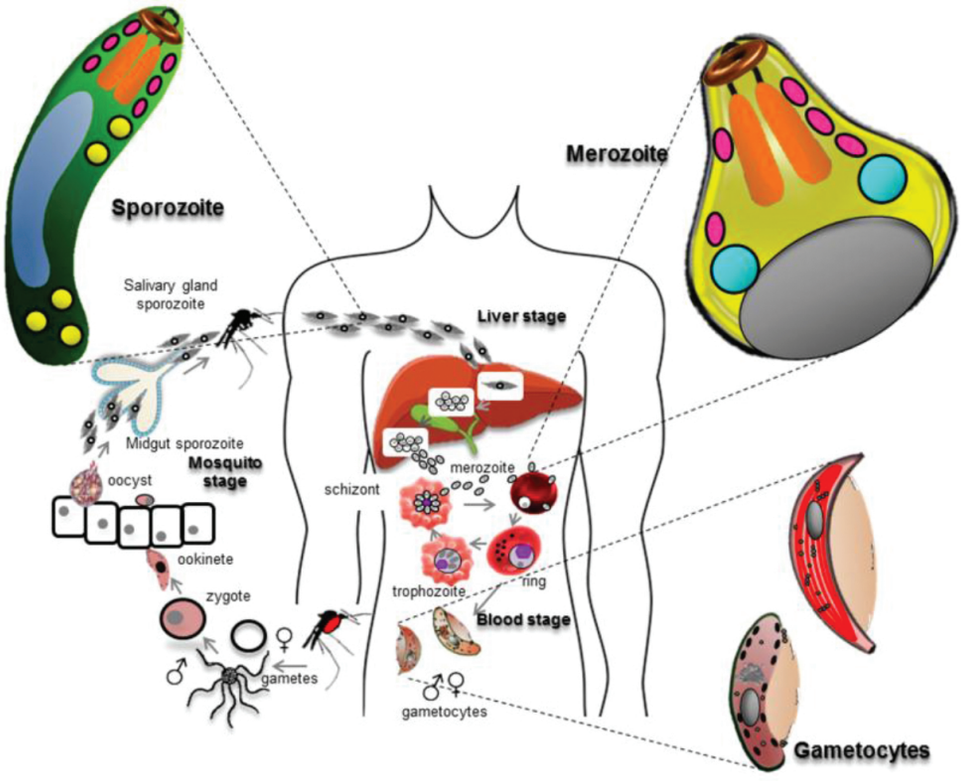

Figure 3. ***Plasmodium*** **life cycle**. Main pre-erythrocyte, blood and sexual stages are denoted.

In the human host, sporozoites are inoculated by the bite of female *Anopheles* spp. mosquitoes, then invade hepatocytes in a time between 5 and 30 min; within hepatocytes each sporozoite

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develops a schizont which release between 10,000 ± 30,000 merozoites to blood stream during a period of 2 ± 10 days depending on the parasite specie [14], in the case of *P. vivax* and *P. ovale* also it produces a different stage in the liver called hypnozoite, which is a silent form responsible for the subsequent relapses [11]. During the travel from the skin to the liver, the parasites cross the capillary epithelium in the dermis and enter to blood circulation, cross the hepatic sinusoids epithelium to enter the parenchyma, and this process as the hepatocyte infection are given by activity of the myosin-actin engine located in the plasma membrane of the parasite and its rhoptries, dense granules and micronemes [15].

The invasion of erythrocytes occurs after several steps with multiple interactions between receptor membrane proteins of host cells and parasite protein ligands expressed in its surface as well as in rhoptries and micronemes [16]. The parasite grows and divides in about 72–48 h according to the specie to the schizont stage which contains more than 30 merozoite particles, which are released with the subsequent invasion and replication in healthy erythrocytes [17]. Acquired immune response induced by malaria parasites is complex and varies depending on the level of endemicity, epidemiology, genetic, age of the host, parasitic stage and parasite species. Repeated infections and continued exposure are required to achieve clinical immunity with symptom reduction and reduced number of parasites in an infected individual or inhibition of parasite replication [18].

5. The murine model in the search for vaccine candidates against malaria

The mouse model has been widely used in the study on malaria, and it has been regarded as a practical model for experimental studies since its genetical features regarding human beings such as homology and similarity at the protein structure level, physiology and life cycle besides of owning a malaria transmission vector (*Anopheles stephensi*) that can be maintained under defined laboratory [19].

Due to this, there are several *Plasmodium* strains that infect rodent models by malaria (*P. berghei, P. yoelii, P. chabaudi, P. vincker*), and their experimental behavior can be extrapolated due to the fulfillment of a standard life cycle under controlled conditions. The two most commonly employed strains in malaria vaccine discovery are the *P. yoelii* and *P. berghei*, which have high similarity with the clinical symptoms and pathology developed by *P. falciparum* in relation to those stages of cerebral malaria, placental malaria, severe malaria and organ damage as liver, kidney and lung [20]. The *P. berghei* infection model has allowed demonstrating the role of interferon in response to parasite replication in the liver, and this participation was then demonstrated in *P. falciparum* [20].

The rodent malaria infection by *P. yoelii* has allowed to demonstrate that humans immunized with the *Pf*CS protein from the parasite sporozoite stage, induced antibodies that cross-react with *P. yoelii*, as well as mice immunized with *Py*CSP stimulated antibodies that cross-react with *P. falciparum*; therefore, this model evidenced its usefulness as a predictive tool for immune response against certain malarial antigens since there are a 70% of genome similarity between *P. yoelii and P. falciparum* [14]. This similarity associate at least 3300 orthologous

genes of *P. yoelii* with 5268 genes of *P. falciparum* [21]. Although the erythrocytic cycle of *P. berghei* and *P. yoelii* takes place more rapidly (24 and 18 h, respectively) compared to those developed in *P. falciparum* (36–48 h) and *P. vivax* (48 h), differences in tropism for invasion of reticulocytes in the case of *P. yoelii* and *P. berghei* are not presented in *P. falciparum* and the genetic similarity between these *Plasmodium* is quite important, and so the rate of increase in parasitemia levels is similar during the first 3–4 days after inoculation *in vitro* as well as the parasite growth [22]. Also, *in vivo* conditions and clinical symptoms associated to the disease are presented in terms of fever, malaise, splenomegaly and breathlessness related with red blood cells rupture which differ of symptoms present in infections with other strains such as *P. chabaudi* where in contrast the infection is associated with hypothermia [23]. The production and regulation in the cytokines expression are no exception to the similarity between the mouse model and a malaria human infection since *P. yoelii* and *P. berghei* replicate many events that can be correlated between both of the infection types [24, 25].

In studies at the level of liver infection cycle were found that about 654 (92%) of proteins in *P. yoelii* correlated with orthologous sequences present in *P. falciparum* and 66% of the genes in the *P. yoelii* transcriptome have orthologues in *P. falciparum* [26]. Also in this stage of infection, it has been possible to obtain *in vivo* images in models of infection with *P. berghei* in mice, these have shown details of hepatocyte invasion by *Plasmodium* which were not yet known in humans, as well as the fact that sporozoites can recognize heparan-sulfate proteoglycans besides that *P. yoelii* infection models have been conducted to tests the oxidative stress in the liver induced by infected erythrocytic forms [27].

Bearing in mind, the possibilities offered by murine infection models, we have conducted an important amount of experiments in order to test a variety of chemically modified antigens as potential vaccine components.

In spite of impressive economic and political efforts conducted by WHO and other non-government organizations for malaria eradication and control, based on insecticide treatment of bed-nets (mainly DTT), use of new formulations of artemisinin and other antimalarials for treatment of infected patients and teaching about an appropriate water and environment care to inhabitants of malaria high-transmission areas, among other strategies, malaria still remains as one of the most important health problems for developing countries. Contrary to those expectations, most of these strategies have failed for malaria control, mainly due to novel and powerful biological evolution of antimalarials-resistance mechanisms developed by *Plasmodium* parasites, joint to the continuous mosquito adaptation and colonization of new environments and territories, climate changing and global warming due to non-controlled gas emissions to the atmosphere. Therefore, hopes for controlling this lethal disease are based on developing more efficient preventive strategies and highly potent malaria vaccines.

Up-to-date, about 236 including chemoprophylaxis and malaria vaccines clinical trials are being conducted worldwide, most of them have been completed showing a limited success (as shown in **Figure 2**). Most conducted studies have been focused on vaccine candidates aimed to block three different potential targets, being the transmission-blocking approach the first (gametocyte-derived proteins such as Pf25 and Pf125); secondly, those candidates directed against *Plasmodium* liver-malaria stages (considering proteins such as Circumsporozoite

surface protein (CSP), liver stage antigen (LSA), sporozoite and liver stage antigen (SALSA), Thrombospondin-related anonymous protein (TRAP) and others) and vaccine candidates directed against malaria blood stages (classical merozoite protein targets are Merozoite surface proteins 1-10 (MSP-1-10), apical membrane antigen-1 (AMA-1), ring-infected erythrocyte surface antigen (RESA-155), serine repeat antigen (SERA), Erythrocyte binding antigen 175 (EBA-175) among others).

As recently mentioned by Birkett in 2015, the European Medicines Agency announced a positive opinion for the malaria vaccine candidate most advanced in development, RTS,S/AS01, which provides modest protection against clinical malaria in all conducted trials, but in spite of its poor efficacy later in 2016, this product was recommended by WHO for large-scale trials in moderate to high malaria transmission areas [28]. As observed in **Figure 2**, 113 trials of pharmaceutical products among antimalarials and vaccine formulations are being conducted in Africa in high-transmission malaria regions by immunizing mainly with modified or attenuated sporozoite NF54 strain malarial parasites or other products such as the so-named biological *Pf*SPZ vaccine all of them formulated on strong adjuvant systems such as AS01 as can be observed in the web site ClinicalTrials.gov, a service of the U.S. National Institutes of Health [29].

Due to the moderate success conducted in the last three decades of researching for finding highly potent vaccines for preventing malaria, the field is open for new ideas regarding the discovery of strategies for developing structurally modulated molecular probes which address the *Plasmodium* complex molecular mechanisms involved in parasite detection, facing the challenge of demonstrating protective efficacy profiles and parasite clearance capacity, so those would enter the pathway of being regarded as components of novel vaccine formulations.

6. Current status of *P. vivax* vaccine progress

Morbidity to malaria outside of the sub-Saharan Africa still remains meaningful causing more than 50% of malaria cases, especially in the Americas and Pacific-Asia where poverty and public health systems are associate to multiple problems. The complex *P. vivax* biology and its ability to differentiate into latent forms called hypnozoites which appear longtime later to produce erythrocyte infective forms, prompt occurrence of macro and micro gametes previous to clinical manifestations are seeming, and thus a short evolution cycle into the mosquito makes useless using standard tools to control *P. vivax*. Simultaneously to decreasing in global incidence, some dramatic changes in pathogen infective species have been reported by *P. vivax* being currently the prevalent *Plasmodium* spp. in those mentioned world regions.

For multiple reasons, the epidemiologic spreading of malaria due to *P. vivax* is being regarded as careless. However, turning on attention to malaria caused by *P. vivax* has to be a priority when thinking in a vaccine against malaria. Most approaches for a *P. vivax* malaria vaccine candidate have considered orthologous sequences among the most predominant *Plasmodium* species as being *P. falciparum* and *P. vivax* especially antigens of both pre-erythrocyte and

erythrocyte stages. Among a number of vaccine candidates, the VMP001/AS01 targets the CSP antigen of *P. vivax*. This has been assessed in controlled human malaria infection (CHMI) studies but proven to be unsuccessful with poor protection capacity. Another candidate, which is a recombinantly expressed on appropriate virus, targets the TRAP antigen and currently is currently in study and another prototype vaccine candidate is based on using the strategy of attenuated sporozoites.

On the other hand, the most focused *P. vivax* erythrocyte-stage antigen is the Duffy binding protein (DBP), which is considered crucial for red blood cell (RBC) invasion; however, the DBP non-conserved character establishes an important hindrance. Importantly, time of protection against malaria would be more relevant for a *P. vivax* vaccine regarding a *P. falciparum* vaccine due to *P. vivax* disease incidence is not focused in given populations as it is for *P. falciparum*. Hepatic *P. vivax*-stages also contribute to reinforce this problem complexity [30, 31].

Therefore, developing potent *P. vivax* vaccines would depend on several key aspects among establishing a continuous *P. vivax*—culture in enriched reticulocyte media or specific growth factors aimed to reproducing parasite infections, also appropriate animal models for vaccine candidate testing and most importantly the right selection of multi antigen formulations in human adjuvants and delivery systems, thus peptido-mimetics would play a role in this pursuit.

7. The meaning of being non-visible to α/β-TCR of T lymphocytes

It is well known the fact that the T-cell receptor sees antigen on the surface of cells associated with an MHC class I or II molecule. Therefore, activating humoral and cell-mediated immune responses requires factors such as cytokines and costimulatory molecules expressed by Th cells. A fine and specific regulation of Th has to be highly regulated in order to avoid any self-reactivity would conduct to auto-immune disorders. In order to ensure the Th-cells activation and regulation, these have to recognize a given antigen that is being presented in the MHC class-II context which is located on an antigen presenting cell (APC) surface. As it is known, these professional presenting cells among macrophages, dendritic cells and B lymphocytes harbor two relevant features: (1) surface expression of class-II (MHC-II) molecules, and (2) recruitment of costimulatory molecules as signals for activation of Th-cells.

Antigen-presenting cells first internalize antigen, and then display a part of that antigen on their membrane bound to a MHC-II molecule. The TH cell recognizes and interacts with the antigen–MHC-II molecule complex on the membrane of the antigen-presenting cell. Immune system is prepared for antigen presentation by stabilizing MHC-II molecules in the endoplasmic reticulum bound to an endogen invariant Ii chain which is later cleaved to a small peptide called class II-associated invariant chain peptide (CLIP) which remains bound to the MHC-II molecule to be then replaced by a given antigen-peptide assisted by a chaperone molecule named HLA-DM in endosomal compartments. Therefore, the antigen-MHC-II bimolecular complex will travel to the APC membrane surface to be presented to T-cell receptors (TCR) of

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T-lymphocytes to establish and stabilize in consequence specific ternary complexes able to trigger $CD4^{+}$TH cell proliferation and so an immune response.

As mentioned one of the main functions of CLIP is to prevent the binding of self-peptide fragments prior to the MHC II localization within the endosome-lysosome, a consensus primary structure of CLIP is ^{87}PVSKMRMATPLLMQA^{101}, which is able to a proper interaction with a HLA-II molecule by anchoring-specific residues to the so-named pockets 1, 3, 4 and 9 of the MHC-II molecule in such a way that its entire structure will remain buried into the HLA-II molecule. The CLIP-HLA-II (CLIP: HLA-DR3) molecular complex is shown in **Figure 4**. As observed, the endogenous peptide is hidden into the presenting HLA-II molecule, and the possibility of being recognized by any TCR is completely abolished, and so an auto-reactive immune response will not take place, thus if a given pathogen can develop immune response evasion mechanisms based on its ligands structure features, it would be desirable to its convenience to resemble the most relevant structure characteristics of CLIP to avoid be recognized by TCRs [32].

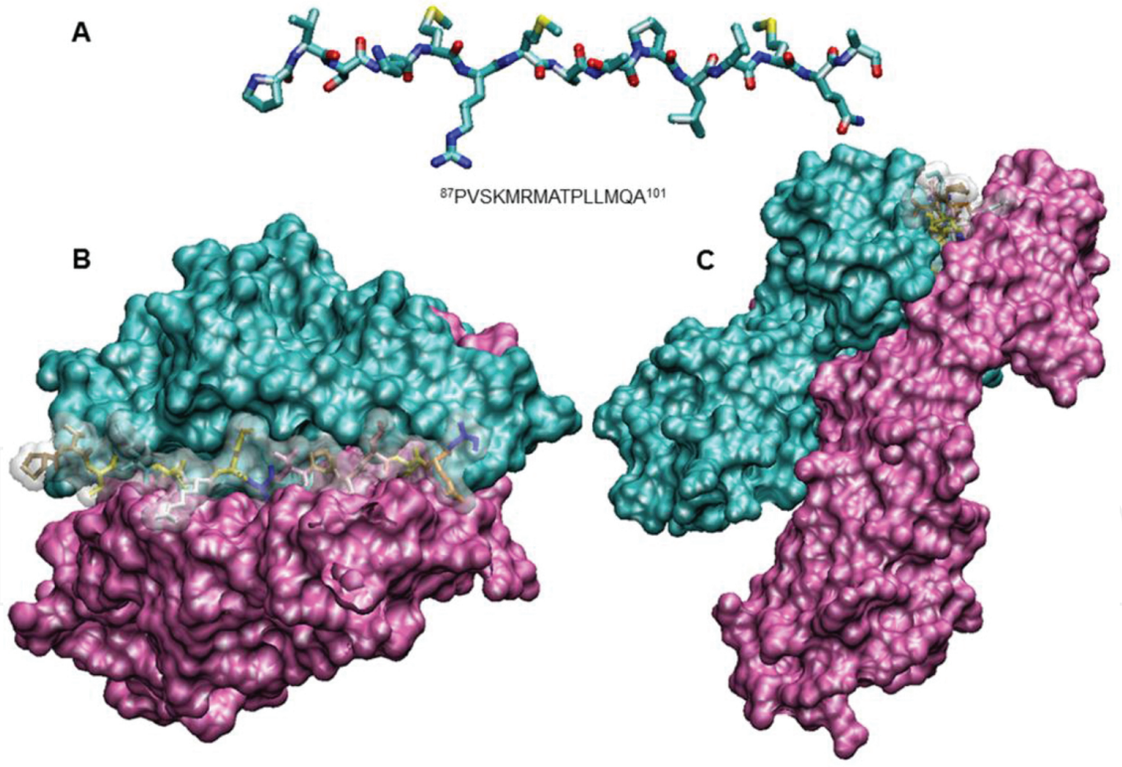

Figure 4. An endogen peptide-MHC-II bimolecular complex. The endogenous invariable Ii-chain cleaved CLIP product (^{87}PVSKMRMATPLLMQA^{101}) complexed to a HLA-DR3 allele. Coordinates of the CLIP-HLA-DR3 complex from the protein data bank (PDB) coded 1A6A corresponded to the structure determined by X-ray diffraction at a 2.75 Å resolution, was downloaded and molecular modeled with the visual molecular dynamics (VMD1.7ή) software of the University of Illinois at Urbana-Champaign. Color code representation for HLAII α-chain in purple, β-chain in cyan, HA peptide is represented in amino acid id code [32].

8. How to become recognized by a TCR-T lymphocyte

Influenza hemagglutinin (HA) or hemagglutinin is a glycoprotein found on the surface of influenza viruses. Its role is to bind the influenza viruses to their target cells through sialic acid, specifically to red blood cells and upper respiratory tract cells [33, 34]. Once the pH has been decreased, a second role of HA is to join the viral cover to formed endosomes. HA is an integral membrane glycoprotein expressed as homo-trimers which seem a barrel-like structure having around 13.5 nm in length. HA is confirmed by three monomers built into a alpha-helical core displaying spherical tips containing those sialic acid-binding motifs. HA is synthesized as monomeric units as precursor forms which are glycosylated and processed on protein maturation, to produce two shorter proteins called HA1 and HA2. The HA monomers are long helical chains attached to the cell membrane by HA2 and capped by HA1. Thus, HA has been responsible for stimulation of neutralizing antibodies which are proven to avoid influenza virus infection to its target cells, thus constituting an important molecular tool for infection control using mechanisms associated to ternary complex stabilization of HA-HLA-II α/β-TCR ($CD4^+$) with specific HLA-DR4 alleles such as DRA*0101 and DRB1*0401 [34].

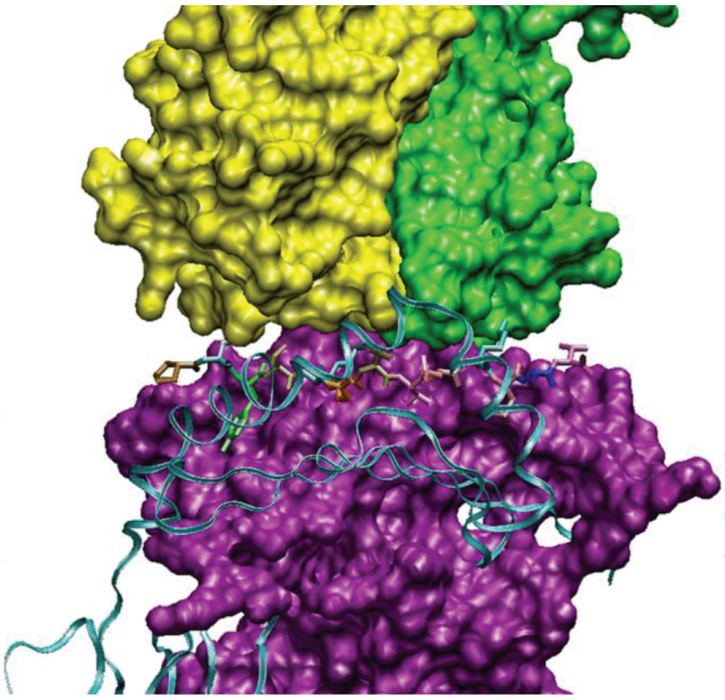

Figure 5. Reactive and T-lymphocyte proliferation upon a stabilized ternary HA-hemagglutinin-HLAII-TCR complex. Coordinates of the human T-cell receptor (TCR) HA1.7, influenza hemagglutinin peptide, and major histocompatibility complex class II molecule, HLA-DR4 (DRA*0101 and DRB1*0401) were downloaded from the protein data bank (PDB) whose code 1J8H corresponded to structure obtained by X-ray diffraction at a 2.40 Å resolution, and then was molecular modeled with the visual molecular dynamics (VMD1.7ή) software of the University of Illinois at Urbana-Champaign. Color code representation for HLAII α-chain in purple, β-chain in cyan, HA peptide is represented in amino acid id code, TCR α-chain in yellow and β-chain in green [34].

Figure 5 recreates the 3D structure of the HA-hemagglutinin-HLAII-TCR ternary complex. As observed, the HA306–318 peptide backbone whose amino acid sequence is PKYVKQNTL-KLAT anchor-specific residues into the HLA-II 1, 3, 4 and 9 pockets and clearly expose some residues in positions 5, 8 and 7 to be recognized by α/β-TCR chains and so stabilizing the molecular complex. However, the HA peptide binds promiscuously and can be presented by most of the frequently occurring DR alleles. Therefore, $CD4^+$ T-lymphocyte proliferation would lead a subsequent neutralizing antibody production able to block the influenza virus infection, being this molecular interaction an effective mechanism effectively used for the immune response. As this a number of similar immuno-reactive complexes have been described [35–40].

9. New modified vaccine components containing non-natural elements considering chiral and topochemical constraints

Current prototype vaccines against malaria are failed to the goal of protecting any individual living in high risk malaria transmission areas even the most promising such as RTS,S which have less than 30% effectivity. As discussed above, the complex life cycle of *Plasmodium* parasites besides human genetic restrictions and immune mechanisms associated with protection makes obtaining an effective malaria vaccine a challenge. Therefore, obtaining new immunogens as vaccines and other molecular systems having potential application in malaria therapy would have to be with precisely selecting relevant antigens which would be submitted for steps of redesigning, production, formulation and *in vitro* tested before assayed in animal models; thus, after proven to be effective have to cross the border line for human clinical trials.

The aim of our research is to produce back-bone modified immunogenic antigens which included non-natural elements such as chiral and peptide-bond substitutions directed to modulate the antigen 3D structure and stimulation of neutralizing antibodies. Our approach is based on low-polymorphic sequences of *Plasmodium* which are then modified into immunological relevant motifs as well as strategically including lysosomal substrates to stimulate processing and presentation steps on vaccinated individuals. Therefore, we have analyzed and produced representative modified immunogens based on a number of native sequences belonging to different stages of *Plasmodium* which upon vaccination of animal models have proven to be effective against malaria infection and parasite clearance [41–43].

One of our first approaches for antigen peptide backbone modification consisted in introducing topochemical elements into the selected antigen primary structure, which consisted in two key features, first the amino acids chirality and second the peptide backbone space orientation. As represented in **Figure 6A**, the N-terminus low polymorphic region of the *Pf*MSP1$^{42\text{-}61}$ native sequence (GYSLFQKEKMVLNEGTSGTA) was the base for evaluating the above described considerations. Thus, chirality impact on immunogenic properties was tested by introducing local and global *L*- and *D*-amino acid substitutions, and the peptide backbone orientation influence was assessed by reversing the primary structure but upholding its native composition. Therefore, a set of peptido-mimetic analogues were designed and synthesized and subsequently tested. As observed, the experimental group consisted in the *Pf*MSP1$^{42\text{-}61}$ native

sequence, entirely made of L-amino acids; its chiral D-analogue which preserved the sequence and was built with D-amino acids (represented in lowercase letters); another so-named ***Retro*** analogue sequence, which was constructed with D-amino acids and reversing the sequence orientation and an analogue called ***Retro-inverso***, which was synthesized with L-amino acids and reversing the peptide sequence orientation. Also punctual or partially D-substitutions were include in the experimental group. CD profiles for the four molecules reflected interesting spectrophotometric properties, thus when comparing the native L-sequence (black line) with its D-enantiomer (red line), their CD profiles behave as specular images of one another as displayed in **Figure 6B**. Similarly, CD profile for the L-native sequence compared with that of the **Retro-inverso** analogue (green line), behave as mirror images of each other bearing in mind that both were made with only L-amino acids, but having opposite peptide backbone orientation.

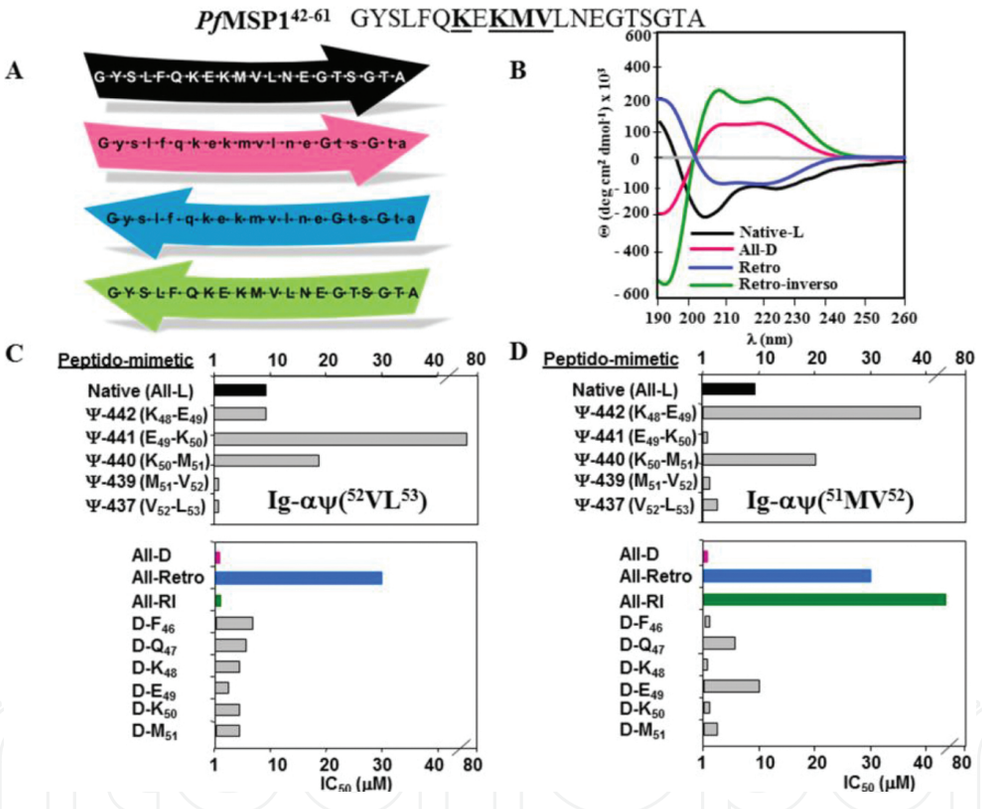

Figure 6. Chiral and topochemical peptido-mimetic designing. **(A)** Molecular peptido-mimetic design based on the N-terminus *Pf*MSP1$^{42\text{-}61}$ peptide. The native sequence is represented in capital letters for L-amino acids, D-enantiomer represented by lowercase letters purple highlighted, Retro-sequence is constituted by D-amino acids in lowercase letters blue highlighted and the so-named Retro-inverso peptidomimetic is confirmed by L-amino acids green highlighted. **(B)** Circular dichroism secondary structure patterns for *Pf*MSP1$^{42\text{-}61}$ peptido-mimetics. Used color code was black line for the native sequence, purple for its D-enantiomer, blue line for the Retro-analogue and green line for the Retro-inverso peptido-mimetic. Competition ELISA was used for the *Pf*MSP1$^{42\text{-}61}$ peptide mapping with monoclonal antibodies coded Ig-αψ-437(^{52}V-L^{53}) and Ig-αψ-439(^{51}M-V^{52}) as shown in **(C)** and **(D)**, respectively. IC50 μM values represent the average of data obtained in triplicate.

CD patterns for the D-enantiomer and the ***Retro-inverso*** analogue had a close relationship regarding the all-***L*** native sequence. On the other hand, CD profiles for the all-D-enantiomer

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(red line) and the ***Retro***-analogue (blue line) behaved as specular images of each other, keeping in mind that these molecules are made of only ***D***-amino acids, having the second a reversed backbone regarding the first. Interestingly, CD patterns of both, the L-***native*** sequence and ***Retro***-analogue resemble each other but are opposite to the CD profile of the D-enantiomer. Therefore, a partial conclusion emerged from these findings, independently of the amino acid composition; backbone orientation seemed to play a key role for the 3D structure properties of the antigen molecule.

In order to test the unique recognition of an antibody stimulated by a reduced amide peptido-mimetic in which the oxygen atom of the carbonyl group (–R–CO–NH–R′–) of a relevant peptide-bond was replaced with two hydrogen atoms to lead an analogue being the reduced form of it and herein named reduced amide peptido-mimetic ψ(–R–CH_2–NH–R′–). Thus, two monoclonal immunoglobulins (mAb) were produced, one directed to *Pf*MSP1$^{42\text{-}61}$ modified between the -^{52}V-L^{53}- and another directed to the -^{51}M-V^{52}- amino acid pairs, respectively, which herein are named as **Ig*α*-ψ-VL$^{52\text{-}53}$** and **Ig*α*-ψ-MV$^{51\text{-}52}$**. Both mAbs possess inhibitory capacities in both *in vitro* and *in vivo* malaria infections by *P. falciparum* as well as *P. yoelii* and *P. berghei* as elsewhere published [42]. Also our experiments allowed to a fine mapping of the MSP-1 N-terminus portion using monoclonal and polyclonal antibodies induced by reduced amide peptido-mimetics of the *Pf*MSP1$^{42\text{-}61}$ peptide and so we identified the antigen epitope whose amino acid sequence is ^{51}MVLNEGTSGTA^{61} as elsewhere reported [44].

Therefore, a whole set of *Pf*MSP1$^{42\text{-}61}$ modified analogues were used in competition assays for their ability to bind these mAbs. As observed in **Figure 6C** and **D**, the non-modified native sequence (all-L) bound at a 10 μg/mL while their inducer modified peptido-mimetics did it at a 1 μg/mL concentration. The reduced amide modification between 49E-K50 residues was an excellent competitor for binding to the **Ig*α*-ψ-MV$^{51\text{-}52}$**. Interestingly, the ***All***-D-enantiomer and ***Retro-inverso*** form of the sequence behaved as strong competitors for the **Ig*α*-ψ-VL$^{52\text{-}53}$** binding, both of them are built with ***D***-amino acids. In a similar way, most partially made D-substitutions were strong binders to both tested antibodies. Thus, both chirality and back-bone orientation become critical properties for antigen-antibody recognition, considering that natural features have to be resembled in artificially-modified immunogens, as well as preserving the molecule structure topology but most relevant it is an appropriate side-chain space orientation, which will be crucial for binding and functional effects.

In another set of experiments conducted based on the *C*-terminus low polymorphic portion the MSP1 antigen, the *Pf*MSP1$^{1282\text{-}1301}$ peptide whose primary structure EVLYLKPLAG-VYRSLKKQLE was the basis for protection capacity assays against malaria in Aotus monkeys immunized with reduced amide peptido-mimetics and partially made D-mutations. Animals were treated in agreement with Colombian and environmental regulations, and those individuals that developed malaria infection after being experimentally challenged with controlled doses of *P. falciparum* were subjected to medication to ensure their health conditions.

As observed in **Figure 7**, a few number of animals vaccinated have controlled the *Plasmodium* parasitemia levels, especially two out of ten vaccinated with the reduced amide ψ-^{7}P-L^{8} peptido-mimetic and four out of eight with a partial D-substitution in K^{6} of the *Pf*MSP-1$^{1282\text{-}1301}$ sequence.

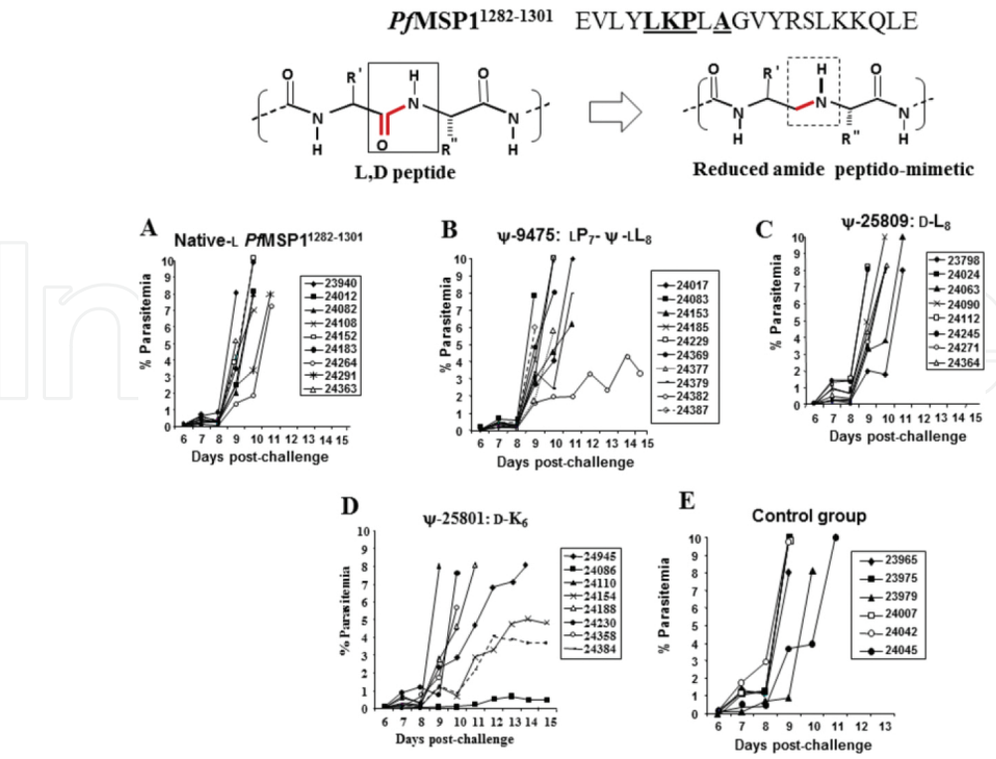

Figure 7. Protection capacity of *Pf*MSP-1/1282-1301 peptido-mimetics. **(A)** Aotus monkeys immunized with the *Pf*MSP-1$^{1282-1301}$ native sequence. **(B)** Animal group immunized with the ψ-9475 (^{7}P-L^{8}) reduced amide peptido-mimetic analogue. **(C)** Animals immunized with the partially substituted ***D***-L^{8} analogue. **(D)** Group of animals immunized with the partially substituted ***D***-K^{6} analogue. **(E)** Aotus monkeys immunized with saline solution as the placebo control group.

On the contrary, animals of the placebo-control and those vaccinated with the native sequence became faster infected and did not control the *Plasmodium* parasitemia. Therefore, the evidence supported the relevance of chiral-space occupancy as well as topochemical modifications as being important elements to be considered for malaria vaccine designing.

In order to be consisting with our proposed molecular models, we decided to focus our attention in another relevant *Plasmodium* antigen, the so-named *Pf*MSP-2 surface antigen. Specifically, we have designed and synthesized some reduced amide peptido-mimetics based on the N-terminus *Pf*MSP2$^{21-40}$ peptide whose amino acid sequence is KNESKYSNTFIN-NAYNMSIR. Thus, two peptido-mimetic analogues coded ψ-128 and ψ-130 which were modified between the ^{30}F-I^{31} and ^{31}I-N^{32} amino acid pairs, respectively, were obtained and characterized. Our experiments for a fine *Pf*MSP2$^{21-40}$ sequence mapping with monoclonal antibodies directed to both modified motifs have revealed a functional epitope whose exact location was ^{25}KYSNTFIN^{32} as previously published [45].

As observed in **Figure 8**, the reactivity patterns of both antibodies by western blot analyses lead to identified native *Pf*MSP2 protein fragments stained at 30.54, 34.21 and 37.90 kDa on a *P. falciparum* FCB-2 membrane protein lysate. Faster polypeptide fragments of *Pf*MSP2 on the SDS-PAGE, could be associated to this antigen cleavage during blood-stage parasite maturation to mature schizonts, previously to merozoite releasing (**Figure 8A**).

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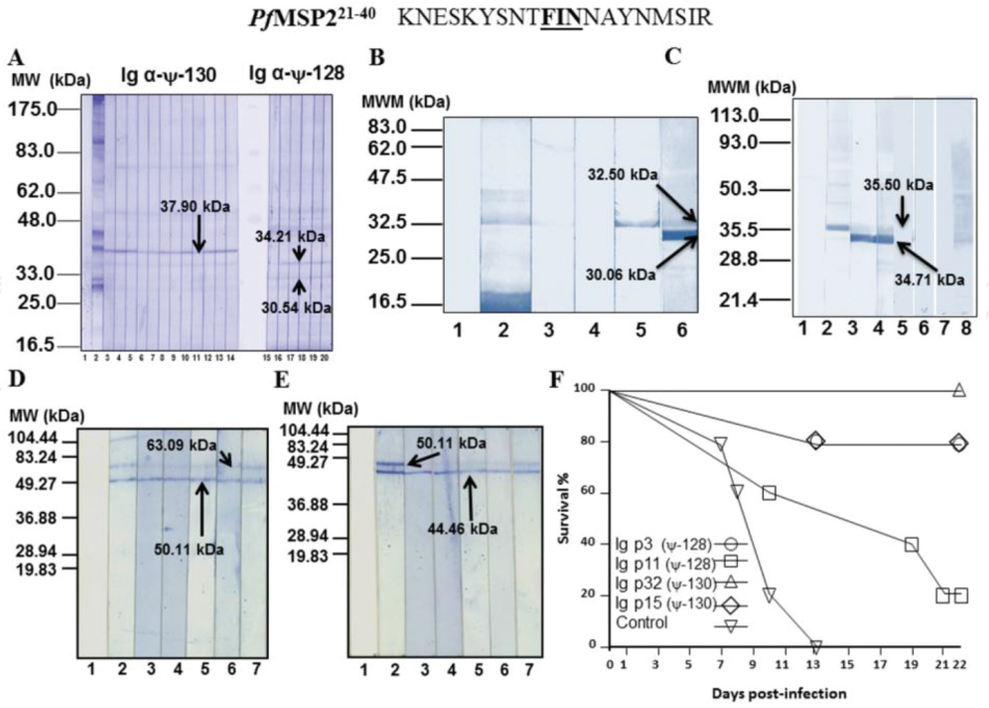

Figure 8. Reactivity and functional properties of *Pf*MSP2$^{21-40}$ peptido-mimetics. **(A)** *Plasmodium falciparum* FCB-2 membrane protein lysed resolved on a 7.5–15% SDS-PAGE gradient system. **(B)** Recombinant MSP2-$(His)_6$ protein treated with the Ig-αψ-128 (^{30}F-I^{31}) monoclonal antibody. **(C)** Recombinant MSP2-$(His)_6$ protein treated with the Ig-αψ-130 (^{31}I-N^{32}) monoclonal antibody. **(D)** Surface membrane proteins from blood stages of *P. berghei* treated with the Ig-αψ-128 and Ig-αψ-130 monoclonal antibodies. **(E)** Surface membrane proteins from blood stages of *P. yoelii* treated with the Ig-αψ-128 and Ig-αψ-130 monoclonal antibodies. **(F)** BALB/c mice infected with lethal doses of rodent malaria treated by passive transference of Ig-αψ-128 and Ig-αψ-130 monoclonal antibodies.

In order to verify the antibody reactivity, an *Escherichia coli* recombinantly MSP2 expressed fragment which contained part of the *Pf*MSP2 N-terminus sequence was employed. Hence, the Ig-αψ-128 antibody detected lysate produced bands at 30.06 and 36.50 kDa and the Ig-αψ-130 antibody recognize bands at 34.71 and 35.50 kDa, all polypeptide bands contained the MSP2 recombinantly expressed fragment as compared with the control raw, as shown in **Figure 8B, C** respectively.

Similarly, a lysate composed by membrane proteins from blood stages of *P. berghei* and *P. yoelii* were analyzed for these mAbs recognition. Therefore both Ig-αψ-128 and Ig-αψ-130 antibodies detected bands at 50.11 and 63.09 kDa mobilities for *P. berghei* ANKA and 44.46 and 50.11 kDa for the *P. yoelii* 17XL strain, respectively, as observed in **Figure 8D** and **E**. Besides, functional *in vivo* activity of these antibodies was tested by passive transferring experiments of both into *P. berghei* and *P. yoelii* infected BALB/c mice groups. Most animals survived to the lethal challenging with *Plasmodium* strains and efficiently have controlled parasitemia levels due to the antibody therapeutic activity as it was published and observed in **Figure 8F** [45]. This set of experiments revealed the importance of performing peptide-backbone strategic modifications by introducing non-natural elements into the immunogen primary structure, but its amino acid sequence identity has to be preserved to avoid any non-specific or non-desirable cross-reactive effects.

To obtain a complete landscape of this novel scenario, further scopes of the strategy of obtaining next generations of malaria vaccine candidates based on introducing non-natural elements into immunogens, trials performed in selected antigens of other *Plasmodium* stages, such as those called pre-erythrocytic as well as sexual forms on macro and micro-gamete particles have to be conducted. Hence, the circumsporozoite surface protein (CSP) expressed on pre-erythrocyte forms offers a classical interesting target for vaccine candidate development.

As reported before, a class-I restricted *Pb*CSP$^{252\text{-}260}$ epitope was identified in the CSP primary structure of *Plasmodium berghei*, a rodent malaria specie [46–49]. Thus, in order to test our hypothesis, we conducted experiments by introducing reduced amide peptide-bond isosteres in a systematic fashion and their subsequent evaluation regarding antibody stimulation and their reactivity was performed. This epitope whose amino acid sequence is SYIPSAEKI was the basis for the molecular designing. Thus, a set of peptido-mimetic analogues were synthesized and characterized. As shown in **Figure 9B** and **C**, antibodies induced by the *Pb*CSP$^{252\text{-}260}$ native sequence have some reactivity for both sporozoite and gametocytes as analyzed by indirect immunofluorescence assays (IFA) experiments while a stronger reactivity of the modified ^{257}A-E^{258} amino acid pair of *Pb*CSP$^{257\text{-}258}$ peptido-mimetic-induced antibodies, become evident regarding both sporozoites and gametocytes as observed in **Figure 9E** and **F**. Antibodies of pre-immune sera did not show any reactivity as seen in **Figure 9A** and **D**.

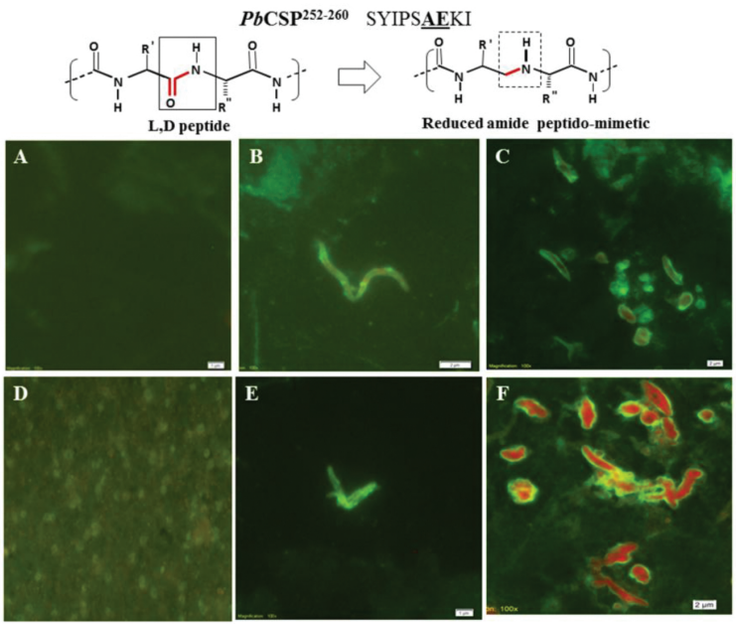

Figure 9. Exploring immunological properties of the restricted class-I *Pb*CSP$^{252\text{-}260}$ epitope by indirect immunofluorescence assays (IFA). **(A)** Pre-immune serum of mouse 2. **(B)** *Plasmodium falciparum* sporozoites detected by antibodies induced by the *Pb*CSP$^{252\text{-}260}$ native peptide post-third boost (mouse 2), image captured at 2 μm. **(C)** Detection of *Plasmodium falciparum* (NF54 strain) gametocytes by antibodies induced by the *Pf*CSP$^{252\text{-}260}$ native sequence post-third boost (mouse 2), and image recorded at 2 μm. **(D)** Reactivity of pre-immune serum of mouse 23. **(E)** Detection of *Plasmodium falciparum* sporozoites by antibodies to the *Pb*CSP (A–E) peptido-mimetic obtained post-third boost (mouse 23), image recorded at 1 μm. **(F)** Detection of *Plasmodium falciparum* (NF54 strain) gametocytes by antibodies directed to the *Pb*CSP (A–E) peptido-mimetic (mouse 23), and image captured at 2 μm.

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The proposed hypothesis has been confirmed by challenging it in different molecular scenarios, all based on analysis of different antigens derived from different *Plasmodium* species and stages and proved in *in vivo* and *in vitro* assays. Thus, an emerging conclusion states the importance of a careful and strategic molecular designing of potential malaria vaccine candidates which consider the antigen global structure modulation but preserving their specific fingerprint represented by their amino acid sequence.

Aimed to understand a possible structure-immunological activity relationship, a subsequent set of nuclear magnetic resonance NMR and molecular dynamic *in silico* experiments lead us to compare in all cases, both the native antigen, as well as their modified derivatives regarding their 3D structure properties. Thus, generated data are presented in **Figure 10**, in which either native antigen backbone conformation is overlapped with that of its functional representative peptido-mimetics or polypeptide conformations of homologue proteins in two *Plasmodium* species are compared.

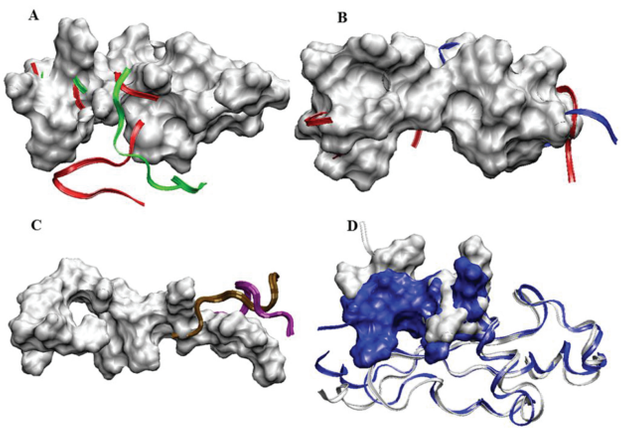

Figure 10. Structure-immunological activity relationship. Overlapped backbone of native and modified peptido-mimetic analogues were organized as follow. **(A)** Accessible solvent surface of the *Pf*MSP1$^{42\text{-}61}$ native sequence in white and ψ-437 in red ribbons and ψ-439 in blue ribbons. **(B)** Accessible solvent surface of the *Pf*MSP1$^{1282\text{-}1301}$ native sequence, ψ-9473 in blue ribbons and ψ-9475 in red ribbons. **(C)** Accessible solvent surface of the *Pf*MSP2$^{21\text{-}40}$ native sequence, ψ-128 in purple ribbons and ψ-130 in ochre ribbons. **(D)** Accessible solvent surfaces in white for *Pf*CSP and blue for *Pb*CSP. The ^{308}SYIPSAEKI^{316} class-I restricted epitope is highlighted.

As observed in **Figure 10A**, overlapped 3D conformations of the *N*-terminus *Pf*MSP1$^{42\text{-}61}$ peptide whose solvent accessible surface depicted in white, and two of its peptido-mimetics those coded as ψ-437 (^{52}V-L^{53}) and ψ-439 (^{51}M-V^{52}) represented by red and green ribbons, revealed deep structure differences between them. A highly compact α-helix structure present in the native sequence became flexible in its two peptido-mimetic analogues due to a single peptide bond modification, in which the oxygen atom of the –CO–NH– amide function of a specific peptide-bond, was replaced with two hydrogen atoms so leading –CH_2–NH– surrogates.

Similarly, backbone of the low polymorphic *C*-terminus of the same *Pf*MSP1 antigen specifically the *Pf*MSP-1$^{1282\text{-}1301}$ peptide fragment (solvent accessible surface in white) was overlapped with its two derive ψ-9473 (^{6}K-P^{7}) and ψ-9475 (^{7}P-L^{8}) peptido-mimetics (red and blue ribbons)

[50]. As in the first analyzed case, a highly compact α-helix structure present in the native sequence became flexible in its two peptido-mimetic analogues due to single peptide bond modifications which surely have introduced new freedom degrees to the molecule, as seen in **Figure 10B**.

On the other hand, backbone structural analyses for the *Pf*MSP2$^{21\text{-}40}$ regarding its two ψ-128 (^{30}F-I^{31}) and ψ-130 (^{31}I-N^{32}) reduced amide peptido-mimetics, revealed a close-related behavior regarding the couple of the above discussed examples for a different *Plasmodium* protein, the *Pf*MSP1. **Figure 10C**, displays the β-stranded conformation of the native sequence (solvent accessible surface depicted in white) regarding the more flexible conformations of both of its peptido-mimetics (represented by purple and ochre ribbons).

An interesting observation become evident when backbone of two homologue proteins are overlapped regarding a class-I epitope region, as it was the case of the *Pf*CSP and *Pb*CSP, as shown in **Figure 10D**; consisted in that those overlapped polypeptide conformations, suggest an intermediate molecular state among them, which could be represented by a peptido-mimetic structure probe. Thus, the peptido-mimetic coded *Pb*CSP$^{252\text{-}260}$ which represents a peptide-bond surrogate located between the ^{257}A-E^{258} amino acid pair, thus this strategic peptide-bond replacement could be responsibly of the stimulated cross-reactive antibodies.

Further experiments in this pursuit will explore hypothesis on *in vivo* protection against malaria regarding CSP peptido-mimetics and will be conducted in order to assess the functional inhibitory activity of peptido-mimetics and their antibodies on malaria-infected mice through *Anopheles albimanus* mosquito bites.

The family of the herein presented structural modified compounds constitute molecular tools to be considered for new generations of functional protective vaccines against malaria, as such, future vaccine candidates could be based on this knowledge and outstanding findings.

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