**Medicinal application of long synthetic peptide technology**

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This review covers the latest developments of long synthetic peptide technology for the rapid identification and development of malaria vaccine candidates and immunological modulators. A brief description of the two most common solid-phase synthetic procedures, together with the latest advances in optimisation of peptide chain assembly and analytical instrumentation, is given, with special attention to non-specialists. Several examples of vaccine candidates developed in the authors’ or their collaborators’ laboratories are also provided.

Keywords: allergy, bee venom, long synthetic peptides, malaria, Plasmodia, vaccine

**1. General considerations**

The utilisation of peptide synthesis technology in solving health problems of worldwide importance was mainly dictated by the desire to obtain stable products devoid of the various contaminants often seen in recombinant material (glycosylation variants, proteases, DNA, endotoxins) in record time and in reasonable quantities to perform preclinical testing. Due to the purity of the products obtained, these results would be reproducible when Good Manufacturing Practice (GMP) products required for clinical application will be subjected to quality assurance testing. In fact, it is common that preparations obtained through recombinant technology may vary in their potency from batch to batch, most likely due to the presence of endotoxins and/or other impurities. The other non-negligible advantage of the synthesis protocols the authors have adopted and continue to improve is the speed of execution. It is now possible to obtain milligram quantities of polypeptides of ≥100 amino acid residues within a few weeks. A further consideration is the capacity to focus the biological activity of most interest on the chosen segment. For example, it is known that not all segments of a protein will confer protection against a given pathogen, such that a specific fragment may indeed be more potent in eliciting a protective immune response than the intact protein, as observed for *Plasmodium falciparum* merozoite surface protein (MSP)-3 [1]. In addition, a fragment may not only be less efficient in eliciting the appropriate protective response, but it may actually hinder the development and/or activity of the protective response, as seen in the case of the *P. falciparum* MSP-1 response [2]. The advantage of long synthetic peptides (LSPs) over a mixture of short peptides is that LSPs can cover the sequence of a protein domain, such that the threedimensional conformation of the polypeptide closely mimics that of the parent protein, with concomitant display of the appropriate biological activity. Finally, the length of the polypeptide is such to assure that no genetic restriction of the immune response is observed.

In summary, the advantages of peptide synthesis technology reside in:

- improved focus of the immune system upon carefully chosen epitopic regions
- precise tailoring by combining multi-restricted helper T lymphocytes (HTLs) and cytotoxic T lymphocytes (CTLs) or B cell epitopes, or both

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• speed of production and purification of polypeptides devoid of an extraneous ‘fusion partner’
• lack of contamination by irrelevant foreign proteins, leading to improved stability (due to the absence of traces of proteases) and safety
• reproducibility in the chemical process
• lack of genome integration, as in the case of genetic immunisation

2. General considerations of peptide synthesis

Search for subunit vaccine candidates for malaria and allergen-derived peptide immunotherapy in the authors’ laboratory required the synthesis and analytical characterisation of many different molecules that could activate the immune system in the population at large without encountering problems due to genetic restriction. In addition, when necessary, they could assume the correct three-dimensional conformation in order to obtain conformational-dependent neutralising antibodies. Thus, the aim was to synthesise small proteins, or pre-order to obtain conformational-dependent neutralising antibodies, they could exist using the peptide synthesis technology that existed ~ 10 years ago.

Attention was initially focused on the practical problems of how a large number of such complex polypeptide chains could be obtained quickly, reliably and economically in an academic biochemistry laboratory with robust, easy-to-use protocols.

During the late 1970’s, the solid-phase method, introduced by RB Merrifield, had begun to dominate peptide chemistry, facilitating the rapid preparation and biological evaluation of many long peptides and proteins [3]. Merrifield’s solid-phase peptide synthesis (SPPS) is illustrated in Figure 1. In brief, the peptide C-terminal amino acid with α-NH2 blocked by the t-butyloxycarbonyl (Boc) protecting group is attached to an amine or diisopropylethylamine, a second activated protected bimolecular reaction. By-products of the reactions and excess reagents will maximise yields of every repeated assembly until the polypeptide sequence is complete. Once the desired sequence is assembled, the polypeptide is cleaved from the resin, with simultaneous removal of the side chain protecting groups.

The resin swollen in the reaction solvent helps solvate the attached peptide so that both small volumes and high molar excess of reagents will maximise yields of every repeated bimolecular reaction. By-products of the reactions and excess reagents are eliminated simply by washing the peptide-polymer with the same solvent. The solid-phase technique, being a rapid cyclic process, has been also automated. Even if polypeptide intermediates cannot be characterised and purified, a single operator can easily assemble good quality crude products using fully automated commercial instruments based on batch wise or flow mode of operation.

Since their introduction, SPPS chemistry and technology have been constantly optimised in such a way as to maximise the efficiency of coupling and minimise side reactions of all the sequential chemical reactions involved in amino acid addition. One such major improvement is the introduction, by Sheppard and collaborators, of an alternative strategy based on a base labile α-amino-protecting group (fluorenylmethyl-oxycarbonyl [Fmoc]), which renders the synthesis more attractive for inexperienced operators and institutional core facilities (Figure 2) [4].

Merrifield’s Boc and Sheppard’s Fmoc α-amino-protecting group strategies are the most commonly used approaches for the assembly of polypeptides. Although both strategies employ similar coupling chemistry, the α-amino group and side chain deprotection chemistries differ. The stepwise removal of Boc, achieved by TFA, requires TFA-resistant side chain protecting groups and linkers (e.g., phenylacetamido-methyl or Pam). Hence, strong acid, typically hydrogen fluoride, is necessary for their removal following completion of chain assembly. In Sheppard’s strategy, the use of Fmoc avoids the neutralisation step and repetitive acid treatments. Side chain protecting groups and linker are removed simultaneously by TFA at the end of the chain assembly. Hence, Sheppard’s method is milder and more flexible. Difficult couplings (Boc chemistry) and difficult deprotection/couplings (Fmoc chemistry) originate either deletion polypeptides (chains lacking one or more amino acid residues) or truncated polypeptides (chains shorter than the target sequence). These side products and several additional impurities produced mainly during the final acidolytic cleavage of peptide-resin (e.g., side chain alkylation of Trp, Tyr, Cys and Met, and impurities due to aspartimide formation) are present in the crude together with the target polypeptide.

A row/stretch of amino acid residues in a polypeptide deprotection and/or coupling reaction(s) that do not undergo completion, or proceed in low yield, are said to constitute a ‘difficult sequence’. The ‘difficult sequences’ are usually characterised by 2 – 5 residues in a row/stretch and occur typically at 5 – 15 residues from the C-terminus of a sequence being synthesised. They originate from intermolecular aggregation of the resin-bound polypeptides. The α-amino groups of the growing chains are partially buried so that amide bond formation through reactions with the incoming activated amino acids is more difficult. As shown by Fourier transform infrared spectroscopy on the sequence 80 – 99 of HIV-1 protease during stepwise assembly on resin, the problem has its origin in α-sheet formation [5]. Indeed, polypeptide chains have a maximum tendency to form intermolecular hydrogen-bonded β-sheet-type aggregates at 5 – 15 amino acid residues, and α-helical or random-chain structures beyond 15 or 20 residues in length.
After the initial difficult stretch, subsequent steps in the assembly of the target sequence may proceed in high to quantitative yield, or be hindered again by aggregation of the growing peptide chains when 'difficult sequences' are met again.

For all of the problems mentioned above, primarily slow and incomplete couplings due to aggregations and/or to contiguous sterically hindered amino acid residues in the sequence, it was felt by early critics that the level of completeness required to obtain target peptide chains containing > ∼ 30 residues was beyond attainability. Theoretically, in the synthesis of a protein of 101 residues, a reaction efficiency per cycle of 99% would give 36% of resin-bound polypeptide with the correct amino acid sequence. The remaining 64% would consist of a mixture of polypeptide contaminants, most of them lacking just a few amino acid residues from the target sequence. The impurities would be 100 polypeptides with a single residue deletion, 5000 with two deletions, and the bulk of the remainder made up of three- to four-residue deletions (> 4,000,000 possibilities). For example, to obtain ∼ 80% crude product purity, overall efficiencies of 98.8 – 99.9% per reaction cycle would be required.

Although the above calculations highlighted an almost hopeless situation, new synthetic protocols and new separation methods have continually been developed toward greater efficiency in peptide assembly, cleavage from the polymeric support and final purification of polypeptide crude products. For example, deletion products are essentially eliminated by irreversibly blocking all free α-amino termini after each single coupling step with acetic anhydride (capping step), and alkylation (t-butylation) of sensitive Trp, Tyr, Cys and Met residues during the final TFA peptide–resin cleavage is greatly reduced by suitable scavengers cocktails.

Reverse phase (RP)-high performance liquid chromatography (HPLC), a breakthrough in liquid chromatographic techniques for peptide purification far greater than could have been anticipated, has made major impacts on the purification of long peptides prepared by stepwise SPPS [6]. RP systems were capable of separating even the closest deletion and truncated peptides from the target product. In the late seventies, both RP-HPLC and SPPS gradually changed the peptide chemistry from being a specialist area, into a field where any scientist was in the position to assemble polypeptide chains containing many more than 30 – 40 amino acid residues, the Pillars of Hercules of stepwise SPPS. The refinements of both Merrifield’s and Sheppard’s methods, and the development of newer and better stationary phases for RP-HPLC, with time have amply demonstrated that the initial critic’s view had indeed been too pessimistic.

The continuous refinements of Boc-based synthetic protocols [7,8] allowed Kent and co-workers to assemble the 99-amino acid residue monomer of the HIV-1 protease analogue [amino-butyric-acid residue (Aba)65,95]-HIV-1 in only 33 h. The synthesis of [Aba65,95]-HIV-1 protease by Fmoc chemistry, reported in 1991, demonstrated for the first time...
that Sheppard’s method can also be used to prepare large polypeptide chains containing up to ~ 100 amino acid residues [9]. The optimised protocols developed over the years for both Boc- and Fmoc-strategies resulted in a significant increase in the efficiency of chain assembly so that, in skilled and experienced hands, either method can provide good results in the synthesis of long peptides and proteins containing > 100 amino acid residues. The authors have, however, judged Fmoc-strategy to be far more accessible and safe for the mildness of the reactions involved. In the authors’ laboratory, the use of highly toxic hydrogen fluoride and expensive laboratory apparatus in the final cleavage step of Boc-strategy was considered not only less convenient, but also too problematic to be handled routinely with due care.

Long peptides can also be obtained by chemical ligation (see review by Dawson and Kent, [10]). The authors opted for a stepwise synthetic approach, as it is faster and easier to handle than other strategies. Routinely, no exploratory run was undertaken and single coupling reactions induced by diisopropylcarbodiimide/1-hydroxy-benzotriazole were implemented. When needed, double-couplings and/or couplings with other more powerful, but costly, reagents at a few chain positions, notably at ‘difficult sequences’, were introduced in successive syntheses if it was thought the final yield could be substantially increased. The work-up of the ‘ crude polypeptide’ obtained after final TFA treatment consisted in the separation of the product at ~ 5°C, followed by lyophilisation from ~ 10% acetic acid. The lyophilised crude products were either directly purified by RP-HPLC or subjected to a preliminary purification step by exclusion chromatography on a G-50 column equilibrated in 50% acetic acid before final purification by RP-HPLC. Prepurification of polypeptides was also obtained by Ni-NTA (nitritolriacetic moiety) affinity chromatography of chains carrying an hexa-histidinylmethionyl extension at their N-terminus. The His tail could be selectively cleaved by CNBr treatment if a Met was introduced before the His tail and the internal Met residues were introduced as methionine sulfoxide [11].

The polypeptide final purification was achieved by RP-HPLC. When necessary, purified polypeptides were folded according to various protocols, one of which was developed in the authors’ laboratory [10]. Using similar synthetic and folding protocols, different biological active chemokines were obtained [12].

The authors have used mass spectroscopy, specifically matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (MS) as an integral part of the synthetic process [13]. High-mass, non volatile samples of polystyrene resin without exploratory runs. At the end of a 100-amino acid chain assembly, ~ 3 – 10 grams of peptide material per gram of insoluble support was obtained. The concomitant application of Fmoc chemistry, Barany’s/Fields’ scavengers cocktails [15,16] and pseudoproline units, together with powerful MALDI-TOF mass spectrometry and RP-HPLC, allowed the authors to routinely and successfully make milligrams of many required/desired target long peptides or proteins.

The charge is generally due to the addition of variable numbers of protons to the long peptides or proteins.

The authors have found MALDI-TOF MS ideally suited to the total covalent structural characterisation of synthetic long peptides and proteins prepared using the Fmoc-strategy. The technique has been used for the examination of the crude product immediately after deprotection and cleavage from the resin, the monitoring of fractionation in the course of purification, and the precise and complete structural characterisation of the purified product. In addition, during the assembly of problematic sequences, mass spectrometry provided feedback for control and optimisation of the Fmoc protocol.

Mass measurements definitely deny the hypothetical objection that stepwise solid-phase methods, when applied to the synthesis of long peptides and proteins, would produce only a microheterogeneous mixture of polypeptide chains. In the MALDI-TOF spectra of crude products, the authors have generally observed strong, major signals corresponding to defined molecular ions of the expected masses, together with signals of the ubiquitous t-butylated polypeptide contaminants. In some instances, several by-products were present in trace amounts; aspartimide, piperidide or Pmc derivatives have been also detected.

Modified coupling protocols (longer coupling/deblocking times or double/triple couplings), partially trytltated Asn and Gln, and low resin loadings have been used synergistically with RP-HPLC and MALDI-TOF MS to minimise the troublesome and continuous problem of ‘difficult sequences’. As aggregation of growing polypeptide chains built up on an insoluble matrix can occur from as early as the coupling of the fourth residue, the authors have also used Fmoc-oxazolidine dipeptides (pseudoprolines, Figure 3) to alleviate, or even eliminate, aggregation in polypeptides containing Ser and Thr residues, both at the beginning of chain assembly and at internal positions. When inserted into a peptide chain, these pseudoproline units were able to disrupt aggregation in the same manner as proline and α-alkyl amino acids [14]. The effects were usually long range, with the onset of aggregation often postponed for as many as six residues. Regeneration of the Ser or Thr residues from the oxazolidine unit occurs during the final cleavage/deprotection by the normal treatment with TFA-scavengers.

From the early 1990s, it was common practice in the authors’ laboratory to carry out the assembly of chains containing ≥ 100 residues at resin loadings of 0.1 – 0.5 mmole/g of polystyrene resin without exploratory runs. At the end of a 100-amino acid chain assembly, ~ 3 – 10 grams of peptide material per gram of insoluble support was obtained. The concomitant application of Fmoc chemistry, Barany’s/Fields’ scavengers cocktails [15,16] and pseudoproline units, together with powerful MALDI-TOF mass spectrometry and RP-HPLC, allowed the authors to routinely and successfully make milligrams of many required/desired target long peptides or proteins.
The first attempt to develop malaria vaccine candidates was to produce the C-terminal fragment of the *P. falciparum* circumsporozoite protein (CSP) [17], which was, at that time, the leading malaria vaccine candidate (see section 3.1). The first synthesis of the 102-amino acid peptide representing the C-terminal fragment of CSP was initiated with some cautious optimism. Expectations were not only met, but indeed surpassed, as the synthesis was completed with a reasonable yield of the final crude material. After purification, a product of satisfactory quality, as judged by HPLC and mass spectrometry, was obtained. Thus, the initial goal and challenge of rapidly obtaining a preclinical product of satisfactory quality was fulfilled. It remained to be proven that this approach could be reproducibly applied to other molecules and did not represent merely a stroke of luck. Synthesis and cleavage protocols (scavenger cocktails) were constantly modified and improved to increase the success rate of long peptide SPPS. In this regard, the impressive improvement of mass spectrometry instrumentation that occurred in the last ten years was fundamental in guiding us toward reaching this objective. After years of experience with long peptide SPPS, it is fair to say that stepwise, machine-assisted synthesis of polypeptides containing ≥ 100 amino acid residues is possible in most cases. Problems in obtaining the target product most likely arise at the purification level, in part due to the presence of contaminants that co-purify with the final material or to the lack of signal of the target product in the mass spectrometry MALDI-TOF instrument, which does occur, even if rarely.

In addition, except for rare cases, peptides are stable in water at 4°C and 37°C for an extended period of time, as judged by MS and HPLC analysis.

**3. Preclinical development of long synthetic peptide malaria and allergen vaccine candidates**

**3.1 Immunological and protective properties of the C-terminal fragments of *Plasmodia* circumsporozoite proteins**

It has been shown by different groups, including the authors’, that CSP is implicated in the protective effect observed in the vaccination with irradiated sporozoites. The CSP is an abundant surface molecule, which is composed of 3 domains, an N-terminal region of ∼ 100 amino acids, a repeat region of 100 – 200 amino acids and a C-terminal region of ∼ 70 – 100 amino acids. The recombinant CSP has been shown to bind to liver cells and to inhibit sporozoite liver invasion. The region of the CSP responsible for binding to heparan sulfate glycoproteins present on the surface of hepatocytes has been located in the C-terminus [18,19]. In particular, the region II segment (RII; sequence 342-363 of the NF-54 *P. falciparum* strain) from different parasite strains or species is highly conserved and is homologous to other malaria proteins (thrombospondin-related adhesive protein) and mammalian proteins, such as thrombospondin and properdin. Peptides representing RII exhibit inhibitory properties with regard to sporozoite liver invasion.

The preclinical data obtained can be summarised as follows. *P. berghei* (Pb) CSP C-terminal fragment 242-310 is immunogenic in mice when injected together with incomplete Freund’s adjuvant. In particular, in BALB/c mice, the sequence contains a T helper epitope 273-292, a CTL epitope 245-253, and elicits a high antibody response. Remarkably, a CD8+-dependent protection is obtained (50 – 100% depending on the oxidation state) [20]. Immunisation with other adjuvants, OM-174 and
QS-21, also leads to a high degree of protection [21]. To this regard, it is striking to note that a high number of CD8+ T cells specific for the epitope 245-253 is obtained in BALB/c and BDF1 mice following immunisation of the Pb CSP C-terminal peptide in QS-21, as determined by specific tetramer staining. This response (4 and 7% of total CD8+ T cells in spleen and liver, respectively) is similar in magnitude to that obtained by viral immunisation (manuscript submitted).

The corresponding C-terminal fragment 282-383 of P. falciparum was shown to be immunogenic in inbred and wild mice, rabbits and Aotus monkeys [17,22,23]. Antibodies to this fragment can effectively inhibit liver sporozoite invasion [17]. In addition, it can be recognised by peripheral blood lymphocytes and sera from humans living in endemic areas [24] and contains several human leukocyte antigen (HLA)-A2.1-restricted CD8+ T cell epitopes [25,26]. Data obtained from seventeen A2.1 donors from Burkina Faso indicated that the sequences 299-308, 327-335 NF-54 and 7G8 were recognised by 6, 23 and 41% of the donors, respectively [26]. CTL clones, which recognised NF-54 325-333 peptide and cross-reacted with the homologous segment derived from WEL, It2G1, T-9-101, T4R and 366b strains, were obtained. These lines recognised human and murine HLA-A2.1 transgenic liver cells infected with CS recombinant vaccinia [27].

3.2 Plasmodium vivax circumsporozoite protein

In collaboration with Drs M and S Herrera (University del Valle, Cali, Colombia), peptides spanning the amino (N, 20-96) and carboxyl (C, 301-372) flanking regions of the P. vivax CSP, together with a repeat-peptide corresponding to a trimer of the first variant repeat GDRADGQPA, co-linearly linked to a universal tetanus toxin T cell epitope (ttp-30) [28], were synthesised and used to assess their potential as vaccine candidates. Antigenicity studies were carried out using human blood samples from residents of a malaria-endemic area of Colombia, and immunogenicity was tested in Aotus monkeys. All three peptides had been shown previously to contain several B, HTL and CTL epitopes [29-31]. Human serum samples were found to contain specific antibodies to the central region more frequently than to the flanking regions of the protein as seen before for P. falciparum CSP (61, 35 and 39% of the samples had antibodies against the repeat, N- and C-peptides, respectively). Human peripheral blood mononuclear cells showed higher levels of IFN-γ than IL-4 when stimulated with peptides containing HTL epitopes. Aotus monkeys immunised with the peptides formulated in either Montanide ISA720 or Freund’s adjuvants produced strong antibody responses that recognised the peptide immunogens and the native P. vivax CSP on sporozoites. These results led to the testing of the three peptides in human volunteers at the University del Valle, Cali, Colombia.

3.3 Other malaria vaccine candidates

Table 1 lists the malaria vaccine candidates synthesised in the authors’ laboratory on which preclinical development was initiated, followed by clinical studies for the most advanced candidates. Some of these antigens represent different examples of the antigen selection strategy allowed by the flexibility of peptide synthesis and which is likely to bring a strong contribution to vaccine development.

The first example is given by P. falciparum exported protein 1 (Exp-1; Table 1), where the transmembrane segment is located in the middle of the protein, thus structurally separating the N- and C-terminal domains. Analysis of the human immune response to these two fragments indicated that the antibody response is almost exclusively focused on the C-terminal region, whereas the T cell response is located on the N-terminal part [32]. The response to C-terminus has been associated with an increased level of protection (manuscript submitted). Given the relatively short length of these two domains, a vaccine candidate consisting of a single peptide covering the two regions could be further developed.

MSP-2 is an example in which the selection of the fragment to be synthesised is dictated by the presence two allelic families (3D7 and FC-27) composed of a quasi-constant C-terminal region of 120 and 90 residues, respectively. Analysis of human antibody response to these two fragments revealed that 100% of the individuals recognised these two regions with high antibody titres. Monoclonal antibodies to 3D7 fragment recognised the native protein, as determined by immunoprecipitation and immunofluorescence on infected erythrocytes. Analysis of the association of protection and antibody response is now under way in collaboration with Dr I Felger (Swiss Tropical Institute, Basel, Switzerland).

MSP-3 (∼350 amino acids) and glutamic rich protein (GLURP; 1150 amino acids) are examples where location, length and parasite polymorphism of the fragments to be synthesised had to be collectively considered. Studies on human subjects with recombinant and synthetic fragments indicated the possibility that only the MSP-3 fragment 186-281 [1] and several fragments for GLURP were possible targets of protective immunity in humans [33-35]. Protection is associated to the presence of cytophilic IgG1 and IgG3 antibodies.

Finally, the immunological property of different regions of the liver-stage antigen-3 (LSA-3), composed of 1860 amino acids, was analysed by using the LSP approach [36]. 17 LSPs spanning the entire antigen were synthesised, and their immunogenicity in BALB/c mice and antigenicity in individuals living in a hyper-endemic malaria area were analysed. The findings show that both specific antibodies and T cell proliferation against most LSA-3-LSP develop in malaria-exposed adults. All individuals studied had detectable antibodies against a minimum of 6 and a maximum of 15 polypeptides. It is noteworthy that antibody prevalence and titres were as high against non-repeat as repeat regions. Although the extent of T cell reactivity was lower than that observed for B cells, most of the sequences contained at least one HTL epitope, indicating that the majority of LSA-3-LSP contain both B and T cell epitopes within the same sequence [36]. Injection of LSA-3-LSP with ASO2 adjuvant in
mice showed strong immunogenicity for most of them, eliciting both T cell responses and specific antibody production. Although all the peptides were immunogenic for B cells, different patterns of T cell responses were induced. Importantly, antibodies against some of the LSPs were able to recognise LSA-3 native protein on *P. falciparum* sporozoites.

### 3.4 Bee venom phospholipase A

Induction of tolerance to allergen is one of the major goals of present trends in the therapy of allergic diseases. T cell tolerance induction has been well characterised in specific immunotherapy protocols based on native allergens, and involves T cells as one of the major players. Tolerance induction has been associated with T cell hyporesponsiveness to antigen, enhanced IL-10 antigen-specific production, as well as antigen-specific IgG4 rise [37,38]. However, one of the major drawbacks of classical immunotherapy with native allergens is the risk of triggering severe systemic adverse events following injection, as native allergens are, by definition, the target of specific IgE antibodies [39]. IgE antibodies recognise mostly three-dimensional structures. It was thus considered potentially useful in this respect to break conformational epitopes by using adequately tailored LSPs. Another advantage of overlapping LSPs was their ability to cover all potential T cell epitopes of the allergen of choice. Furthermore, LSPs could be synthesised in prevision of clinical trials, in optimal conditions of purity, following GMP guidelines. The authors, therefore, first designed a murine model of systemic allergy to phospholipase A2 (PLA2), a major bee venom allergen [40]. Mice were immunised repetitively subcutaneously with PLA2 in alum and mounted a strong IgE response. Once injected intraperitoneally with PLA2, mice were able to develop a marked decrease in rectal temperature, an expression of systemic anaphylaxis in these animals. To downregulate the allergic response in this model, the authors considered two different approaches: an immunotherapy protocol based on soluble LSP administration, either via the subcutaneous [40] or mucosal (nasal) routes [41]. In both cases, evidence of T cell tolerance was obtained, as suggested by marked T cell hyporesponsiveness specific to the whole allergen, as well as to each of the three LSPs covering PLA2 (LSP 1-60, 47-99, 90-134). Furthermore, allergen-specific IgE levels were markedly reduced and PLA2-specific IgG4 enhanced. Remarkably, whereas sensitised animals developed anaphylactic reactions at each intraperitoneal administration of native PLA2 in control animals, mice treated with the PLA2-derived LSP did not develop any significant change in their rectal temperature, indicative of an excellent tolerance of LSP and of the absence of activation of the immediate allergic reaction [40]. Furthermore, following provocation with native PLA2, LSP-treated animals did not decrease their rectal temperature, in contrast to untreated mice. Taken together, these models strongly support the setting of an immunotherapy protocol in bee venom-allergic patients based on the use of LSP covering the major bee venom allergen, PLA2.

### 4. Clinical trials

#### 4.1 *Plasmodium falciparum* circumsporozoite protein C-terminal fragment 282-383

A Phase I clinical trial was designed to determine the safety and immunogenicity of the C-terminal region of *P. falciparum* CSP 282-383 using a Good Laboratory Practice preparation that was sterily conditioned [42]. This procedure was allowed at the time the trial was planned. The initial synthetic protocol was optimised by selecting adequate protecting groups of the two cysteines present at the N-terminal part of the peptide, where a drop of 80% in the coupling efficiency was observed in the initial synthesis. The material was then oxidised in air. The LSP was formulated in alum and Montanide ISA 720 at two doses – 100 and 300 µg – and injected intramuscularly. The preparation was well-tolerated and few minor adverse reactions were recorded. Strong sporozoite-specific antibody responses were elicited in the volunteers who received the Montanide formulation at the 100 µg dose. In addition, robust lymphocyte proliferation responses were equally elicited with concomitant *in vitro* production of IFN-γ, crucial in the elimination of the parasite in almost all volunteers. Most importantly, as seen in mice, the authors also observed the development of CD8+ T lymphocyte responses, which are important in immunity to malaria. In contrast to other malaria preparations, notably the recombinant RTS,S [43] (which remains, at present, the only product giving some protection), recombinant CS vaccinia [44] or naked CS DNA [45], this was the first example in which a malaria candidate could simultaneously activate specific CD4+ and CD8+ T and B cells.

### Table 1. Long synthetic peptides in preclinical and/or clinical trials.

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<tr>
<th>Malaria antigens</th>
<th>Preclinical studies</th>
<th>Clinical trials</th>
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<tbody>
<tr>
<td>PfExp-1</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>CS-likeTRAP</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>CS 282-383</td>
<td>+</td>
<td>Phase I/la</td>
</tr>
<tr>
<td>GLURP 85-211</td>
<td>+</td>
<td>Phase Ia</td>
</tr>
<tr>
<td>MSP-2 3D7 strain</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>MSP-2 FC-27 strain</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>PvCS 23-99, 301-379</td>
<td>+</td>
<td>Phase Ia</td>
</tr>
<tr>
<td>PvCS repeat-tip30</td>
<td>+</td>
<td>Phase Ia</td>
</tr>
</tbody>
</table>

CS: Circumsporozoite; GLURP: Glutamic rich protein; MSP: Merozoite surface protein; ND: Not done; Pf: *Plasmodium falciparum*; Pla: Phospholipase; Pv: *Plasmodium vivax*.
A new Phase I clinical trial using a GMP preparation was then designed to determine the optimal dose and adjuvant that will be tested for efficacy studies using live sporozoites in a Phase II clinical trial. Results from both clinical studies should be available at the end of 2004. For this material, a further optimisation of the synthesis protocol was performed to allow a better control of the oxidation step of the final purified product by choosing the proper protecting groups of the four cysteines according to the authors’ oxidation protocol [101].

4.2 Plasmodium vivax circumsporozoite protein N, R and C fragments

A randomised, double-blind, single-site Phase I clinical trial was conducted in healthy and malaria naive adult volunteers. The vaccine consisted of three LSPs (N, R, C, see above) covering the full-length of the P. vivax CSP. In this case, no modification of the original synthetic protocols was introduced. Peptides were tested individually in a dose escalating fashion, at 10, 30 and 100 µg/dose formulated in Montanide ISA720. Vaccines were administered by intramuscular injection on the deltoid muscle on days 0, 60 and 180. Sixty-nine subjects were vaccinated; no severe adverse events were recorded and the vaccine was well-tolerated. The specific antibody response and the induction of IFN-γ production were assessed. High antibody titres to the N, R and C peptides and antigen-specific IFN-γ-producing T cells were elicited. Antipeptide antibodies recognised the CSP in incomplete Freund’s adjuvant tests (Herrera et al., submitted). Phase II clinical trials are now planned for this vaccine against P. vivax malaria.

4.3 Plasmodium falciparum merozoite surface protein-3 and glutamic rich protein

As discussed in 3.3, the MSP-3 conserved region 186-281 of P. falciparum is the target region of a protective immune response mediated by cytophilic antibodies [1]. Even in this case, no modification of the original synthetic protocols was performed. An open, randomised, two-adjuvant (Montanide ISA 720, Alum) Phase I clinical trial was set up to evaluate the safety and immunogenicity of increasing doses (10, 20, 30 and 100 µg) of a LSP construct spanning the conserved region of MSP-3 targeted by biologically active antibodies (MSP-3-LSP). Thirty-five healthy volunteers were randomised to receive 3 injections at day 0, 30 and 120. No vaccine-related serious adverse events occurred in the trial. After the third injection, volunteers displayed marked specific antibody response mediated by cytophilic antibodies (23/30) (mainly IgG1), anti-native MSP-3 response by immunofluorescence (19/30), T cell proliferative response (26/30) and IFN-γ production (25/30), as analysed per protocol. In conclusion, MSP-3-LSP vaccine was safe and immunogenic even at the lowest dose of antigen employed. The potential of the vaccine candidate is supported by the induction of a strong, protective cytophilic response, that is, the type of antibodies involved in protective mechanisms in endemic areas ([46] and Drulhe et al., manuscript submitted).

Similar studies were conducted with GLURP synthetic peptide 85-213 and similar conclusions as those found for MSP-3 candidate were obtained (Sauerwein et al., manuscript submitted)

Phase Ib studies are now under way in Burkina Faso for MSP-3 vaccine candidate, while similar studies are being planned for GLURP.

4.4 Bee venom phospholipase A

The authors first established in vitro that the three overlapping LSPs derived from PLA2 were able to efficiently stimulate bee venom-allergic patients’ peripheral blood mononuclear cells in terms of T cell proliferation, as well as T helper type 2 cytokine production, typical of the atopic status [37,47]. Moreover, T cell proliferation obtained with the mixture of the three long peptides was better than the one obtained with a mixture of small, 20-amino acid, overlapping peptides mapping the entire PLA2 allergen. This strongly suggested that LSPs were able to recruit several potential CD4 T cell epitopes in a more effective way than shorter peptides. Furthermore, in comparison with other strategies based on the use of a restricted number of T cell epitopes, LSPs were spanning all potential T cell epitopes of PLA2 and did not require, in these conditions, a prescreening of patients with adequate major histocompatibility complex class II presentation [47]. LSPs were thus considered as potential tolerising agents for PLA2-sensitive patients. As a caveat, and like any other approaches of specific immunotherapy (with recombinant allergens for instance), it will have to be postulated that an immunotherapy formulation based on a few major allergen-derived LSPs may be sufficient to significantly decrease patient hypersensitivity and reactivity to allergen exposure. It is indeed inconceivable to prepare mixtures of proteins such as those present in natural extract.

The authors designed a Phase I clinical study with the three LSPs covering PLA2 [48]. Nine bee venom-hypersensitive patients were compared to a placebo group of seven bee venom-sensitive patients. In the active group, LSPs were administered over a 3-h period, from a starting dose of 0.1 µg to a maximal dose of 100 µg, representing a cumulative dose of 254 µg of PLA2 – that is, a dose > 20-fold superior to the dose of native PLA2 than can be administered to hypersensitive patients during classical immunotherapy protocols. No systemic anaphylactic reactions or local reactions occurred in any of the patients during this short incremental dose period. Patients were then immunised with 100 µg of the three LSP mixture at day 4, 7, 14, 42 and 70. Clinical tolerance was generally excellent. In only two patients, mild, late (> 2 h) local reactions (erythema) occurred after peptide injection at days 14, 42 and 70, and disappeared after ∼ 1 h. In these two patients, after the last injection at day 70, hand palm pruritus and transient erythema of the upper part of the trunk occurred > 3 h after LSP injection, where symptoms were compatible with T cell activation, but not immediate anaphylactic reactions. There were no severe adverse (life-threatening) events.
Immunological end points revealed a marked hyporesponsiveness of T cells to LSP stimulation after an initial phase of activation at day 14. One month after the start of immunisation, peptide-specific T cells were secreting markedly enhanced amounts of IL-10 and IFN-γ, whereas IL-4 was low and unchanged, as compared with the control group. There was a significant decrease in PLA2-specific IgE at day 80 and a parallel rise in PLA2-specific IgG4. In preliminary studies, despite their relatively long sequence, LSPs were binding IgE in only 1 patient out of 10. After the course of immunisation in bee venom-allergic patients, there was no significant increase in PLA2-specific IgG4. In preliminary studies, safety was excellent and strongly supported the use of LSPs for further immunotherapy studies in allergic patients.

5. Expert opinion and conclusions

The experience accumulated in 10 years of long peptide SPPS indicates that this approach can be used as a valuable alternative/complement to other vaccine discovery approaches.

SPPS is certainly unique in the rapid progress from preclinical to clinical testing through a faster selection of possible candidates, thus reducing the cost of overall vaccine development.

On the other hand, this approach to vaccine development has been restricted to academic institutions/small–medium biotech companies and has barely attracted major pharmaceutical companies nor has been supported by major donors. We feel that this limitation could be mainly attributed to the lack of proper understanding by the scientific and industrial community at large of the enormous potential and versatility that this platform can offer. Synthetic polypeptide products of ~40 amino acids are already commercially available. We feel that the great progress in synthesis, purification and analysis protocols/instrumentations achieved in the recent past will undoubtedly further demonstrate that this length does not represent an insurmountable stumbling block. If synthetic vaccine candidates were found to be highly protective in Phase II clinical trials, we have no doubt that the necessary financial and technological resources will be mastered to produce the quantity necessary to cover the world population, as was the case with the malaria synthetic candidate vaccine developed by Patarroyo and collaborators [50]. Unfortunately, after several Phase III trials, it was concluded that it was not efficacious [51,52]. Thus, it is our hope and conviction that more bold choices and investments will be made by the pharmaceutical companies and fund providers to take full advantage of the potential of the long peptide SPPS platform in bringing safe and efficacious vaccines/therapeutic molecules to the needed populations in record time. In addition, SPPS coupled with DNA recombinant technology should also provide a unique opportunity to screen entire genomes for molecules/fragments of interest and to rapidly test the candidate vaccines in clinical trials.

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Medicinal application of long synthetic peptide technology


Patent


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