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Applications of Protein Epitope Mimetics in Vaccine Design.

A New Supersecondary Structure in the Circumsporozoite Protein of *Plasmodium falciparum*?

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Abstract: An approach to synthetic vaccine design is illustrated, focusing on the immunodominant $(NPNA)_n$ repeat region of the circumsporozoite (CS) protein of the malaria parasite *Plasmodium falciparum*. Modelling suggests that the NPNAN motif may adopt a helical β-turn, which is tandemly repeated in the CS protein to generate a novel supersecondary structure. Cyclic peptidomimetics of this NPNAN motif were synthesized and shown by NMR to adopt helical turns in aqueous solution. When incorporated into Immunopotentiating Reconstituted Influenza Virosomes (IRIVs), humoral immune responses were generated in mice that cross-react with native CS protein on sporozoites. IRIVs are a human-compatible delivery system that appear generally suitable for inducing antibody responses against conformational epitopes using constrained peptidomimetics. This approach may offer great potential for the design of molecularly defined synthetic vaccines, including those targeted against multiple antigens and development stages of *P. falciparum*, or against other infectious agents.

Keywords: Peptide · Peptidomimetic · Protein folding · Secondary structure · Synthetic vaccine

Introduction

Malaria is one of the world's most debilitating diseases, with more than 2 billion people currently at risk worldwide [1], and a toll of several hundred million illnesses, and 1.5–2.7 million deaths annually (WHO, World Health Report, 1998). There is presently no effective vaccine against the parasite, and older established drugs like chloroquine are rapidly losing their effectivenesss due to resistance. On the brighter side, ongoing efforts to sequence the 14 chromosomes and around 7000 genes in the malaria parasite [2] will no doubt provide many exciting opportunities for the discovery of new drug targets [3][4], as well as highlighting new antigens as potential malaria vaccine candidates [5][6].

A vaccine against the extracellular stages of the parasite should function by stimulating the immune system to produce antibodies that recognize the intact parasite. This can, in principle, be achieved with either an attenuated or irradiated parasite, or a subunit such as a recombinant surface protein, or a specific surface epitope in the form of a synthetic peptide. One of the many difficulties in designing an effective malaria vaccine is the complex life cycle of the parasite [7][8]. When an infected *Anopheles sp.* mosquito bites a human host, thousands of threadlike sporozoites enter the bloodstream. Within a matter of minutes, the sporozoites can invade liver cells, where they are hidden from the antibody-arm of the immune system. Within the liver cell, the sporozoite develops into a schizont containing more than 10 000 uninucleated merozoites. The growing parasite causes the hepatocyte to rupture, releasing the merozoites into the blood stream. The merozoites can invade blood cells, and undergo further multiplication. Alternatively, merozoites can develop into a sexual stage known as gametocytes, which can reinfect mosquitoes. In the mosquito gut, an oocyst is formed, out of which new sporozoites emerge, thereby completing this remarkable life cycle. Not surprisingly, the surface proteins on sporozoites that stimulate an immune response are not the same as those found on merozoites, or indeed on other developmental stages of *P. falciparum*. Hence an effective malaria vaccine will most likely need to be composed of several immunogens targeting multiple developmental stages of the parasite.

It has been known for some time that sporozoites attenuated by X-irradiation can induce a protective, immune response against malaria challenge [9]. The dominant antibody target on these attenuated sporozoites is the major surface pro-

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tein, the circumsporozoite (CS) protein [10]. The central portion of the CS protein ($M_r \approx 44$ kDa [11][12]) contains 41 tandem repeats of a tetrapeptide, 37 of which are Asn-Ala-Asn-Pro (NANP) and four of which are Asn-Val-Asp-Pro (NVDP). In fact, sequencing studies have shown that many proteins and genes of malaria parasites contain extensive arrays of tandemly repeated amino acid motifs [13].

It was shown that linear, tandemly repeated NANP peptides can elicit antibodies in mice and rabbits that recognize the native CS protein and block sporozoite invasion of hepatocytes [12][14–16]. These results were a prelude to vaccination studies in humans with synthetic $(NANP)_3$ peptides conjugated to tetanus toxin, which proved that an anti-sporozoite immune response could be generated in this way, but the efficacy was not good enough for use as a malaria vaccine [17]. Subsequently, a number of studies were initiated to optimise the immune response to $(NANP)_n$ peptides (*e.g.* [18–21]). It is noteworthy in all these efforts, that the conformation(s) of the NANP repeats in the CS protein was not known, and so could not be taken into account in the design process. It seemed most likely that short linear $(NANP)_3$ peptides would be largely unstructured in aqueous solution, and be susceptible to rapid proteolytic degradation in serum. A later study also suggested that a significant part of the immune response against a linear $(NANP)_3$ peptide is directed against the chain terminii [22], which of course are not present in the native CS protein.

Conformation plays a key role in the ability of peptides to elicit antibody responses against folded proteins. Linear peptides often elicit antibodies that bind well to denatured proteins, but less frequently recognize conformational epitopes in native protein structures. Unfortunately, the structure of the (NANP)-repeat region in the CS protein is still unknown. It is likely, however, to adopt a stable and repititious structure. Early theoretical studies led to models involving various helical-like structures [23–25]. It was also suggested that the NPNA repeat unit (**1**) might adopt a stable type-I β-turn (Fig. 1) [24]. This is important, since it implies that the repeating structural motif is formed by the turn-forming $(NPNA)_n$ cadence rather than by $(NANP)_n$. Experimental support for this idea was obtained later from NMR studies of peptides in aqueous solution with both the NANP and NPNA cadences [26]. These studies provided evidence that linear $(NPNA)_n$ peptides exist in a dynamic equilibrium between unfolded and β-turn-like hydrogen-bonded, folded conformations, with the folded forms encompassing the structural unit NPNAN, rather than the more commonly quoted four residue β-turn NPNA.

Stabilization of β-turns in the NPNA motif has been achieved both by C(α)-backbone methylation of proline (**2**) [27][28] and by incorporation of NPNA motifs into template-bound cylic peptidomimetics (**3**) [29] and (**4**) [30], without abolishing the ability of these analogues to elicit sporozoite cross-reactive antibodies in mice. Interestingly, the mimetic **4** exhibits in NOESY spectra strong $d_{NN}(i,i+1)$ connectivities between Asn^5 and Ala^6 as well as Ala^6 and Asn^7, indicative of a helical β-turn within the NPNAN motif, of the type suggested earlier by Dyson and coworkers [26]. A molecular model of **4**, consistent with the NMR data, predicts a helical turn for the NPNAN unit comprising a type-I β-turn, with the Asn^3 CO in H-bonding distance of the Ala^6 HN, and in addition the possibility of an i (Asn^3 CO) to i+4 (Asn^7 HN) hydrogen bond, *i.e.* with Ala^6 in the α-region of φ/ψ space.

We are exploring an approach to synthetic vaccine design, which involves using protein epitope mimetics (PEMs), in the form of cyclic peptidomimetics, coupled to a human compatible adjuvant, for the induction of antibody responses against conformational epitopes. Allied with the use of combinatorial chemistry

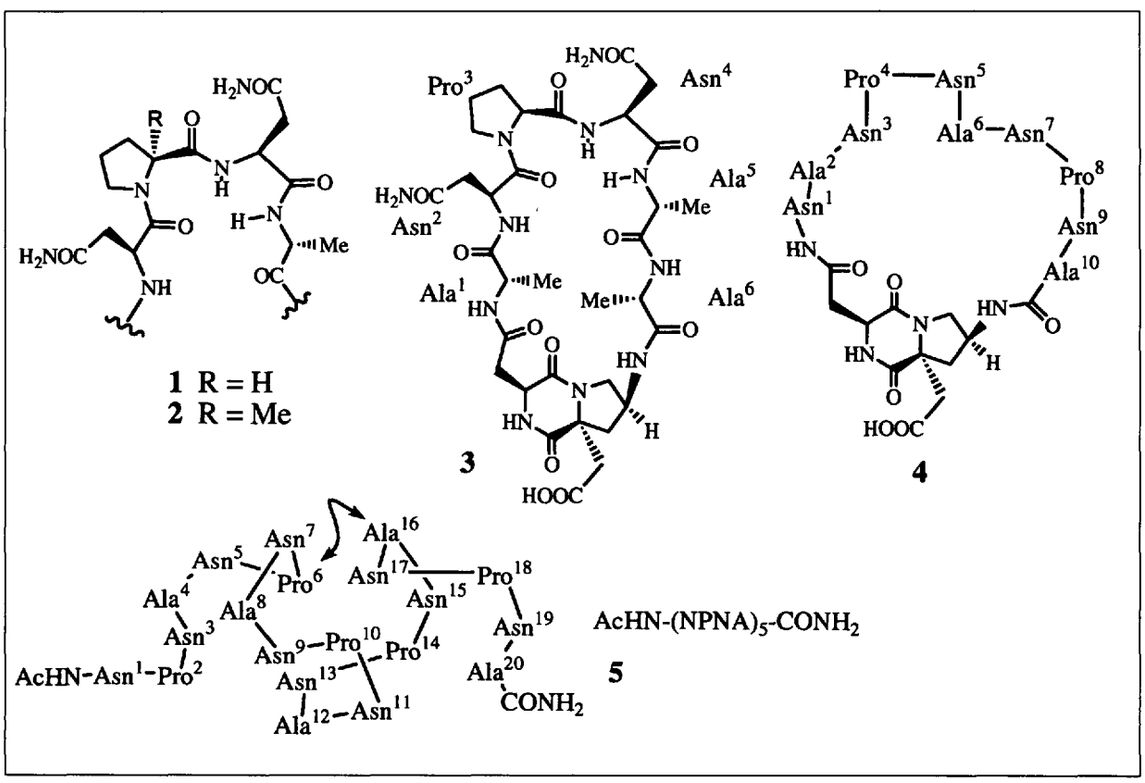

Fig. 1. Structures of the NPNA motif and related peptidomimetics. The arrow on structure **5** indicates the position for the cross-link (see text)

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methods, this approach may have great potential for the identification and optimization of molecularly defined synthetic vaccine candidates, in a form directly suitable for human clinical trials. We describe below some results of these studies, focusing on the NPNA-repeats of the malaria parasite, which lead to a new proposal as to how this region might fold in the native CS protein. This information may be of value in attempts to design more effective anti-sporozoite vaccines.

Molecular Modelling

Using the backbone ϕ/ψ angles for Asn^3-Asn^7 taken from earlier models of **4** [30], a linear peptide was built with the sequence Ac-$(NPNA)_5$-NH_2 (**5**) wherein the helical turn conformation (with the appropriate backbone ϕ/ψ angles) was also tandemly repeated. The resulting model of **5** (Fig. 2) was stable in MD simulations in water solvent, and adopted the expected repititious supersecondary structure shown in Fig. 3A. Conceivably, this supersecondary structure may be close to the preferred conformation of the NPNA-repeat region in the native CS protein.

To explore this idea further, we set out here to stabilize this supersecondary structure by appropriate cross-linking of the peptide backbone in **5**, and by examining the ability of the resulting cross-linked peptidomimetic to elicit antibodies that recognize the native CS protein on sporozoites. Examination of molecular models suggested that a suitable cross-link could be formed by introducing an amino group at the β-position of Pro^6 and amide coupling to the spatially adjacent side chain carboxyl of Glu as a replacement for Ala^{16}, *i.e.* as indicated in Fig. 1–3. A model of this cross-linked peptidomimetic was constructed, and the model also remained in the expected conformation during MD simulations in water (Fig. 3B).

Synthesis of the Peptidomimetic

The required orthogonally protected (2*S*,3*R*)-3-aminoproline (**9**) was prepared from the known β-lactam **6** [31] as shown in Scheme 1. The chemistry is straightforward, and the synthesis proceeds in good yields. The required cross-linked peptidomimetic was prepared by solid-phase peptide synthesis methods, as outlined in Scheme 2. The 20-mer peptide **10** was assembled using Fmoc-chemistry. Cleavage from the resin and removal of side-chain protecting groups proceeded in one step to afford **11**. The key backbone coupling of the $Apro^6$ and Glu^{16} side chains was then achieved in a remarkably clean and high yielding cyclization in DMF with HATU. Monitoring the reaction by HPLC showed essentially quantitative cyclization of the precursor (data not shown). This high efficiency probably reflects the fact that the required conformation is strongly preferred by the peptide backbone. Finally, the 20-mer **12** was acetylated to give **13** for conformational studies by NMR, and **12** was also coupled *via* a succinate linker to a regioisomer of phosphatidyl ethanolamine (PE', 1-palmitoyl-3-oleoyl-phosphatidylethanolamine) to afford the conjugate **14** ready for incorporation into an IRIV (see below).

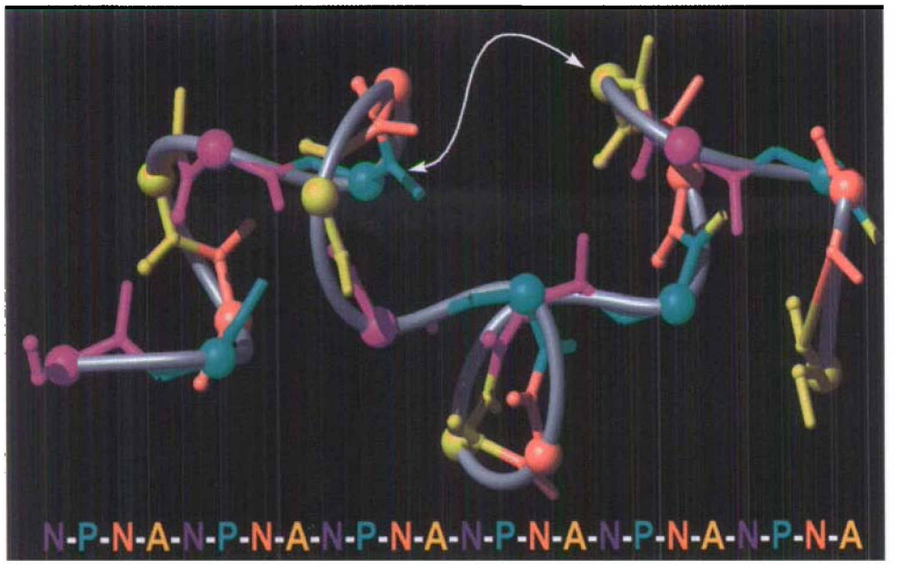


Fig. 2. A computer model of **5** in a stable conformation in which each NPNAN motif adopts a helical β-turn (see text). The extended $(NPNA)_n$ sequence in the CS protein may adopt a repeated helical β-turn supersecondary structure related to that shown here. The arrow indicates the position for the cross-link (see text). Colour coding; Asn = pink, Pro = cyan, Asn = coral, Ala = yellow. The C(α) atoms are marked with a ball

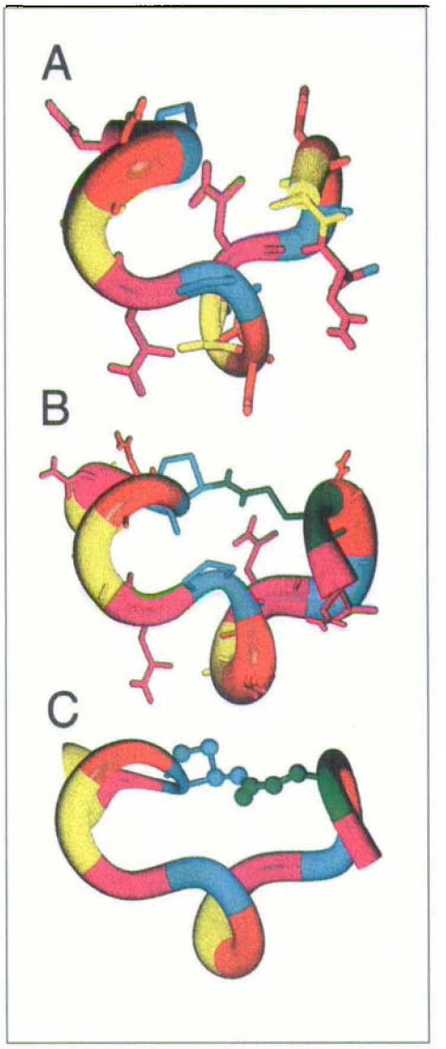
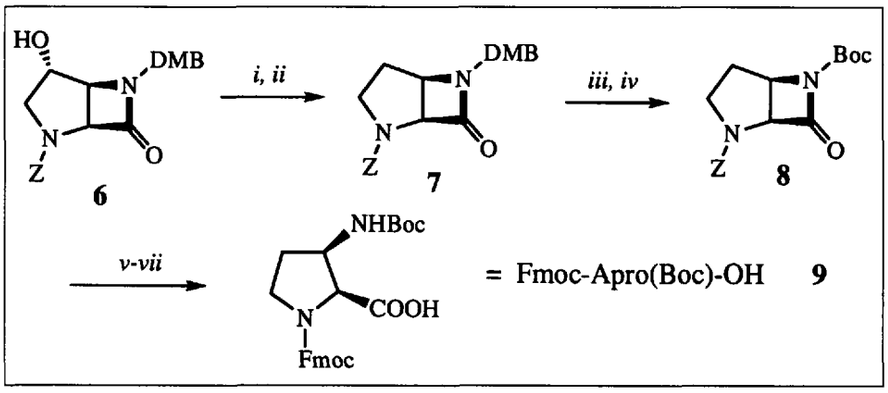


Fig. 3. **A**, Representation of superimposed structures taken from an MD simulation in water of peptide **5** residues Asn^5-Ala^{16} with the repeated helical β-turn supersecondary structure (see Fig. 2). The sausage was calculated using the average displacement of the C(α) atoms, which is represented by the spline radius. The same colour coding used in Fig. 2 applies here. **B**, As in **A**, except the molecule is a model of peptidomimetic **13**. The $Apro^6$ residue is in cyan and Glu^{16} is in green. **C**, As in **B**, except the molecule is the average NMR structure of **13** deduced in water by NMR and dynamic simulated annealing. The sausage now represents the average displacement of the C(α) atoms of four NMR structures. The cross-linked residues are shown in ball-and-stick. The N- and C-terminal NPNA motifs are omitted for clarity. This Fig. was prepared using MOLMOL [34]

Conformational Studies

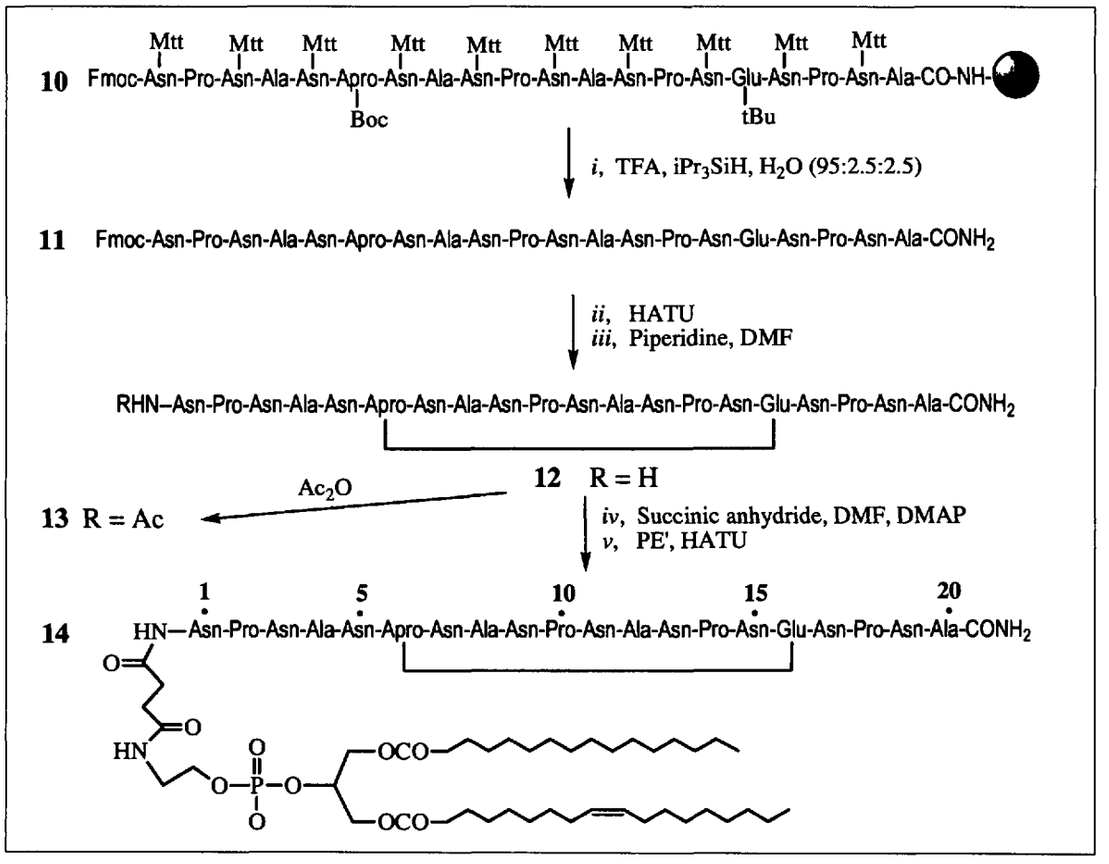
The preferred solution conformation of **13** was studied by NMR and MD methods in aqueous solution at pH 5 and 293K, in analogy to previous studies of **3** [29]. The 1D ^{1}H NMR spectra indicated the presence of a major conformer and two minor ones (ratio 80:14:6), the latter two most likely arising due to *cis-trans* isomerism at Asn-Pro peptide bonds, in analogy to earlier studies [26][27][29]. The minor forms were not considered further. A full assigment of the ^{1}H spectrum of the major form was complicated by chemical shift overlap, particularly of the Asn H-C(β) resonances. However, the peptide backbone HN, H-C(α) resonances could be assigned unambiguously.

2D NOESY spectra showed strong $d_{NN}(i,i+1)$ connectivities between the peptide NH groups of Asn^7 and Ala^8 as well as Ala^8 and Asn^9 in the first helical turn, Asn^{11} and Ala^{12} as well as Ala^{12} and Asn^{13} in the next helical turn, and between Asn^{15} and Glu^{16} as well as Glu^{16} and Asn^{17} in the last helical turn. These together with the observation of long range NOEs between Pro H-C(α) (i+1) and Ala HN (i+3), provide evidence for three relatively stable helical turns formed by the residues Asn^5-Asn^9, Asn^9-Asn^{13}, and Asn^{13}-Asn^{17}.



Scheme 1. *i*, $(Tf)_2O$, CH_2Cl_2, pyridine (98%); *ii*, $NaBH_4$, THF/DMF (58%); *iii*, $K_2S_2O_8$, Na_2HPO_4, MeCN/H_2O (80%); *iv*, $(Boc)_2O$, CH_2Cl, DMF, Et_3N (76%); *v*, LiOH, THF, H_2O (99%); *vi*, Pd-C, MeOH, H_2 (93%); *vii*, Fmoc-Osucc, iPr_2NEt, CH_2Cl (68%)

Average solution structures for **13** were calculated using NOE-derived distance restraints by dynamic simulated annealing and MD simulations, using methods described earlier [29]. The resulting average structures reveal a common core comprising the anticipated three helical turns from Asn^5-Asn^{17}, with higher flexibility in the regions of the N- and C-termini (Fig. 3C). The backbone conformation of the central region, however, corresponds closely to the expected supersecondary structure deduced for models of **5** (Fig. 3A and 3B). We conclude, therefore, that although the mimetic is not rigid, it can adopt a supersecondary structure comprising three interlinked helical turns, each based on the (NPNAN) motif. In future work we will seek to strengthen this conclusion, through the synthesis and conformational analysis of other cross-linked peptidomimetics.



Scheme 2. Synthesis of the peptidomimetics **13** and **14**

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Immunological Studies

For immunizations, the constrained peptidomimetic was coupled to PE' (**14**), and then incorporated into an IRIV. IRIVs are spherical, unilammelar vesicles, prepared from a mixture of phospholipids and influenza virus surface glycoproteins [32]. The hemagglutinin membrane glycoprotein of influenza virus plays a key role in the mode of action of IRIVs. This major antigen of influenza virus is a fusion-inducing component, which facilitates antigen delivery to immunocompetent cells. The IRIV technology has been licensed already for human clinical use. In the case of the hepatitis A vaccine Epaxal-Berna™ [33], the first IRIV-based non-influenza vaccine to receive a product license for human use from a national authority, the hepatitis A antigen spontaneously binds to the IRIV. In this study, we have coupled the peptidomimetic covalently to PE' (**14**) and incorporated the mimetic-PE' conjugates into the IRIV. Since IRIVs have been licensed already for human use, the peptidomimetics can be tested in a format that directly allows human clinical studies.

Antibody responses elicited by IRIV loaded with **14** were studied in BALB/c mice. After a pre-immunization with the influenza vaccine Inflexal Berna™ (Berna-Products, Bern, Switzerland), and three doses of IRIV-**14**, the sera of all immunized mice contained mimetic-specific antibodies, as demonstrated by ELISA with **14** coated on ELISA plates (Fig. 4A). The cross-reactivity of these anti-sera with the template-bound peptidomimetics **3** and **4** was also analysed by ELISA. The sera from three of four mice immunized with **14**-IRIV cross-reacted with the mimetic **4** (Fig. 4B), but none reacted with **3** (Fig. 4C). It is interesting to note that a helical NPNAN turn is possible in **4** but not in **3** [30]. That a significant part of the antibody response to **14**-IRIV cross-reacts with **4** also means that these cross-reacting antibodies should not recognize alone the ends of the peptide chain in **14**, but rather the novel helical-turn supersecondary structure in the central part of the molecule. However, structural studies with monoclonal antibodies will be necessary to confirm this conclusion.

The binding of anti-**14**-IRIV antisera to the CS-protein was analysed by an indirect immunofluorescence assay using *P. falciparum* sporozoite preparations. In all immunized animals a significant anti-sporozoite antibody response was detected (Fig. 5A). The specificity of the cross-reaction was demonstrated by a competition experiment. Incubation of the antiserum with the sporozoites in the presence of **13** completely abolished immunostaining (Fig. 5B). The IRIV formulations thus elicited a significant proportion of parasite binding antibodies among the total anti-mimetic immune response. Future studies will focus on more detailed analyses of cross-reactivity of monoclonal anti-**14**-IRIV antibodies and structural studies to determine the conformations of bound antigen.

Significance

Synthetic linear peptides are often compromised as vaccine candidates due to their inherent flexibility and susceptibility to proteolysis. Linear peptides often elicit antibodies that bind well to denatured proteins, but less frequently recognize conformational epitopes in native protein structures. A further problem is the weak immune responses elicited by linear peptides, even conjugated to carrier proteins, when administered in alum,

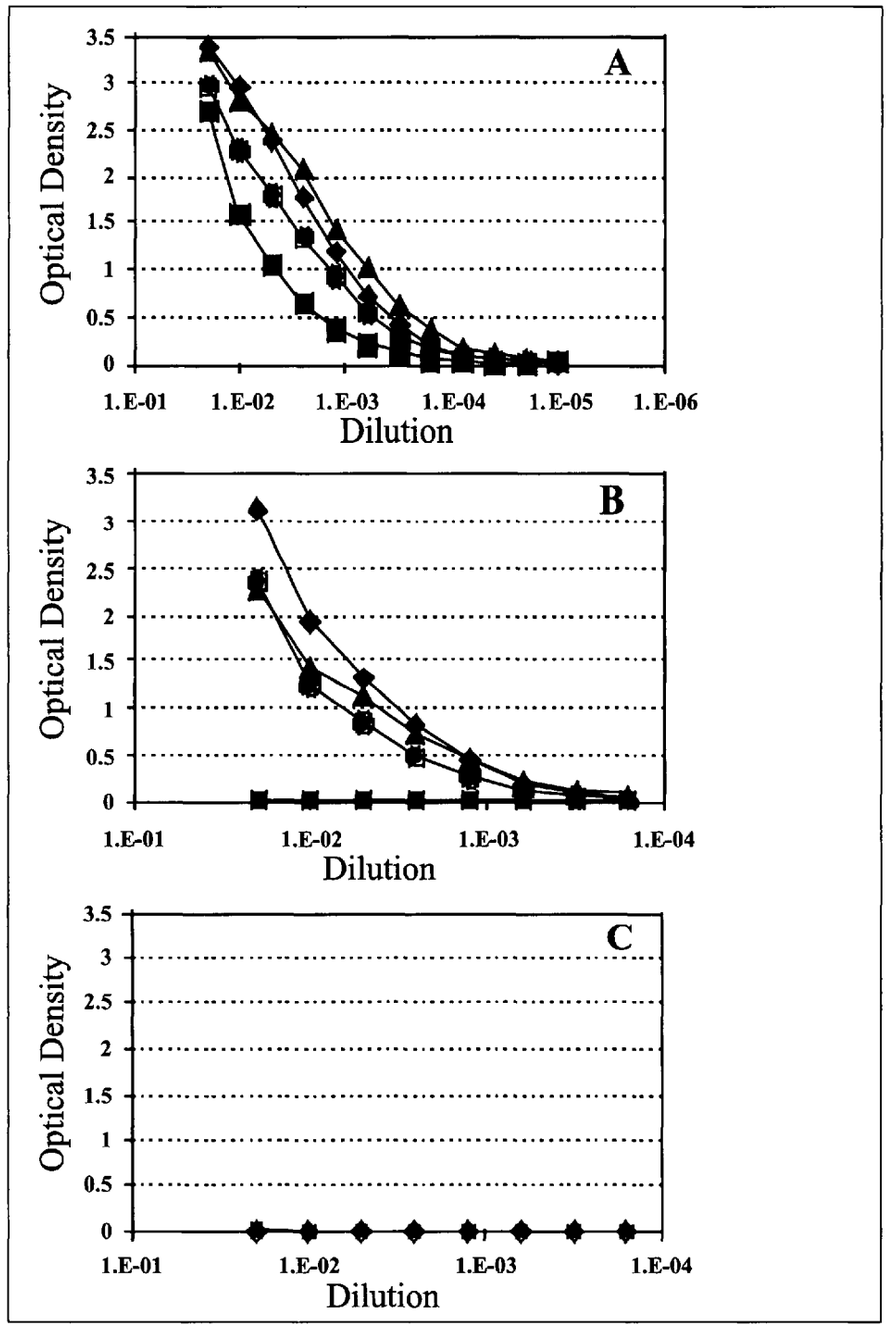

Fig. 4. Serum IgG titres of BALB/c mice immunized three times with **14**-IRIV. ELISA was performed in ELISA microtiter plates coated either with **14** (A), a PE-conjugate of **4** (B) or with **3** conjugated to a multiple antigen peptide for coating on the ELISA plates (C) and incubated with serial dilutions of the sera of individual mice. Bound IgG was detected using alkaline phosphatase-conjugated antibodies specific for mouse gamma heavy chains

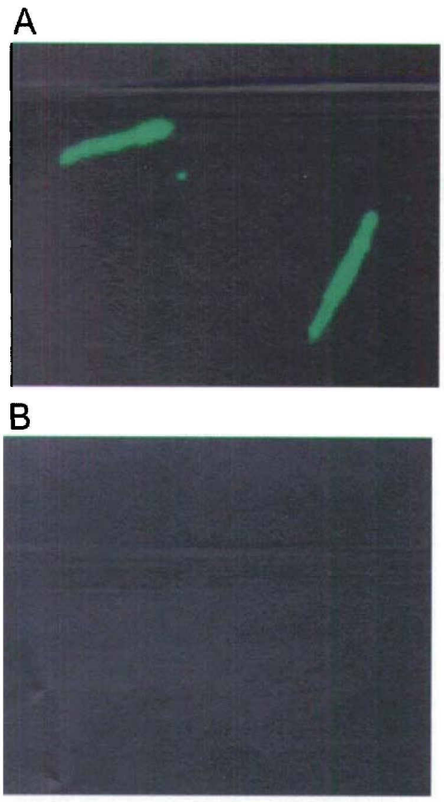

Fig. 5. Immunofluorescence staining of *P. falciparum* sporozoites by mouse anti-**14-IRIV** antiserum, using a FITC-labelled secondary anti-mouse IgG antibody (A). Incubation of the primary antibody with the parasites in the presence of the mimetic **13** (50 μg/ml) abolished staining of the sporozoites (B)

the commonly used human compatible adjuvant.

In this work, an approach to synthetic vaccine design is followed, in which cyclic peptidomimetics are presented to the immune system in multiple copies on the surface of Immunopotentiating Reconstituted Influenza Virosomes (IRIVs). These virosome particles contain also influenza virus proteins that facilitate uptake of the virosome by immunocompetent cells. IRIVs have been licensed already for human use, so the peptidomimetics can be tested in a format that directly allows human clinical studies.

Here, peptidomimetics of the central $(NPNA)_n$ repeat region of the circumsporozoite (CS) protein of the malaria parasite *Plasmodium falciparum* have been studied. Previous NMR and modelling studies suggests that NPNAN units in this region adopt a helical β-turn, which may be tandemly repeated to form a novel supersecondary structure. To test this proposal, a cyclic mimetic was prepared, and shown by NMR methods to adopt a preferred conformation having three tandemly repeated helical turns. Antibodies raised against the mimetic were shown to cross-react with the native CS protein on *P. falciparum* sporozoites. Further studies are now planned with a small library of related mimetics, to provide further support for the biological relevance of this new supersecondary structure, and to further optimise the immunological response with a view to application as a potential anti-sporozoite vaccine candidate.

In general, this approach appears to offer great potential for the design of molecularly defined combined synthetic vaccines, including those targeted against multiple antigens and development stages of *P. falciparum*, and against other infectious agents.

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