Exploiting Conformationally Constrained Peptidomimetics and an Efficient Human-Compatible Delivery System in Synthetic Vaccine Design

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Peptide and protein mimetics are potentially of great value in synthetic vaccine design. The mimetics should function by stimulating the immune system to produce antibodies that recognize the intact parasite. Also the mimetics should be presented to the immune system in a way that leads to efficient antibody production. Here we investigate the application of cyclic peptidomimetics presented on immunopotentiating reconstituted influenza virosomes (IRIVs), a form of antigen delivery that is licensed already for human clinical use, in synthetic vaccine design. We focus on the central $(NPNA)_n$ repeat region of the circumsporozoite (CS) protein of the malaria parasite Plasmodium falciparum *as a model system. Cyclic peptidomimetics of the NPNA repeats were incorporated into both an IRIV and (for comparison) a multiple-antigen peptide (MAP). Both IRIV and MAP delivery forms induced mimetic-specific humoral immune responses in mice, but only with the mimetic-IRIV preparations did a significant fraction of the elicited antibodies cross-react with sporozoites. The results demonstrate that IRIVs are a delivery system suitable for the efficient induction of antibody responses against conformational epitopes by use of cyclic template-bound peptidomimetics. Combined with combinatorial chemistry, this approach may have great potential for the rapid optimization of molecularly defined synthetic vaccine candidates against a wide variety of infectious agents.*

KEYWORDS:

antibodies · immunochemistry · peptides · peptidomimetics · virosomes

Introduction

There is great interest in the use of peptide and protein mimetics in the design of novel synthetic vaccine candidates. Due to their inherent flexibility, linear peptides often elicit antibodies that bind to denatured proteins but that less frequently recognize conformational epitopes in native protein structures. This, together with their often weak ability to elicit antibody production when administered as conjugates in human-compatible adjuvants (for example, alum), has so far limited the application of peptides as synthetic vaccine candidates. We investigate here a new approach to synthetic vaccine design, through the use of conformationally constrained cyclic peptidomimetics displayed on the surface of influenza virus like particles, called immunopotentiating reconstituted influenza virosomes (IRIVs). IRIVs have been used already to present viral proteins to the immune system, for example, the IRIV-based hepatatis A vaccine Epaxal-Berna was the first liposomal vaccine to receive a product license for human use from a national authority.[1, 2] We set out here to show that IRIVs, as a human-compatible adjuvant, are also capable of presenting peptidomimetics in multiple copies to the immune system and of eliciting efficient pathogen cross-reactive antibody responses.

As a model system we have chosen the central $(NPNA)_n$ repeat region of the circumsporozoite (CS) protein of the malaria parasite *Plasmodium falciparum*. Sporozoites attenuated by X irradiation can induce a protective humoral immune response against a malaria challenge.[3] The dominant antibody target is the approximately 45 kDa CS protein,[4–6] which includes a central region containing 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). Linear, tandemly repeated NANP peptides were shown earlier to elicit antibodies in mice and rabbits that

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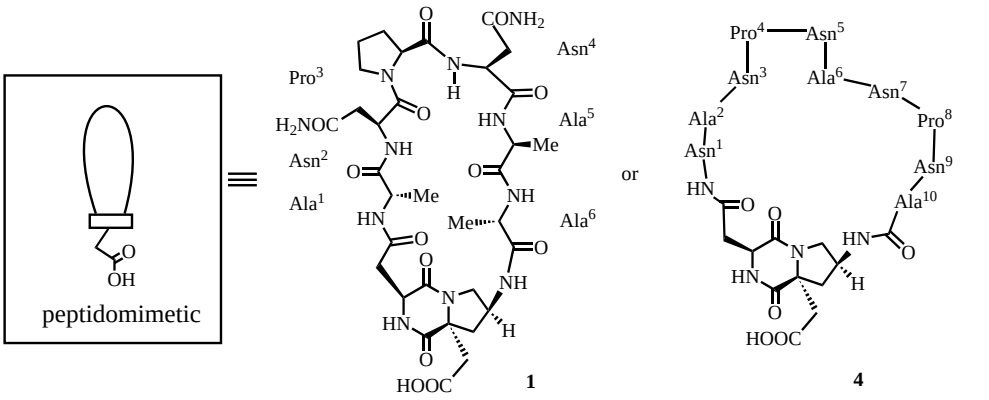
recognize the native CS protein and block sporozoite invasion of hepatocytes.[6–9] These findings were a prelude to vaccination studies in humans with linear synthetic $(NANP)_3$ peptides conjugated to tetanus toxin. However, the immune response generated in this way was not strong enough for these conjugates to be useful as a malaria vaccine.[10] Presently, the structure of the $(NANP)_n$ repeat region in the CS protein is still unknown, although theoretical studies suggest it is likely to adopt a stable and repetitious conformation,[11–13] possibly based on β-helical turns or similar structures.[14]

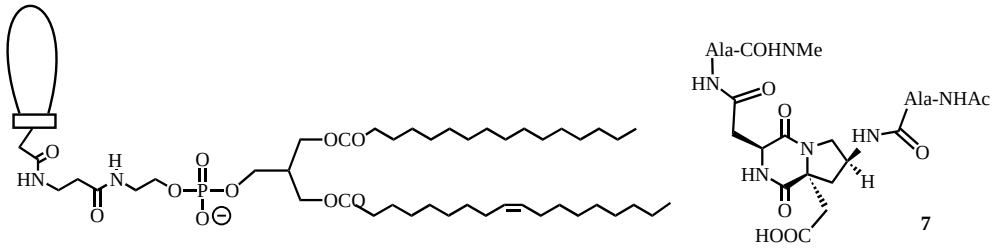
Conformation plays a key role in the ability of peptides to elicit antibody responses against folded proteins. In this work, we have prepared cyclic peptidomimetics of the NPNA repeats, and covalently linked them to IRIVs. IRIVs consist of spherical, unilammelar virus-like particles prepared from a mixture of phospholipids and influenza virus surface glycoproteins.[2, 15] However, no nucleic acids are present. The hemagglutinin membrane glycoprotein of the influenza virus plays a key role in the mode of action of IRIVs. This major antigen of the influenza virus is a membrane fusion inducing component, which facilitates antigen delivery to immunocompetent cells.[16] We show that, at least in this case, compared to alum-adjuvanted constructs of the peptidomimetic with a multiple-antigen peptide (MAP), the IRIV delivery system more efficiently elicits parasite cross-reactive antibodies. The approach appears to be well suited for the design of molecularly defined synthetic vaccine candidates, in a form that is directly suitable for human clinical testing.

Results

Design and synthesis of mimetics: The mimetic **1**, described in earlier work,[17] contains an NPNA motif linked through flanking alanine residues to a template. This mimetic was coupled through a succinate linker to a regioisomer of phosphatidylethanolamine (PE′, 1-palmitoyl-3-oleoyl-phosphatidylethanol-



1 **4**



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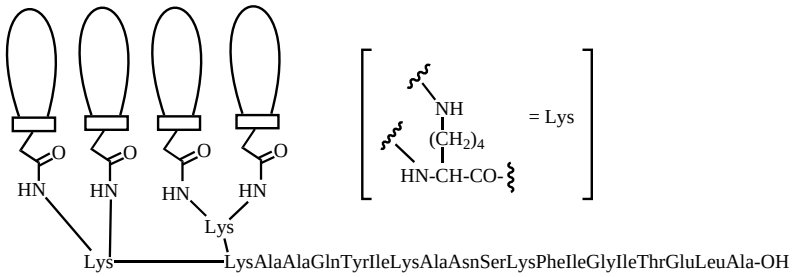
2, with peptidomimetic **1**
5, with peptidomimetic **4**



3, with peptidomimetic **1**
6, with peptidomimetic **4**
8, with peptidomimetic **7**

amine), to afford the conjugate **2**, ready for incorporation into IRIVs. In addition, **1** was incorporated into the MAP construct **3**, as described previously.[17] The mimetic **4** contains a larger loop with two intact NPNA units. The mimetic **4** was also coupled through a linker to PE′ to give **5**, and to a MAP to give **6**.[18] To determine how strongly immunogenic the template is in the mimetics **1** and **4**, the molecule **7** was prepared and incorporated into a MAP to give **8**.

Antibody responses elicited by a peptidomimetic formulated with alum or with IRIVs: First, antibody responses elicited by the MAP construct **3** adsorbed onto alum, and by IRIVs loaded with **2**, were compared in BALB/c mice. After three immunizations, sera from all immunized animals contained mimetic-specific antibodies, as demonstrated by enzyme-linked immunosorbent assays (ELISAs) with immobilized **3** (Table 1). None of the sera exhibited cross-reactivity with the template – MAP construct **8** (see Figure 1), which indicates that the immunogenicity of the template itself was negligible.

The binding of antibodies to the CS protein was analyzed by immunofluorescence assays (IFAs) with *P. falciparum* sporozoite preparations (Table 1). The IRIV formulation elicited significant anti-sporozoite responses in all animals immunized. In contrast, half of the animals immunized with the alum formulation generated no detectable anti-sporozoite antibody response and in the others the IFA titers were very low. The IRIV formulation thus elicited a higher proportion of parasite-binding antibodies among the total antimimetic antibodies than the alum formulation (compare Figures 2 A and B).

Table 1. *Antibody responses in mice immunized with peptide mimetics coupled to IRIV or adsorbed onto alum.*

Mouse no.	Immunogen	Sporozoite IFA titer	ELISA titer[a]
group a[b]	**3** – alum		
1		10	6386
2		10	4004
3[c]		–	1651
4[c]		–	3445
group b	**2** – IRIV[d]		
5		200	1052
6		400	1126
7		200	1258
8		400	1658
9		1000	1187
group c	**2** – IRIV[d]		
10		400	591
11		200	1094
12		40	482
13		400	1073
14		500	1803
group d	**5** – IRIV		
15		400	2966
16		4000	4209
17		4000	684
18		4000	1810
19		2000	3032
20		4000	1112
21		1000	881
22		2000	1357
23		4000	1694
24		4000	1088

[a] ELISA titers were calculated at effective dose 20% values with GENESIS LITE software. [b] One animal died after the second immunization. [c] Animals 3 and 4 showed no IFA reactivity. [d] In group b the phospholipid to **2** ratio was 38:3, in group c it was 7.6:3.

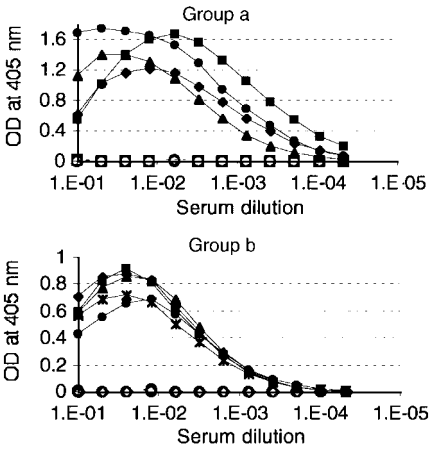


Figure 1. *Anti-peptidomimetic IgG ELISA responses against template-bound peptidomimetics (closed symbols) and template **8** (open symbols) in mice immunized three times with **3** – alum (group a) or **2** – IRIV (group b). Mice were preimmunized with Inflexal and received three doses of mimetic (50 μg) intramuscularly. Each curve represents the data from an individual mouse.*

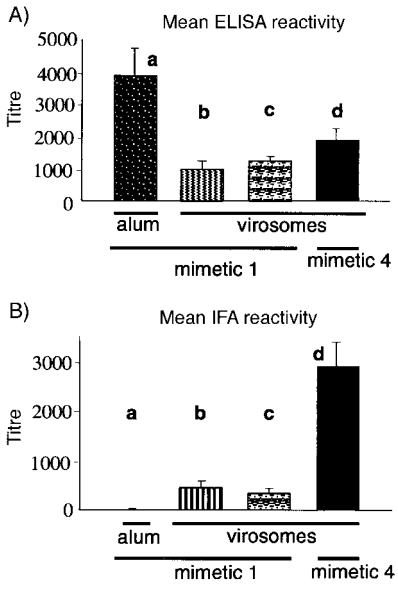


Figure 2. *Mean ELISA and IFA (A and B, respectively) IgG serum responses in mice immunized three times with peptidomimetics. Group a received **3** – alum, groups b and c received **2** – IRIV, and group d received **5** – IRIV. Bars represent the mean reactivity plus SE. SE = Standard error.*

In a second series of experiments the immunogenicity of cyclic peptidomimetic **4** was analyzed. After three immunizations with IRIV-loaded **5**, significant ELISA and IFA titers were seen in sera from all immunized mice (Table 1). While the ELISA titers were only slightly higher than those elicited by IRIV-loaded **2**, the IFA titers were significantly higher (compare Figures 2 A and B). The mimetic **4** was thus superior to **1** in eliciting a high proportion of parasite cross-reactive antibodies. None of the anti-**5** – IRIV

antisera cross-reacted with the template – MAP conjugate **8** in an ELISA (data not shown).

Binding properties of mimetic-specific mAbs: ELISA titers of sera of individual mimetic – IRIV immunized mice did not correlate strictly with IFA titers (Table 1; Figure 3). This suggested that only a subset of antibodies elicited against the peptidomimetics cross-reacted with the CS protein on the cell surface of

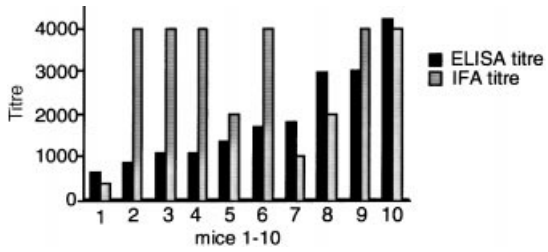


Figure 3. *Distribution of anti-peptidomimetic IgG ELISA and anti-sporozoite IgG IFA titers in ten mice (group d) immunized three times with* ***5*** *– IRIV after one preimmunization with Inflexal. ELISA titers correspond to effective dose 20% values calculated with GENESIS LITE software. Every pair of bars represents one animal.*

the sporozoites. To investigate this further, we generated monoclonal antibodies (mAbs) against both the mimetics **1** and **4**. Three hybridoma cell lines secreting anti-**2** mAb and six lines secreting anti-**5** mAb were isolated. The cross-reactivities of the mAb produced by the three anti-**2**-specific clones (designated mAbs 1.7, 1.15, and 1.26) and by the six anti-**5**-specific clones (designated mAbs 2.1, 3.1, 3.2, 3.3, 3.4, and 3.5) were analyzed (Table 2). One of the anti-**2** mAbs (mAb 1.26) and four of the anti-**5** mAbs (2.1, 3.1, 3.2 and 3.3) cross-reacted with *P. falciparum* sporozoites in IFAs (Figure 4). All mAbs bound to the peptidomimetic used for immunization but not to the respective second mimetic or to the template structure in **8** (Table 2).

Conformations of the mimetic 4: The solution conformation of **1** has been described earlier.[17] The conformation of **4** in aqueous solution was investigated by NMR spectroscopy with

Table 2. *Binding properties of peptidomimetic-specific monoclonal antibodies.*

mAb[a]	Mimetic[b]	Isotype	Binding to			
			2[c]	**5**[d]	**8**[e]	sporozoites[f]
1.26	**2**	IgM	+	−	−	+
1.7	**2**	IgG	+	−	−	−
1.15	**2**	IgG	+	−	−	−
2.1	**5**	IgG	−	+	−	+
3.1	**5**	IgG	−	+	−	+
3.2	**5**	IgG	−	+	−	+
3.3	**5**	IgG	−	+	−	+
3.4	**5**	IgG	−	+	−	−
3.5	**5**	IgG	−	+	−	−

[a] mAbs were derived from three separate fusion experiments. [b] Mice were immunized with IRIV loaded with the respective mimetics. [c] ELISA reactivity to conjugate **2**. [d] ELISA reactivity to conjugate **5**. [e] ELISA reactivity to conjugate **8**. [f] IFA reactivity to *P. falciparum* sporozoites.

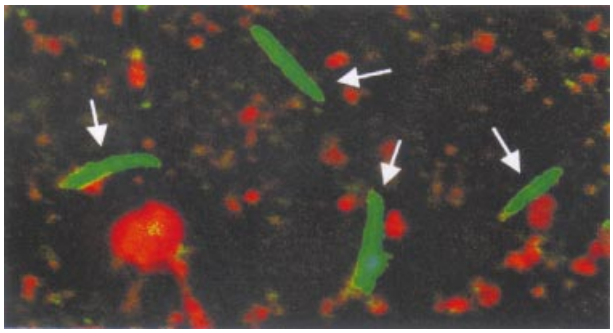


Figure 4. *mAb 1.26 immunofluorescence labeling of* P. falciparum *sporozoites with an FITC-labeled secondary antibody. Sporozoites are indicated by white arrows.*

the same methods (at 290 and 300 K and pH 5.0). The one-dimensional NMR spectra indicated the presence of one major conformer and two minor ones (in a ratio of 77:15:8), with the latter two most probably arising due to *cis – trans* isomerism at Asn – Pro peptide bonds, in analogy to earlier studies.[14, 17] The minor forms were not considered further. Although the peptide backbone groups (NH, C(α)H) could be assigned, extensive overlap prevented residue-specific assignments of side-chain Asn resonances. This together with a sparcity of long-range NOEs thwarted attempts to calculate solution structures based on NOE restraints.

Nevertheless, NOESY spectra revealed strong $d_{NN}(i,i+1)$ connectivities between Asn^5 and Ala^6, as well as between Ala^6 and Asn^7. These, together with high field shifted resonances and a relatively low temperature coefficient for the Ala^6 NH group ($\delta = 7.82$ and $\delta/\Delta = -3.7$ ppb K^{-1}) suggest a β turn is formed by the four residues Asn^3-Pro^4-Asn^5-Ala^6. A β-turn structure, however, may not be the whole story. The $d_{NN}(i,i+1)$ connectivities show that the Ala^6 NH group is close to the peptide NH group of the preceeding Asn (as expected in a β turn) and the following Asn residue. This could occur if the Ala^6 residue is in the α region of ϕ/ψ space, with the Asn^3 CO moiety within (or close to) hydrogen-bonding distance of both the Ala^6 NH and the Asn^7 NH groups as shown in a model in Figure 5. This leads to the intriguing possibility that conformations are present in which a (perhaps distorted) β turn is extended by one residue to create a five-residue conformational unit (NPNAN) with Ala in a helical state. Additional studies are needed with new peptidomimetics to confirm these hypotheses. It should be noted that similar conclusions concerning a five-residue NPNAN motif were reached earlier, in studies of linear tandemly repeated NPNA peptides.[14] This type of helical turn was not observed earlier for **1**.[17]

Discussion

The inherent flexibility of linear synthetic peptides is a well-known drawback in their use as vaccine components. Antibodies elicited by linear peptides often bind linear epitopes in the denatured protein but do not recognize conformational epitopes in native protein structures. There is, therefore, increasing interest in the design of constrained synthetic peptide and

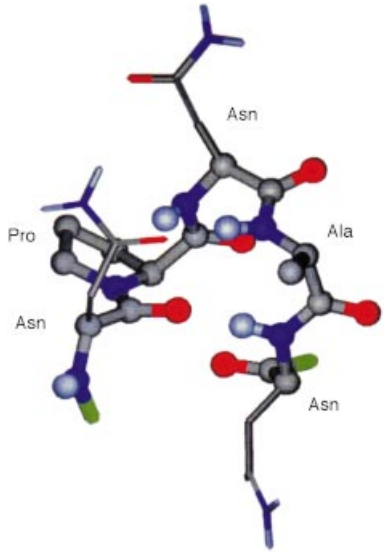


Figure 5. *A model of the postulated helical β-turn region from Asn³ to Asn⁷ in* ***4****. Color code: Nitrogen atoms, blue; oxygen atoms, red; hydrogen atoms attached to nitrogen atoms, light blue. The chain termini are green. The Asn⁷ backbone HN group is shown within hydrogen-bonding distance to the Asn³ CO group.*

protein mimetics, which mimic conformational B-cell epitopes. One approach in the design of β-turn and β-hairpin mimetics is to attach the turn or hairpin of interest to a template, to generate a cyclic constrained peptidomimetic.[19] Precedence suggests that such cyclic peptidomimetics may also be more resistant to proteolytic degradation. In this work, we have prepared template-bound cyclic peptidomimetics of reverse-turn conformations that seem to be important in the conserved central $(NPNA)_n$ repeat region of the CS protein of the malaria parasite *P. falciparum*.

Short linear peptides containing tandemly repeated $(NPNA)_n$ motifs adopt only transiently β-turn conformations based on the NPNA cadence in aqueous solutions.[14] Stabilization of the β turn has been achieved both by C(α)-backbone methylation of proline[13, 20] and by incorporation into cyclic peptidomimetics,[17, 21] without abolishing the ability of the analogues to elicit sporozoite cross-reactive antibodies. However, it is still uncertain how multiple tandemly repeated reverse turns based on the NPNA cadence might fold into a supersecondary structure in the native CS protein. In this respect, the possibility that the repeat conformational unit is not just the β-turn-forming tetrapeptide NPNA, but rather a five-residue NPNAN unit (see above and Dyson et al.[14]), with the Ala residue in the helical region, deserves mention, since this could form the basis for a tandemly repeated conformational unit in the folded CS protein.

We have shown previously that the template-bound cyclic peptide **1** containing the sequence ANPNAA elicits sporozoite cross-reactive antibodies under conditions where a linear peptide containing the same sequence failed to induce a detectable cross-reactive immune response.[17] While this established the feasibility of using cyclic peptidomimetics to induce CS protein cross-reactive antibodies, it became clear that a suitable delivery system is required to induce high titers of cross-reactive antibodies. The presentation of the peptidomimetic on the surface of an IRIV, in an undistorted conformation, and in multiple copies, seemed ideally suited to allow cross-linking of surface Ig receptors and generate an efficient antiparasite directed immune response. Furthermore, the IRIV technology has been licensed already for human clinical use.[2, 15]

In this work, we show that immunization of mice with the **2**–IRIV formulation induced anti-sporozoite responses that were superior to those elicited by an alum-adjuvanted mimetic–MAP construct **3**. The alum-adjuvanted **3** formulation induced high titers of antimimetic antibodies but hardly any sporozoite cross-reactive immune response. Alum, the adjuvant most commonly used for vaccines in humans, had thus apparently favored the generation of antibodies against conformations of the NPNA motif that do not resemble the native CS protein.

The **5**–IRIV immunogen elicited significantly higher sporozoite cross-reactive IFA titers than the **2**–IRIV immunogen. However, antimimetic ELISA titers were comparable with both constructs. This demonstrates that **4** elicits a higher proportion of sporozoite cross-reactive antibodies in the total antimimetic antibody population than **1** does. This conclusion is strengthened by the properties of the antimimetic mAbs isolated here, which demonstrate that only a portion of the antimimetic antibodies are sporozoite cross-reactive and confirm that peptidomimetic **4** has superior immunogenic properties. Although the small (**1**) and large (**4**) loop mimetics analyzed are closely related in sequence, none of the antimimetic mAbs generated cross-reacted with both structures. This indicates, that the relevant conformational epitopes presented by the two structures are significantly different from each other, but close enough to the conformation(s) of the CS-protein repeat unit to elicit sporozoite cross-reactive antibodies. Interestingly, NMR studies of the mimetic **4** suggest that helical-turn conformations (Figure 5), based on the five-residue NPNAN motif, may be present in **4** but not **1**.[17] Finally, the results also demonstrate that the template structure used had negligible immunogenicity.

The IRIV could in principle be loaded simultaneously with several different peptidomimetic B-cell epitopes and with linear peptides as T-cell epitopes.[22] Based on these results, IRIVs appear to have great potential in the design of molecularly defined combined synthetic vaccines, including those targeted against multiple antigens and development stages of *P. falciparum*, or indeed against other infectious agents. Furthermore, an IRIV-based synthetic peptide vaccine would be expected to be safe, since IRIV-based protein vaccines have already shown a very good safety profile in humans.[2] The concerted application of combinatorial peptidomimetic chemistry with the use of IRIVs as an efficient human-compatible delivery system, may prove to be of great value in the design of molecularly defined synthetic peptide vaccines against a wide variety of infectious agents.

Experimental Section

Synthesis of mimetics: The mimetic **1** and the MAP conjugate **3** were prepared as described earlier.[17] The mimetics **4** and **7** were prepared as described for **1**, by solid-phase peptide synthesis with the same building blocks and with macrocyclization in solution. For the preparation of **2** and **5**, the mimetic (10 mg) was coupled to PE′

(11 mg, 2 equiv) with HATU (5.8 mg, 2 equiv), HOAt (2.1 mg, 2 equiv), and iPr$_2$EtN (7.8 μL, 6 equiv) in NMP (700 μL) overnight at room temperature. The solvent was then evaporated, and the product was purified by chromatography (silica gel, $CHCl_3$/MeOH/AcOH/H_2O = 9:6:0.5:0.5) to afford product (11 mg). MS (ESI): for **2** m/z: 1599.4 $[M+Na]^+$; for **5** m/z: 2039.5 $[M+Na]^+$. The MAP constructs **6** and **8** were prepared by the same method used for **3**.[17] HATU = 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOAt = 7-aza-1-hydroxy-1*H*-benzotriazole, NMP = *N*-methylpyrrolidine.

Preparation of mimetic-loaded virosomes: For the preparation of PE′–mimetic–IRIV, a solution of purified Influenza A/Singapore hemagglutinin (4 mg) in PBS was centrifuged for 30 min at 100 000 g and the pellet was dissolved in phosphate buffered saline (1.33 mL) containing 100 mM octaethyleneglycolmonodecylether (PBS-OEG). Phosphatidylcholine (32 mg; Lipoid, Ludwigshafen, Germany), phosphatidylethanolamine (6 mg) and the PE–mimetics **2** or **5** (4 mg) were dissolved in a total volume of 2.66 mL of PBS-OEG. The phospholipids and the hemagglutinin solution were mixed and sonicated for 1 min. This solution was then centrifuged for 1 hour at 100 000 g and the supernatant was filtered (0.22 μm) under sterile conditions. Virosomes were then formed by detergent removal with BioRad SM BioBeads (BioRad, Glattbrugg, Switzerland). Mice in groups a and b received **2**–IRIV that differed in the ratio of phospholipids to mimetic–PE conjugate. In group a the phospholipid to **2** ratio was 38:3 and in group b the ratio was 7.6:3 (w/w).

Mouse immunogenicity studies: BALB/c mice were preimmunized intramuscularly with commercial whole virus influenza vaccine (0.1 mL; Inflexal Berna, Berna Products, Bern, Switzerland) on day 21. Starting on day 0, they received at three-weekly intervals three doses of either **3**–alum (Alhydrogel 85), **2**–IRIV, or **5**–IRIV intramuscularly at doses of 50 μg of mimetic. Blood collected two weeks after the third immunization was analyzed by ELISA and IFA.

Generation of antimimetic monoclonal antibodies. Mice, which had been immunized intramuscularly twice with **2**–IRIV or three times with **5**–IRIV, received an intravenous booster injection three days prior to fusion. Spleen cells were fused with PAI mouse myeloma cells by use of polyethylene glycol, $M_w = 1500$, according to the methods described.[23] The hybrids were selected in HAT medium and cells secreting peptidomimetic-specific antibodies were identified by ELISAs.

ELISAs: ELISA microtiter plates (Immunolon 4B, Dynatech, Embrach, Switzerland) were coated at 4 °C overnight with a solution (50 μL, 5 μg mL^{-1}) of mimetic–MAP constructs in PBS (pH 7.2). Wells were then blocked with 5% milk powder in PBS for 1 h at 37 °C, and washed three times with PBS containing 0.05% Tween-20. Plates were then incubated with two-fold serial dilutions of mouse serum or hybridoma cell supernatants in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at 37 °C. After washing, the plates were incubated with alkaline phosphatase conjugated goat antimouse IgG (γ-chain specific) antibodies (Sigma, St. Louis, MO) for 1 h at 37 °C and then washed. Phosphatase substrate (1 mg mL^{-1} *p*-nitrophenyl phosphate (Sigma)) in buffer (0.14% Na_2CO_3, 0.3% $NaHCO_3$, 0.02% $MgCl_2$; pH 9.6) was added and incubated at room temperature. The optical density (OD) of the reaction product was recorded at 405 nm after an appropriate time with a microplate reader (Titertek Multiscan MCC/340, Labsystems, Finland). Titration curves were registered and analyzed with GENESIS LITE 2.16 software (Life Sciences Ltd., Basingstoke, UK). Effective dose 20% values (ED20%) were calculated for each curve and the corresponding titers were set as endpointtiters.

Immunofluorescence assays: Air-dried unfixed *P. falciparum* salivary gland sporozoites (strain NF54) attached to microscope glass slides were incubated in a moist chamber for 20 min at 37 °C with serum diluted in PBS. The slides were then washed five times with PBS containing 0.1% bovine serum albumin (PBS-BSA) and dried. Fluorescein isothiocyanate (FITC) labeled goat antimouse IgG (Fab-specific) antibodies (Sigma), optimally diluted in PBS containing Evans-Blue (0.1 g L^{-1}; Merck, Germany), were added. After incubation for 20 min at 37 °C the slides were again washed five times with PBS-BSA, dried, mounted with glycerol, and covered with a cover slide. A Leitz Dialux 20 microscope with 12.5/18 ocular and a 40x/1.30 oil fluorescence 160/0.17 objective was used to detect fluorescence staining at 495 nm excitation and 525 nm emission wavelengths.

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