Structural Modifications Enable Conserved Peptides to Fit into MHC Molecules thus Inducing Protection against Malaria

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Developing a rational methodology for obtaining vaccines against *P. falciparum* malaria (the disease's most lethal form, afflicting more than 250 million people around the world per year and killing about 2 million of them)[1] has become one of the main objectives of public health authorities around the world.[2] A multiantigenic vaccine, containing molecules from the parasite's different developmental stages, is required due to the parasite's remarkable complexity and adaptability.[3] The first such approach (the SPf66 synthetic vaccine),[4,5] which used peptides from molecules from different parasite stages, conferred limited protective efficacy in *Aotus* monkey studies and in field trials carried out on human volunteers around the world.[6]

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Since SPf66 was made up of peptides with high red-blood-cell (RBC) binding ability,[7] the data suggested that amino acid sequences and peptides from membrane proteins and/or molecules mediating host-cell invasion (RBCs or hepatocytes) represented the best targets for inhibiting or destroying the parasite. Conserved high-activity binding peptides (HABPs) were identified in an attempt to avoid the parasite's great genetic variability/adaptability.

However, conserved HABPs are poorly immunogenic[8] and poor protection inducers[9] against experimental challenge with a *P. falciparum* strain 100% infective for the *Aotus* monkey experimental model.

Hundreds of modified HABP analogues (with which thousands of *Aotus* have been immunised) were synthesised when searching for this rational methodology. A great number of these peptide analogues were found to be immunogenic and protection-inducers when some of the critical residues in RBC binding had been substituted for amino acids of similar mass but different polarity. It was these induced conformational, charge distribution and hydrophobic changes in key positions in the modified HABPs that rendered them immunogenic and protection-inducing.[8–16]

It has been suggested that such changes enabled these modified peptides to fit better into class II major histocompatibility complex (MHC) molecules, thus forming the MHC II-peptide–T cell receptor (TCR) (MHC II–peptide-TCR) complex.[8] The interaction of these HABPs with MHC class II (HLA-DRβ1*) purified molecules was thus analysed in this work to test such a hypothesis, and correlations were sought between their immunological activity, their ability to bind to these molecules and their three-dimensional structure as determined by prior ^{1}H NMR studies.[8–16]

The chemical synthesis and molecular characterisation of native peptides D,[10] E,[11] F,[9] G,[8] H,[12] I,[13] J,[14] L[15] and M[16] and their analogues as well as their immunogenic and protection-inducing capacity in the *Aotus* monkey against experimental challenge have already been described,[8–16] as have their three-dimensional structures determined by ^{1}H NMR.[8–16] The methodology used for isolating HLA-DRβ1* 0101, 0301, 0401 and 1101 molecules and the binding of these peptides to them has also been previously described.[17] The peptide being studied by this methodology has been shown to be able to compete or not with a biotinylated peptide for binding to purified HLA-DRβ1* molecules.[18]

The immune responses and HLA-DRβ1* molecule-binding capacity of peptides C, B,[19] and A[21] and their analogues have also been analysed.

The peptide HLA-DRβ1* binding register was modified when designing the molecules in some of these peptides (i.e. F and F1,[9] J and its J2 analogue[14] and G and its G2 analogue[8]), based on the peptide motifs for the HLA-DRβ1* alleles to which they were found to bind experimentally.

Analysing conserved HABP binding to the purified HLA-DRβ1* (0101, 0301, 0401 and 1101) molecules studied in this work (Table 1), for which there are almost identical molecules in *Aotus*,[18] supports our original hypothesis:[8] that most conserved HABPs' absence of immunogenicity and/or protection-inducing capacity could be partly due to their poor ability to form the MHC II-peptide–TCR complex, thus requiring specific modification. It was found that 10 out of 13 conserved unmodified HABPs did not bind to the HLA-DRβ1* molecules studied here (Table 1). Only RESA protein native peptide D[10] and *P. falciparum* SERA protein I[12] peptide specifically bound to HLA-DRβ1* 0401 and HLA-DRβ1*0301 molecules, respectively. MSP-1 protein native peptide J[14] bound promiscuously, simultaneously and with high capacity to HLA-DRβ1* 0301 and HLA-DRβ1* 1101 molecules.

Certain modifications made to the conserved HABPs, which allowed them to bind specifically to HLA-DRβ1*, made some of these peptides (i.e. peptides A1, A2, B1, C1, D1, D2, E1, E2, F1, G1, G2, G3, H1, I1, J1, J2 and K1) immunogenic and protection-inducing (Table 1). However, some of the peptides that bound to HLA-DRβ1* molecules (i.e. peptides C2, D5, D6, D7, D8, D9, F2, H2, I2 and K3) became immunogenic but not protection-inducing; this suggests that additional modifications are required, probably to those residues theoretically making contact with the TCR (as shown later).

Some immunogenic and protection-inducing modified HABPs (D3, D4, E3, K2, L1, L2 and M1) did not bind to any of the HLA-DRβ1* molecules analysed here (Table 1); this suggests that they could be binding to other HLA-DRβ1* molecules not included in the present study or to *Aotus* class II exclusive molecules.[22]

Table 1 confirms a finding from the study,[15] totally the opposite of what was expected, in which native peptide J and modified peptides D10, D11, D12, G4, J3 and J4, which are derived from different HABPs and *P. falciparum* proteins that bind promiscuously to several class II molecules, were neither immunogenic nor protection-inducing.

Based on previously described ^{1}H NMR studies,[8–16] Table 2 shows that native nonimmunogenic, non-protection-inducing conserved HABPs show totally random (A, D, F and K), α-helical (B, J and G) or classic β-turn (E) structures, perhaps as an immune evasion mechanism. Their helicity must therefore be substantially shortened (e.g. B1 and J2), displaced (e.g. A1, D2 and G2) or distorted (β-turn induced; e.g. F1, K1 and E2) in order to make them become immunogenic and protection-inducing. These modifications could give them a more appropriate configuration for fitting into the MHC II-peptide–TCR Complex. This was proved when HLA-DRβ1* molecule binding became induced on modifying the HABPs such that some of them became immunogenic and protection-inducing.

It was also observed (Figure 1, right and Table 2) that in all immunogenic and/or protection-inducing peptides, the distance between amino acids that theoretically fit into putative HLA-DRβ1* molecule pockets 1 and 9 is 23.0 ± 3.0 Å. The only exception was the peptide B1 analogue, whose structure was determined in DMSO due to solubility problems.[19] The distance between pockets 1 and 9 is much less (17.0 ± 2.5 Å) in native HABPs or their nonimmunogenic and non-protection-inducing analogues. The exception was peptide F4 (24.29 Å), which has a completely different orientation of residues that fit into pockets 4 and 6. Peptide D5 was immunogenic but not protection-inducing (Table 2). The above data allow us to sug-

Table 1. *These HABPs amino acid sequences, their immunogenic activity (immunofluorescence antibodies against the* P. falciparum *parasite* $\geq$ *1:160), and their protection-inducing activity (complete absence of parasites in Aotus blood 15 days after being challenged) have already been described.[8–16] Peptides L and M and their analogues have not had their peptide-binding motives assigned due to their not binding to specific HLA-DRβ1* molecules.*

Peptide	Peptide sequence P_1 P_4 P_6 P_9	IFA post 3rd	No of protected monkeys	% of HLA-DRβ1* molecule binding 0101	0301	0401	1101	Ref.
A	KSKKHKDHDGEKKKSKKHKD	0	0	−1	29	19	7	[21]
A1	KSKKH**M**D**L**DGE**MMMA**KK**L**KD	**3(1280)**	**1/8**	−3	**53**	34	31	
A2	KSKKH**MI**HDGEKKK**I**KK**L**KD	**1(160)**	**1/4**	−14	**51**	17	2	
A3	KSKKH**M**DHDGEKKK**I**KK**L**KD	**1(160)**	0/9	−14	32	21	0	
A4	KSKKH**MN**HDGEKKK**V**KK**L**KD	**2(320)**	0/6	−5	10	−1	5	
A5	KSKKHKDHDGEKKK**V**KK**L**KD	**1(640)**	0/6	−22	9	21	0	
A6	KSKKH**MI**HD**I**EK**M**K**I**KK**L**KD	**1(320)**	0/8	−11	25	8	20	
B	**YTNQNINISQERDLQKHGFH**	0	0	−2	−10	30	−10	[19]
B1	**L**TNQNINI**D**QE**FN**L**M**KHGFH	**1(640)**	**1/9**	−4	**51**	16	42	
B2	**L**TNQNINI**D**QE**FP**L**M**KHGFH	0	0/5	−9	**55**	29	42	
B3	YTNQNINIS**M**ER**NL**M**K**HGFH	0	0/6	−2	−2	**53**	−4	
C	**KMNMLKENVDYIQKNQNLFK**	0	0	6	15	4	10	
C1	KMNM**HL**ENV**PW**I**M**N**K**QNLFK	**1(320)**	**2/7**	−6	**50**	−9	13	
C2	KMNM**HM**ENV**PW**I**V**KNQNLFK	**3(320)**	0/7	0	**67**	28	20	
C3	KMNM**HM**ENV**P**Y**IM**KNQNLFK	0	0/7	4	**64**	25	8	
C4	KMNM**HL**E**H**V**PW**I**M**KNQNLFK	**1(320)**	0/6	−6	32	−10	34	
C5	KMNM**HM**ENV**AW**I**M**KNQNLFK	**1(1280)**	0/9	6	23	−10	30	
C6	**KE**MNM**HMK**NVDYIQKNQN	0	0/10	−23	44	12	1	
D	**MTDVNRYRYSNNYEAIPHIS**	0	0	24	45	**61**	16	[10]
D1	V**I**RYRYSNNYEAI**D**HIS	**1(640)**	**1/6**	31	12	**65**	16	
D2	MTDV**I**RYRYSNNYEA**SD**HIS	**1(5120)**	**1/6**	−5	33	**50**	16	
D3	MTDVNRYRYSNNYE**EQ**PHIS	**1(1280)**	**2/4**	−4	17	24	0	
D4	MTDVNRYRYSNNYE**QE**PHIS	**1(1280)**	**2/9**	−15	25	28	22	
D5	MTDV**I**RYRYSNNYEA**ES**HIS	**4(5120)**	0/2	6	**83**	32	16	
D6	MTDV**I**RYRYSNNYE**SES**HIS	**1(640)**	0/4	3	**85**	30	24	
D7	MTDV**VF**YRYSNNYE**GQ**PHIS	**1(320)**	0/5	−4	**87**	43	4	
D8	MTDV**I**RYRYSNNYE**SND**HIS	**1(320)**	0/4	−13	**85**	46	24	
D9	TDVNRYRYSN**D**YE**SSDK**	**1(320)**	0/8	−14	**52**	41	2	
D10	MTDV**I**RYRYSNNYE**SSD**HIS	0	0/5	−27	**90**	**57**	24	
D11	MTDV**I**RYRYSNNYE**GSD**HIS	0	0/5	−8	**82**	**52**	4	
D12	MTDV**I**RYRYSNNYE**GND**HIS	0	0/5	−6	**78**	**52**	4	
E	**KNESKYSNTFINNAYNMSIR**	0	0	5	0	38	−60	[11]
E1	SKYSNTF**NI**NAYNM**V**IRRSM	**1(5120)**	**1/8**	9	1	**53**	0	
E2	KNESKYSNTF**EV**NAYNMSIR	**1(5120)**	**1/3**	4	23	0	**50**	
E3	KNESKYSNTF**EV**NAYNM**VN**R	**1(2560)**	**1/10**	−1	2	−12	1	
F	**DAEVAGTQYRLPSGKCPVFG**	0	0	6	25	7	27	[9]
F1	DAEVAGTQY**FH**PSGK**S**PVFG	**1(5120)**	**1/5**	5	20	**60**	20	
F2	DAEVAGTQ**WF**LPSGK**S**PVFG	**2(320)**	0/6	−3	47	0	**60**	
F3	DAEVAGTQ**WFD**PSGK**S**PVFG	**2(640)**	0/5	1	22	−2	22	
F4	DAEVAGTQ**WFN**PSGK**S**PVFG	0	0/6	0	8	1	44	
G	**EVLYLKPLAGVYRSLKKQLE**	0	0	5	18	7	15	[8]
G1	EVLY**HV**PLAGVYRSLKKQLE	**1(640)**	**2/4**	−4	30	−5	**61**	
G2	EVLY**HM**PL**G**GVYR**A**LKKQLE	**1(2560)**	**1/6**	4	26	−6	**63**	
G3	EVLYL**LD**LAGVYRSLKKQLE	**1(2560)**	**1/4**	−12	47	−8	**20**	
G4	EVLYL**MS**LAGVYRSLKKQLE	0	0/5	1	**83**	−1	**91**	
H	**WGEEKRASHTTPVLMEKPYY**	0	0/5	−17	12	−3	−6	[12]
H1	**YS**E**M**KRAS**L**TTPVL**K**EKPYY	**1(320)**	**1/5**	4	19	7	**52**	
H2	**YS**E**M**KRAS**L**TTPVL**K**E**M**P**W**Y	**2(320)**	0/5	−2	29	−1	**53**	
H3	**YS**E**M**KRAS**M**TTPVLMEKPYY	0	0/6	−2	13	−2	46	
I	**YDNILVKMFKTNENNDKSELI**	0	0/5	0	**79**	0	38	[13]
I1	DNI**H**VKMFK**VI**ENNDKSELI	**1(2560)**	**2/9**	−3	4	17	**54**	
I2	DNI**H**VKM**R**K**VIM**NNDKSELI	**1(640)**	0/5	−3	**75**	0	38	
J	**QIPYNLKIRANELDVLKKLV**	0	0/5	1	**88**	−3	**91**	[14]
J1	QIPYNLKIRA**G**GLD**GG**KKLV	**2(2560)**	**1/3**	−1	16	−14	**53**	
J2	QIPYNLKIRANMLDV**D**KKLV	**1(160)**	**1/5**	−3	43	−3	**77**	
J3	QIPYNLKIRA**I**MLD**AH**K**M**LV	0	0	25	**57**	0	**76**	
J4	QIPYNLKIRANMLDV**N**KKLV	0	0	6	**53**	9	**76**	
K	**NNNFNNIPSRYNLYDKKLDL**	0	0/5	2	33	11	6	[20]
K1	FNNIPSRYNLYDK**ML**P**LDD**	**2(640)**	**2/5**	45	5	18	**69**	
K2	NNIPSRYNLYDK**M**LDL**D**	**1(1280)**	**2/10**	0	08	13	24	
K3	NNIPSRYNLYDK**M**LDL**DDL**	**9(320)**	0/9	−6	**52**	15	24	

gest that 23.0 ± 3.0 Å is the range of distance between amino acids 1 and 9 required to fit into the respective pockets of any HLA-DRβ1* molecule in immunogenic and protection-inducing modified HABPs. Therefore, very compact and rigid peptides that have lesser distances (i.e. all those with α-helices), have greater distances (i.e. totally random) or are totally disorientated in their configuration will not fit well into class II molecules and thus not allow formation of the MHC II-peptide–TCR complex for properly activating the immune system. They must therefore become modified.[8–16]

Although side-conformation reliability is limited by relatively high conformational freedom, clear differences were observed in these peptides. The amino acids that form modified HABP binding motifs (P1, P4, P6 and P9, shadowed in Table 1, suggesting that they were fitting correctly into their respective pockets) agreed with the biological results of these peptides binding to their respective HLA-DRβ1* molecules.[23,24] If we consider that the bright pink residue fits into pocket 1, the blue one fits into pocket 4, the orange one fits into pocket 6 and the green ones fits into pocket 9 (based on those motifs experimentally defined as being specific for HLA-DRβ1* 0301, HLA-DRβ1* 0401 and HLA-DRβ1* 1101 alleles,[23,24] which are similar to those alleles most frequently found in *Aotus*),[22] then differences in location and orientation of some of these lateral chains become most marked between protective and non-protection-inducing peptides. These differences are clearly shown in Figure 1, right-hand side, for peptides A1, D2, F1, E2, J2 and G2 in pocket 4 (blue) and pocket 6 (orange) when compared to their native or

Table 1. (Continued)

Peptide	Peptide sequence P_1 P_4 P_6 P_9	IFA post 3rd	No of protected monkeys	% of HLA-DRβ1* molecule binding 0101	0301	0401	1101	Ref.
K4	NNIPSRYNLYDK**M**LPL**DL**	0	0/8	27	29	10	29	
K5	FNNIPSRYNLYDK**M**L**E**L**DD**	0	0/7	38	**63**	23	24	
L	**KSYGTPDNIDKNMSLIHKHN**	0	0/6	6	34	5	38	[15]
L	**M**SYG**SD**DN**N**DKN**K**SL**NN**KHN	**2(320)**	**1/4**	7	−4	−6	19	
L2	**M**SYG**S**DDN**D**DKN**K**SL**D**HKHN	**1(320)**	**2/4**	2	0	2	16	
L3	**MV**YG**SD**DN**N**DKN**K**SL**NN**KHN	**2(640)**	0/5	4	−10	−4	9	
L4	**MA**YG**SD**DN**D**DKN**K**SL**D**HKHN	0	0/5	7	−7	5	1	
M	**MLNISQHQCVKKQCPQNS**	0	0/5	3	5	−12	10	[16]
M	MLNIS**ML**Q**T**V**MMMT**PQ**K**	**1(2560)**	**2/8**	13	3	−13	3	
M2	M**H**NISQ**L**Q**V**VKK**MV**PQ**K**	**1(640)**	0/8	−1	0	−11	11	
M3	MLNIS**ML**Q**T**V**M**K**MT**PQ**K**	0	0/7	−13	4	−16	47	

Table 2. *Summary of structural, biological, and immunological properties of native and modified HABPs.*

Peptide	Structural features	Distance [Å]	HLA-DRβ1 binding	Putative TCR contacts	I[a]	P[b]
A	random	–	–	–	–	–
A3	α-helix (E11–K14)	16.44	–	–	+	–
A1	α-helix (D7–G10) and (M13–K16)	20.32	0301	3,7,8	+	+
B	α-helix (Q4–L14)	17.73	–	–	–	–
B1	α-helix (I6–F12)	19.57	0301	3,7,8	+	+
D	random	–	**0401**	–	–	–
D5	α-helix (M1–Y9)	21.28	0301	–	+	–
D2	α-helix (V4–R8)	24.73	0401	2,3,7,8	+	+
F	random	–	–	–	–	–
F4	classical type III β-turn(T7–F10)	24.29	–	–	–	–
F1	distorted type III′ β-turn(T7–F10)	25.04	0401	2,3,7,8	+	+
K	random	–	–	–	–	–
K4	α-helix (P8–M17)	19.53	–	–	–	–
K1	classical type III β-turn (S9–N12) and classical type III β-turn (Y14–M17)	23.55	1101	3,7	+	+
E	classical type III′ β-turn (S7–F10)	18.97	–	–	–	–
E2	distorted type III′ β-turn Y6–T9 and distorted type III β-turn (A14–M17)	22.18	**1101**	3,7	+	+
J	α-helix (Q1–V20)	16.85	**0301**,**1101**	–	–	–
J2	α-helix (P3–N11)	20.21	**1101**	3,7	+	+
G	α-helix (Y4–Y12)	14.26	–	–	–	–
G2	α-helix (L8–K17)	21.85	**1101**	3,7	+	+

[a] Immunogenicity. [b] Protectivity.

nonprotection analogues (Figure 1, left-hand side). Profound structural modifications in conformation, charge distribution, hydrophobicity and distance were thus observed in modified conserved HABPs, leading to their residues being suitably placed to fit into their respective HLA-DRβ1* pockets, thus appropriately activating the immune system. This was proved by their changed ability to bind to these molecules.

The modifications have also had a great impact on orientating those residues that putatively come into contact with the TCR, such as T2 (red), T3 (turquoise), T5 (pale pink), T7 (grey) and T8 (yellow) as shown in Figure 1. There were marked differences in the orientations of residues T3, T7 and T8 in peptides A1 and B1, which specifically bound to HLA-DRβ1* 0301 molecules, when compared to A3 and B. The same was seen in T2, T3, T7 and T8 in peptides D2 and F1, which specifically bound to HLA-DRβ1* 0401, when compared to D5 (immunogenic but nonprotective) and F4 (nonimmunogenic and non-protection-inducing) the same as in residues T3 and T7 from peptides K1, E2, J2 and G2, which bound to HLA-DRβ1* 1101, when compared to K4, E, J and G (nonimmunogenic and non-protection-inducing).

The above data suggest that, depending on the HLA-DRβ1* allele to which an immunogenic and protective peptide binds, there could be specific selection for those residues that come into contact with the TCR. Thus, when a peptide binds to HLA-DRβ1* 0301, the T3, T7 and T8 residues are allowed to come into contact with the TCR, whilst, with the HLA-DRβ1* 0401 allele, residues T2, T3, T7 and T8 come into contact with the TCR, and, with the HLA-DRβ1* 1101 allele, residues T3 and T7 are the TCR contacting residues. In agreement with our observations, it has been found that MHC II alleles influence the T-cell functions in the mouse system by restricting TCR access to specific I-A bound peptide residues.[25]

The absence of T5 (light pink) residue interaction (suggested as being fundamental in peptide interaction with the TCR)[26] was most striking; we have not been able to explain this to date.

As immunity to blood-stage murine malarial parasites is MHC II dependent,[27] the immunogenic and protection-inducing structural characteristics and HLA-DRβ1* purified molecule binding capacity of the peptides have to be identified. What we have described in this manuscript is that profound structural modifications have to be made to key residues in conserved HABPs so that they can fit properly and form the MHC II-peptide–TCR complex to induce an appropriate protective immune response. Based on the number of HLA-DRβ1* alleles and the number of peptides they can bind to, it has been predicted that a fully protective malaria vaccine, which would protect $>95\,\%$ of the world's population, might need up to 44 different epitopes;[28] this therefore represents the minimum number of immunogenic and protection-inducing modified HABPs that have to be designed.

This article has thus brought together the data from hundreds of in vivo experiments that made use of the *Aotus* exper-

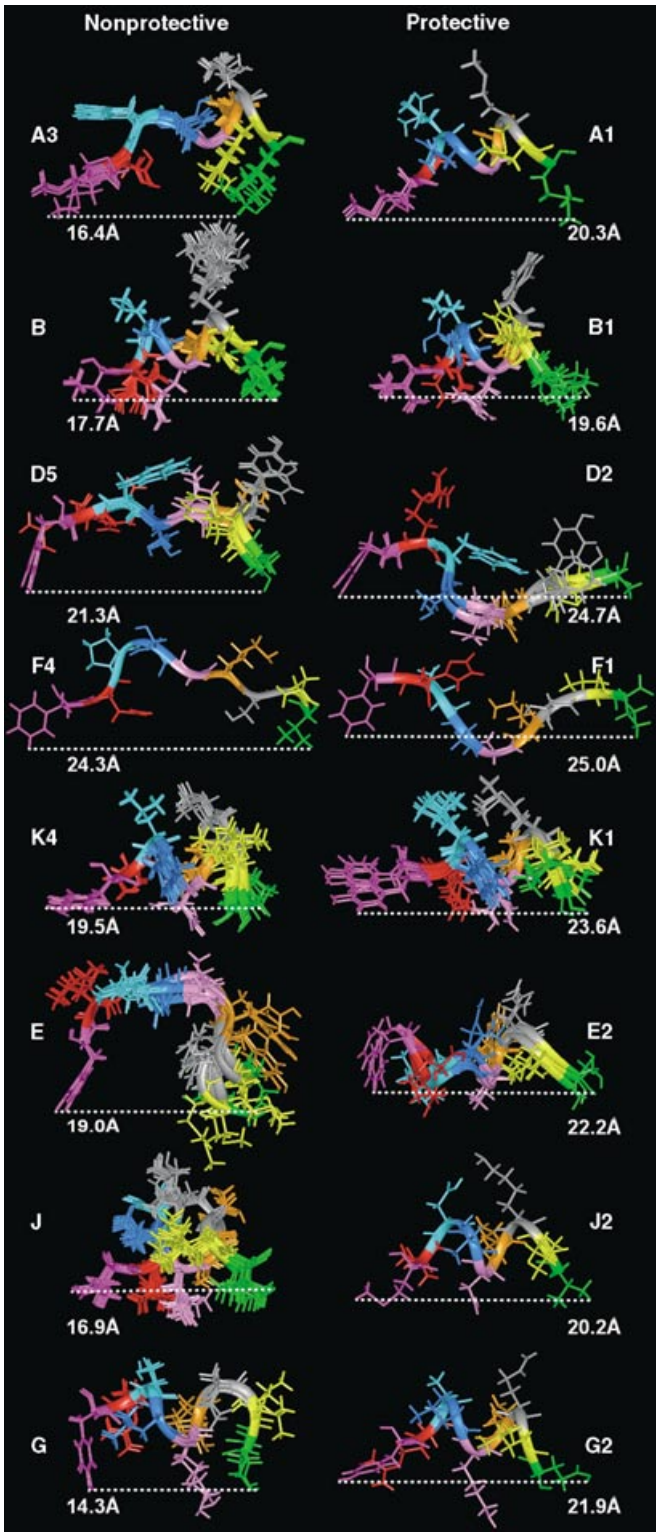

Figure 1. *Nonprotective peptide structures are shown in the left-hand panel and protective peptides in the right-hand panel. Bright pink: pocket 1, red: T2, turquoise: T3, blue: pocket 4, pale pink: T5, orange: pocket 6, grey: T7, yellow: T8, and green: pocket 9. Pockets were assigned according to peptide motif and the characteristics for each HLA-DRβ1* to which these peptides bind. T stands for putative residues making contact with the T-cell receptor. The three-dimensional structures of D,[10] K,[20] E,[11] F,[9] G[8] and J[14] and their analogues have already been published. Work on peptides A and B and their analogues is in press.[19,21] Peptide F4 is immunogenic but not protection-inducing.*

imental model to show how rationally designing a subunit-based, multicomponent, multistage, chemically synthesised malaria vaccine is being developed in attempts at controlling this deadly disease.

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Keywords: conformation analysis · human-leucocyte-associated antigens · malaria · NMR spectroscopy · peptides

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