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Synthesis and Antigenic Properties of Reduced Peptide Bond Analogues of an Immunodominant Epitope of the Melanoma MART-1 Protein

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Abstract: Backbone modifications have been introduced into the melanoma derived peptide MART-$1_{(27-35)}$ to increase its binding to class I major histocompatibility complex HLA-A2 molecule, and ultimately to enhance its immunogenicity. Each analogue was obtained by replacing one peptide bond at a time in the natural epitope by the aminomethylene (CH_2-NH) surrogate. All analogues displayed an increased resistance to proteolysis. Interestingly, the comparative results showed that five analogues bound more efficiently to HLA-A2 than the parent peptide. On the other hand, two pseudopeptide/HLA-A2 complexes were recognized by one melanoma-specific T cell clone. Close examination of the impact of such modifications at the molecular level provides useful supports for the rational design of stable compounds with applications in anti-tumour specific immunotherapy and in vaccine development. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antigenicity; MART-1 antigen; pseudopeptides; reduced peptide bond

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) recognize peptide antigens associated with major histocompatibility complex (MHC) molecules at the cell surface. The recognition of peptide antigens by class I-restricted CTLs can control and eliminate virus-infected or tumoural cells. The identification of several melanoma-associated antigens recognized by $CD8^+$ CTLs have provided new hope in melanoma immunotherapy [1,2]. Since this, several studies have been devoted to elicit efficient CTL response towards tumour-derived peptides [3,4], despite their generally low or intermediate affinity for MHC molecule [5–7]. In the case of viral antigens, a correlation has been demonstrated between immunogenicity and peptide binding affinity to class I MHC molecules [8]. Hence, a promising new approach in peptide-based anticancer vaccine may be the use of peptides which have been altered to increase their MHC Class I binding affinity [9–11].

The future of synthetic peptides for generating new vaccines and immunotherapeutical agents is also strongly impaired by their high susceptibility towards enzymatic degradation and their rapid clearance from circulation. In the last few years, potential applications of pseudopeptides have been explored in immunology. In such pseudopeptides, one or several peptide bonds, but not the residues themselves, are changed, which results generally in an increased enzymatic stability. For example, peptide analogues of B-cell epitopes bearing retro-inverso bonds [12,13] or reduced peptide bonds [14] showed a better antigenic reactivity than the corresponding parent peptides. Moreover, it was reported that a retro-inverso peptide analogue of the major

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antigenic site of the foot and mouth disease virus could elicit high levels of long lasting protective antibodies [15]. Most interestingly, a considerable protection of swine from foot-and-mouth disease was induced with only one dose of this all-D retro peptide [16].

The binding capacity to MHC-I [17–19] and MHC-II molecules [20–23] of such pseudopeptides was also explored, and in several cases, the influence of peptide bond substitutions was studied at the TCR level [21,22,24–27]. In particular, a partially modified retro-inverso peptide analogue of an influenza virus epitope was shown to modify the cytokine profile of specific $CD8^+$ T effector cells [25]. More recently, a significant protection against Lymphocytic Choriomeningitis virus infection was induced by a reduced peptide bond analogue of the H-$2D^b$-restricted $CD8^+$ T cell epitope GP33 when injected in saline solution [28]. Thus, pseudopeptides might represent a new class of altered peptide ligands and promising tools for use in immunotherapy.

The natural epitope 27-35 of the melanoma MART-1 antigen, AAGIGILTV, binds weakly to HLA-A2 [5]. In spite of its low HLA-A2 binding, MART-$1_{(27\text{-}35)}$ can be recognized by HLA-A2 restricted TILs, suggesting that it is an immunodominant epitope [5,29]. Furthermore, *in vitro* melanoma cell lysis was obtained by MART-$1_{(27\text{-}35)}$-specific CTLs [3,4,30]. Recently, singly substituted derived peptides from MART-$1_{(27\text{-}35)}$ and MART-$1_{(26\text{-}35)}$ were shown to bind more efficiently to HLA-A2 and were considered as good candidates for effective immunotherapy [31,32]. In the present study, we synthesized a series of reduced pseudopeptide bond $\Psi(CH_2\text{-NH})$ analogues corresponding to the MART-$1_{(27\text{-}35)}$ epitope, and analysed their resistance to proteases. The synthetic analogues were then tested for their capacity to promote HLA-A2 assembly in lysates of peptide transporter-deficient cells T2, and for their recognition by MART-$1_{(27\text{-}35)}$-specific CTLs. The ultimate goal was to obtain reduced peptide bond analogues which increased their binding to HLA-A2 molecules without significantly altering TCR recognition of the HLA-A2 /MHC complex.

MATERIALS AND METHODS

Chemicals

Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acid derivatives were purchased from Neosystem (Strasbourg, France). *t*Butyl was the side chain protecting group of Thr. Fmoc-Val-p-benzyloxybenzyl alcohol resin (0.7 mmol/g, 200–400 mesh, polystyrene cross-linked with 1% divinylbenzene) was obtained from Novabiochem (Läufelfingen, Switzerland). Trifluoroacetic acid (TFA), *N*,*N*-dimethylformamide (DMF), dichloromethane, acetonitrile and piperidine were from SDS (Peypin, France).

Peptides

The influenza A virus nucleoprotein peptide NP383-391 (SRYWAIRTR) and the peptide MART-$1_{(27\text{-}35)}$ (AAGIGILTV) from the melanoma MART-1 protein, were synthesized by Neosystem (Strasbourg).

Amino Aldehydes

Fmoc-protected amino aldehydes were prepared by the racemization free method of Fehrentz and Castro [33]. Briefly, the protected amino acids were converted to the corresponding *N*,*O*-dimethylhydroxamates, and were then reduced with lithium aluminium hydride to afford the desired aldehydes.

Reduced Peptide Bond Analogues Synthesis

The analogues were synthesized in Fmoc chemistry by the stepwise solid-phase methodology using a multichannel peptide synthesizer [34]. Protected amino acids were coupled by *in situ* activation with (benzotriazol-L-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate and Nα-Fmoc deprotection was performed as previously described [34]. Introduction of the reduced peptide bond required a reductive alkylation reaction between the Nα-amino group of the resin-bound peptide and the appropriate Fmoc-protected α-amino aldehyde (2.5 eq), and employed sodium cyanoborohydride (7.5 eq) in DMF containing 1% acetic acid, as described earlier [35,36]. The completion of the reaction was monitored by the ninhydrin test. Couplings following the introduction of the reduced peptide bond were realized using a two-fold excess of *N*α-Fmoc-amino acids and activating reagents.

Side chain deprotection and cleavage of peptides from the solid support was performed by treatment with reagent K (82.5% TFA: 5% phenol: 5% water: 5% thioanisole: 2.5% 1,2-ethanedithiol) for 2 h 30 min at 20°C [37]. Peptides were purified by reversed-phase HPLC (RP-HPLC) using a Perkin-Elmer preparative HPLC system on an Aquapore ODS 20 μm column (100 × 10 mm). The elution was

achieved with a linear gradient of aqueous 0.1% TFA (A) and 0.08% TFA in 80% acetonitrile, 20% water (B) at a flow rate of 6 mL/min with UV detection at 220 nm.

Enzymatic Resistance

The parent peptide and the reduced peptide bond analogues (200 μg/mL) were incubated at room temperature with pepsin (100 μg/mL) in phosphate-buffered saline (PBS, pH 7.4). The reaction was stopped at intervals by adding TFA (10% of the final volume). The suspension was centrifuged at $10000 \times g$. 100 μL of the supernatant were injected on the analytical RP-HPLC column. Sensitivity of peptides to enzymatic degradation was evaluated by calculating the peak area corresponding to the intact peptide remaining after incubation with pepsin.

Analytical Methods

Analytical RP-HPLC was run on a Beckman instrument (Gagny, France) with a Nucleosil C18 5 μm column (150×4.6 mm) using a linear gradient of 0.1% TFA in water and acetonitrile containing 0.08% TFA at a flow rate of 1.2 mL/min. Matrix-assisted laser desorption and ionization time-of-flight (MALDI-Tof) spectra were obtained on a Protein TOF™ mass spectrometer (Bruker, Wissembourg, France).

Cell Lines

Human T cell clones infiltrating tumours (TILs) were used. LT8 (Vβ5), LT11 (Vβ9) and LT12 (Vβ2) clones were kindly provided by F. Farace (Institut Gustave Roussy, Villejuif, France). They were extracted from melanomas, restricted by the HLA-A2.1 molecule, and specific to the MART-$1_{(27\text{-}35)}$ peptide. Cells were grown in complete medium: RPMI 10% human AB serum, containing 50 IU/mL penicillin, 50 μg/mL streptomycin, non-essential amino acids, 10 mM hepes, 1mM pyruvate, glutamine, 100 U/mL IL-2 (Boehringer) and TCGF 3%.

The mutant T2 cell line was used as target cell in cytolytic assays. T2 is a variant of the cell line T1 produced by fusion of the T lymphoma cell CEM and the B lymphoblastoid cell line 721.174. It expresses reduced amounts of HLA-A2 and no HLA-B or -C molecules at the cell surface [38]. Owing to a lack of peptide transporters, HLA-A2 heavy chains accumulate in the endoplasmic reticulum. T2 cells were grown in complete medium: RPMI 10% FCS containing 50 IU/mL penicillin, 50 μg/mL streptomycin, non-essential amino acids, 10 mM hepes, 1 mM pyruvate and glutamine.

HLA-A2 Assembly

The capacity of peptides to promote HLA-A2 assembly was tested in T2 cell lysates as previously described [39]. The peptide NP383-391(epitope associating with HLA-B27 molecules and not with HLA-A2) was used as a negative control. Briefly, aliquots of 8×10^5 T2 cells were lysed in 65 μL of 10 mM Tris-buffered saline, pH 7.5, containing 1% NP40 plus protease inhibitors (1 mM phenylmethanesulfonyl fluoride (PMSF), 100 μM iodoacetamide, 2 μg/mL aprotinin, 10 μM leupeptin, 10 μM pepstatin and 10 μg/mL trypsin inhibitor). Cells were lysed in Eppendorf microfuge tubes, with different concentrations of exogenous peptide, and incubated at 37°C for 30 min. Lysates (65 μL) were diluted with 135 μL PBS containing 0.05% Tween 20, 3 mM sodium azide, 10 mg/mL BSA and 1 mM PMSF; 100 μL of this solution were placed in each of two wells of a microtiter plate (Nunc Maxisorp) coated with an anti-HLA-A2 monoclonal antibody BB7.2 [40] and incubated overnight at 4°C. Correctly folded HLA complexes captured by BB7.2 were revealed with an anti-β2-microglobulin immunoglobulin M28 [41] coupled to alkaline phosphatase. Phosphatase activity was detected using 4-methylumbelliferyl phosphate as substrate (Sigma), and the resulting fluorescence was measured at 340 nm excitation/460 nm emission in a Microfluor reader. Each peptide was tested in duplicate, and three independent experiments were performed.

Cytolytic Activity Assay

The cytolytic activity was determined in a ^{51}Cr release assay. The T2 cells were labeled with 100 μCi of sodium chromate (Dupont-NEN Research Products, Boston, MA) and were incubated for 2 h at 37°C with 1 μM peptide, or with various peptide concentrations as specified. Target cells were washed twice with 0.9% NaCl medium containing 5% FCS, and dispatched at 1500 cells per well, and were then incubated with effector cells for 4 h. The radioactivity released into the supernatant was measured. Spontaneous release never exceeded 25% of the maximum ^{51}Cr uptake. The percentage of specific lysis was determined using the formula: [(experimental release-spontaneous release)/(maximum release-spontaneous release)] × 100. Each assay was performed in triplicate.

RESULTS

Solid Phase Synthesis of Reduced Peptide Bond MART-$1_{(27-35)}$ Analogues

Table 1 shows the sequence of the antigenic peptide MART-$1_{(27-35)}$ and of the eight pseudopeptides (Ψ(1-2) to Ψ(8-9)) which were used in this study. Each analogue was obtained by replacing one peptide bond at a time in the parent sequence by the aminomethylene (CH_2-NH) surrogate. The reduced peptide bond was obtained by reductive amination of *N*-Fmoc-α-amino aldehydes in acidic dimethylformamide, as described previously [36]. Stepwise elongation of peptide chain using fluorenylmethyloxycarbonyl chemistry led to the fully protected peptide resin. Acidic cleavage and HPLC purification on a C-18 column yielded the final products. All the compounds were identified by matrix-assisted laser desorption and ionization time of flight mass spectrometry and their purity was assessed by analytical HPLC (Table 1).

Enzymatic Stability of the Reduced Peptide Bond MART-$1_{(27-35)}$ Analogues

Sensitivity of peptides to pepsin degradation was performed as described in the 'Materials and Methods' section. Pepsin is known to cleave preferentially after hydrophobic and aromatic residues, but not to cleave peptide bonds containing valine, alanine or glycine. Thus, only peptide bonds in positions 6-7 and 7-8 of the parent peptide MART-$1_{(27-35)}$ can be cleaved by pepsin. However, interestingly, the eight reduced peptide bond analogues showed a better resistance to proteolysis compared with MART-$1_{(27-35)}$. The MART-$1_{(27-35)}$ half-life was about 11 min, while the half-lives of the analogues were higher and ranged from 14 to 116 min (Table 1). Introduction of a reduced peptide bond in the *N*- or *C*-terminal part of the sequence slightly modified the resistance of the resulting Ψ(1-2), Ψ(2-3) and Ψ(8-9) pseudopeptides (Table 1), while isostere bond replacements in the middle part of the sequence significantly increased resistance to pepsin degradation as exemplified by the half-lives of Ψ(3-4), Ψ(4-5), Ψ(5-6), Ψ(6-7) and Ψ(7-8) analogues (Table 1).

HLA-A2 Assembly

The eight reduced peptide bond analogues were tested for their ability to promote HLA-A2 assembly in T2 cell lysates. T2 being deficient in peptide transporters, empty HLA-A2 molecules accumulate in the endoplasmic reticulum. In T2 cell lysates, stable HLA-A2 molecule/peptide complexes are formed upon addition of exogenous peptides, and the assembly is detected using the monoclonal antibody BB7.2, which recognizes specifically the stable complexed form of HLA-A2, as already described [39]. As previously reported, peptide MART-$1_{(27-35)}$ bound only weakly to HLA-A2 [9] and in our test, HLA-A2 assembly in the presence of this peptide at a concentration of 10^{-6} was low (Figure 1). Interestingly, five analogues [Ψ(1-2) to Ψ(5-6)] bound more efficiently to HLA-A2 than MART-$1_{(27-35)}$, while the other ones [Ψ(6-7) to Ψ(8-9)] gave the same binding than the parent peptide.

Table 1 Peptide Sequences, Retention Times (R_t) in Analytical RP-HPLC, Peptide Masses and Half-lives in Enzymatic Degradation

Name	Sequence	R_t (min)[a]	Mass[b]	$t_{1/2}$ (min)[c]
Mart 1	H-Ala^{27}-Ala-Gly-Ile-Gly-Ile-Leu-Thr-Val^{35}-OH	12.71	813.6	11
Ψ(1-2)	H-Ala^{27}-**Ψ(CH_2-NH)**-Ala-Gly-Ile-Gly-Ile-Leu-Thr-Val^{35}-OH	12.53	802.7	19
Ψ(2-3)	H-Ala^{27}-Ala-**Ψ(CH_2-NH)**-Gly-Ile-Gly-Ile-Leu-Thr-Val^{35}-OH	12.58	802.9	14
Ψ(3-4)	H-Ala^{27}-Ala-Gly-**Ψ(CH_2-NH)**-Ile-Gly-Ile-Leu-Thr-Val^{35}-OH	11.85	803.6	34
Ψ(4-5)	H-Ala^{27}-Ala-Gly-Ile-**Ψ(CH_2-NH)**-Gly-Ile-Leu-Thr-Val^{35}-OH	12.03	802.3	75
Ψ(5-6)	H-Ala^{27}-Ala-Gly-Ile-Gly-**Ψ(CH_2-NH)**-Ile-Leu-Thr-Val^{35}-OH	11.29	804.4	116
Ψ(6-7)	H-Ala^{27}-Ala-Gly-Ile-Gly-Ile-**Ψ(CH_2-NH)**-Leu-Thr-Val^{35}-OH	12.05	800.4	99
Ψ(7-8)	H-Ala^{27}-Ala-Gly-Ile-Gly-Ile-Leu-**Ψ(CH_2-NH)**-Thr-Val^{35}-OH	12.77	801.9	60
Ψ(8-9)	H-Ala^{27}-Ala-Gly-Ile-Gly-Ile-Leu-Thr-**Ψ(CH_2-NH)**-Val^{35}-OH	12.46	801.3	15

[a] Linear gradient of A: 0.1% TFA and B: acetonitrile containing 0.08% TFA, 5–65% B in 30 min.
[b] Matrix-assisted laser desorption and ionization mass spectrometry data.
[c] Incubation time at room temperature required for the degradation by pepsin of 50% of the peptide.

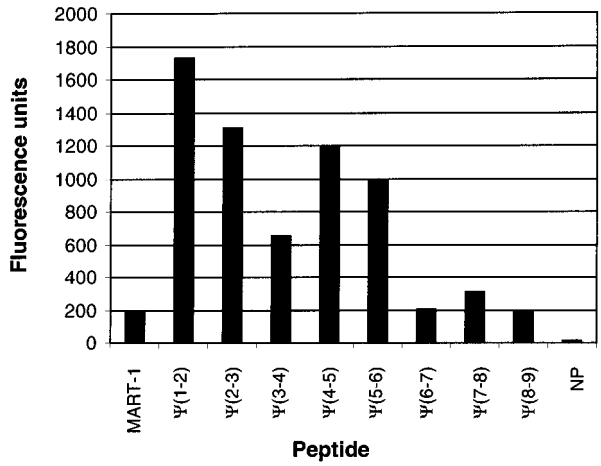

Figure 1 HLA-A2 binding to MART-$1_{(27-35)}$ and the reduced peptide bond analogues. Aliquots of T2 cell lysates were incubated with 10^{-6} M of each peptide. HLA-A2/peptide complexes were retained on BB7.2 Ig-coated microtiter plate well and revealed by an anti-β_{2m} coupled to PAL. Results are expressed as arbitrary fluorescence units at 340/460 nm obtained after 2 h of incubation with the MUP substrate. The spontaneous assembly obtained with T2 cell lysate without adding exogenous peptide was about 20.

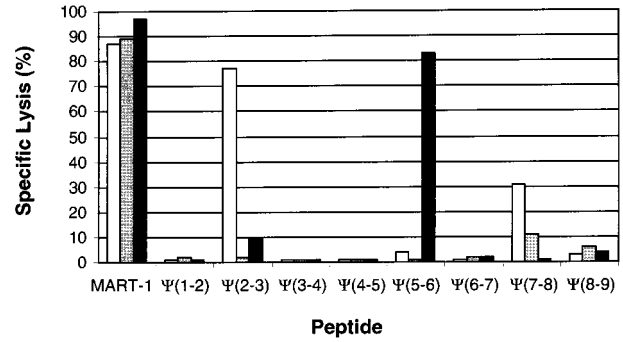

Figure 2 Peptide recognition by HLA-A2-restricted melanoma-reactive TILs. ^{51}Cr-labeled T2 cells were incubated with 1 μM peptide for 2 h at 37°C. Peptides tested were MART-$1_{(27-35)}$ and the reduced peptide bond analogues. After washings, T2 cells were incubated with the LT8 (white bar), LT11 (grey bar) and LT12 (black bar) T cell clones specific for the MART-$1_{(27-35)}$ epitope. Lytic activity was evaluated in a 4-h ^{51}Cr release assay with an effector: target (E:T) ratio of 3:1.

Recognition of Synthetic Analogues by HLA-A2-restricted TILs

As observed previously, five reduced peptide bond MART-$1_{(27-35)}$ analogues were higher HLA-A2 binders than the parent peptide. However, this did not imply that these analogues could be recognized by TCRs reactive with the natural MART-$1_{(27-35)}$ epitope associated to HLA-A2 molecules on the surface of melanoma cells. Peptides were tested for their ability to stimulate three HLA-A2-restricted melanoma-specific CTLs derived from TILs, i.e. LT8, LT11 and LT12 clones (Figure 2). CTL activity was determined in a ^{51}Cr release assay against peptide loaded T2 cells. In most cases, modifications resulted in a loss of recognition by TILs specific for the natural peptide. In spite of a weak recognition of Ψ(7-8), LT11 significantly recognized none of the reduced peptide bond analogues, LT12 only one analogue [i.e. Ψ(5-6)] and LT8 two analogues [i.e. Ψ(2-3), and to a lesser extent, Ψ(7-8)]. However, Ψ(2-3) and Ψ(5-6) were good stimulators for the LT8 clone and the LT12 clone, respectively. With the exception of Ψ(7-8), which required a concentration of up to 10^{-6} M, Ψ(2-3) and Ψ(5-6) could sensitize 50% of the T2 cells for lysis by LT8 and LT12 clone, respectively, at a concentration of about 10^{-7} M which was also the concentration required for 50% lysis of the T2 cells loaded with MART-$1_{(27-35)}$ (Figure 3).

DISCUSSION

Identification of tumour antigens has opened an invaluable opportunity for an anti-tumour specific immunotherapy. Based upon the important role of CTLs in preventing and controlling tumour growth, peptide-based strategies for the elicitation or the modulation of anti-tumour CTL responses are of particular interest. However, the use of peptide agonists or partial agonists in immunotherapy is limited by the high susceptibility of peptides to proteolysis. This problem may be overcome by introduction of amide bond surrogates in synthetic peptides. The immunological impact of peptide backbone modifications in the interaction of peptides with class I or class II MHC molecules was investigated only recently [17–28]. Syntheses of several surrogates are now well established [42], and we chose in a first approach to examine the contribution of the carbonyl counterpart of each peptide bond to the interactions of the melanoma MART-$1_{(27-35)}$ epitope with its biological receptors. Eight analogues of this antigenic peptide were synthesized by replacing one peptide bond at a time by a reduced peptide bond in the native sequence. It is well known that the replacement of a peptide bond

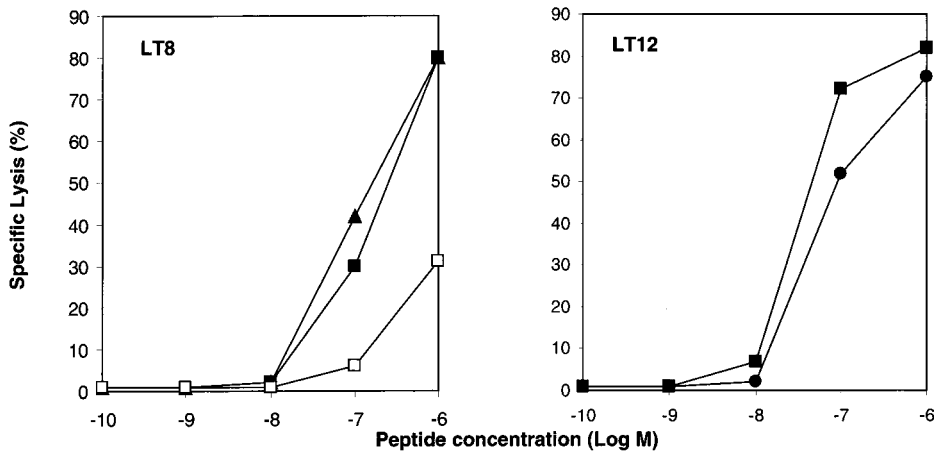

Figure 3 Lysis by LT8 and LT12 T cell clones of T2 cells incubated with different peptide concentrations. ^{51}Cr-labeled T2 cells were first incubated for 2 h with MART-$1_{(27-35)}$ (square), Ψ(2-3) (triangle), Ψ(7-8) (open square) and Ψ(5-6) (circle) at the indicated concentrations before being lysed in a 4-h cytolytic assay with LT8 (left panel) or LT12 (right panel) clone at an E:T ratio of 3:1.

by a reduced peptide bond leads to the modification of the intramolecular interactions. As a consequence, an overall or a locally structural change in the peptide is observed [43,44].

Introduction of an aminomethylene bond in the MART-$1_{(27-35)}$ sequence resulted in an increased stability to enzymatic hydrolysis of the resulting analogues. If the specificity of pepsin is precisely taken into account, peptide bonds in positions 6-7 and 7-8 are preferentially cleaved. Our results show that the enzymatic stability is not only increased when the reduced amide bond is introduced at these positions but also at positions 3-4, 4-5 and 5-6. Such a remote effect of single backbone modifications on the stability of adjacent peptide bonds has been reported in peptide drug development [45]. In this study, we clearly show that introduction of only one peptide bond surrogate in a parent sequence greatly enhances the stability to peptidases of the entire sequence. This is of practical importance when considering that the antigen persistence in the biological fluids can increase the functional half-life of peptide-MHC complexes [28].

The impact of replacement of one peptide bond by an aminomethylene bond on the ability of the resulting MART-$1_{(27-35)}$ analogues to induce HLA-A2 assembly was then analyzed. Interestingly, while in such case, a potential intermolecular hydrogen bonding is lost at the point of replacement of the carbonyl moiety by a methylene group, five reduced peptide bond analogues were more efficient binders than the parent peptide. Cristallographic studies of peptide/HLA-A2 complexes have revealed a major contribution of polar peptide main chain atoms in the binding interaction with the HLA-A2 molecule [46]. For example, one hydrogen bond is observed between the carbonyl oxygen of the residue in position 2 (P2) of the ligand and Lys^{66} of HLA-A2. Another hydrogen bond is observed between the carbonyl oxygen of P8 and Trp^{147}. These bonds are lost in the interaction between the corresponding reduced peptide bond analogue and HLA-A2. However, the Ψ(2-3) analogue binds very efficiently to HLA-A2. This indicates that in the case of MART-$1_{(27-35)}$, the loss of one intermolecular hydrogen bond is not detrimental, but rather may increase the efficiency of peptide/HLA-A2 binding. In a previous study, Guichard *et al.* showed that reduced peptide bond analogues could bind to the soluble recombinant class I K^d molecule [17]. The authors found that five out of eight analogues keep a K^d binding capacity, and that the relative binding affinities of these analogues were three- to ten-fold lower than that of the parent peptide. In the present study, most of the analogues bound more efficiently to HLA-A2 than the parent peptide. This can be explained by the fact that, contrary to the K^d model, interactions between MART-$1_{(27-35)}$ and HLA-A2 are not optimal, as exemplified by the poor binding properties of MART-$1_{(27-35)}$ to HLA-A2.

Influence of reduced peptide bond replacement on the capacity of the resulting analogues to be recognized by MART-$1_{(27-35)}$-specific CTLs has revealed that several substitutions resulted in a loss of recognition by MART-$1_{(27-35)}$-reactive TILs. Crystal structures of HLA-A2-Tax peptide bound to different

TCRs have shown that four contacts connecting the TCR and the peptide backbone are conserved: two of them involve the carbonyl of the fourth and the sixth peptide bond [47,48]. It is tempting to speculate that in the case of Ψ(4-5) and Ψ(6-7), the loss of CTLs recognition results from the absence of these direct contacts between the TCRs and the peptide carbonyls. Another explanation, also valid for Ψ(1-2), Ψ(3-4) and Ψ(6-7), would be that the reduced peptide bond induces a conformational change of the peptide or the MHC molecule. It is well known that the replacement of the carbonyl moiety by a methylene group in a reduced peptide bond leads to the generation of a strong proton donor. The secondary amine of the reduced amide bond is protonated at physiological pH and the resulting ammonium link (CH_2-N^+H_2) has been shown to stabilize folded structures in pseudodipeptide units by acting as a strong hydrogen bond donor [43]. This protonated secondary amine may provide a supplementary hydrogen bond, beneficial for the binding of the analogues to HLA-A2, but detrimental for the interaction between the pseudopeptide/HLA-A2 complex and the TCR.

CONCLUDING REMARKS

In this study, we have introduced aminomethylene bonds into the immunodominant epitope of the HLA-A2 restricted melanoma antigen to enhance enzymatic resistance and class I MHC binding affinity of the resulting analogues. Two reduced peptide bond analogues Ψ(2-3) and Ψ(5-6) bound with high affinity to HLA-A2, and were recognized by one MART specific TIL. These first encouraging results suggest that other peptide bond surrogates could be introduced in this antigenic peptide. It is tempting to speculate that few of them will both increase the binding capacity of the resulting analogue to HLA-A2 and enhance the stimulation of natural epitope specific CTLs, as demonstrated in the case of an influenza virus epitope (25).

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