A tricyclic ring system replaces the variable regions of peptides presented by three alleles of human MHC class I molecules

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Background: Cytotoxic T-lymphocytes (CTLs) recognize complexes of short peptides with major histocompatibility complex (MHC) class I molecules. MHC molecules are polymorphic, and the products of different MHC alleles bind to different subsets of peptides. This is due to differences in the shape of the peptide-binding groove on the surface of the MHC protein, especially the 'pockets' into which anchor residues at each end of the peptide fit. Non-peptidic ligands for class I molecules may be useful clinically.
Results: By applying computer-aided design methods guided by X-ray structures, we designed and synthesized several MHC class I ligands, based on known peptide ligands, in which the tricyclic, aromatic compound phenanthridine replaced the central amino acids of the peptides. These semi-peptidic fluorescent ligands bound with high affinity and with allelic specificity to the peptide-binding groove of different MHC class I molecules, forming crystallizable complexes.
Conclusions: Specificity for binding to different MHC class I molecules can be imparted to the common phenanthridine element by judicious choice of terminal peptidic elements from either nonamer or decamer peptides. The phenanthridine-based ligands have a long bound half-life, as do antigenic peptides.

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Introduction

Major histocompatibility complex (MHC) proteins are central to the cellular immune response. There are two classes of MHC molecules; both present antigenic peptides on the surface of cells, but the different classes have different functions [1]. Class II MHC proteins, which are found on antigen-presenting cells, derive their peptidic ligands from proteolyzed, exogenous proteins and stimulate helper T cells, activating an antibody response to the exogenous protein. Class I MHC proteins, which are found on virtually all cells, instead display peptides derived from proteins made within the cell. Peptides bind to MHC class I proteins in a groove on the upper surface of the protein, forming a composite surface (Fig. 1) that can be recognized by the T-cell receptor (TCR). In the absence of peptide, MHC class I proteins are not stable, and do not survive on the cell surface.

The MHC class I system appears to have evolved to allow the recognition and destruction of virally infected cells. When a cell is infected with a virus, peptides derived from viral proteins are displayed on the cell surface, where they can be recognized by circulating cytotoxic T-lymphocytes (CTLs), initiating a cytolytic response. However, unwanted or inappropriate TCR recognition of MHC–peptide complexes can initiate harmful responses, for example graft rejection and autoimmune disorders [2]. Graft rejection occurs because MHC class I molecules are highly polymorphic, so that cells from grafted tissue that is not matched carry complexes of peptides with foreign MHC class I proteins, causing rejection. Autoimmunity results from a failure of the systems used by the body to ensure that T cells do not bind to complexes of self MHC with self peptide, and therefore do not attack self cells.

The interaction between MHC class I molecules and their peptide ligands is now well understood. Peptides bound to different alleles of MHC class I molecules have

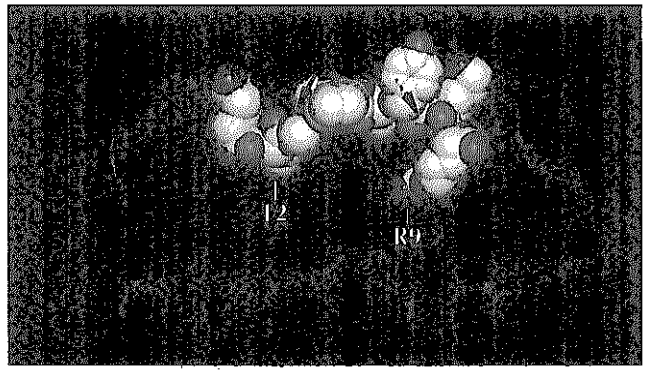

Fig. 1. Structure of the Flu-NP peptide presented by the MHC molecule HLA-Aw68. The Flu-NP peptide (KTGGPIYKR) uses residues T2 and R9 (marked) as anchor residues. The vectors chosen for use in the search for possible ligands were from Cα of G4 to N of G4 (solid arrow) and from Cα of Y7 to CO of Y7 (hidden behind the ring of Y7, and therefore shown as a dotted arrow).

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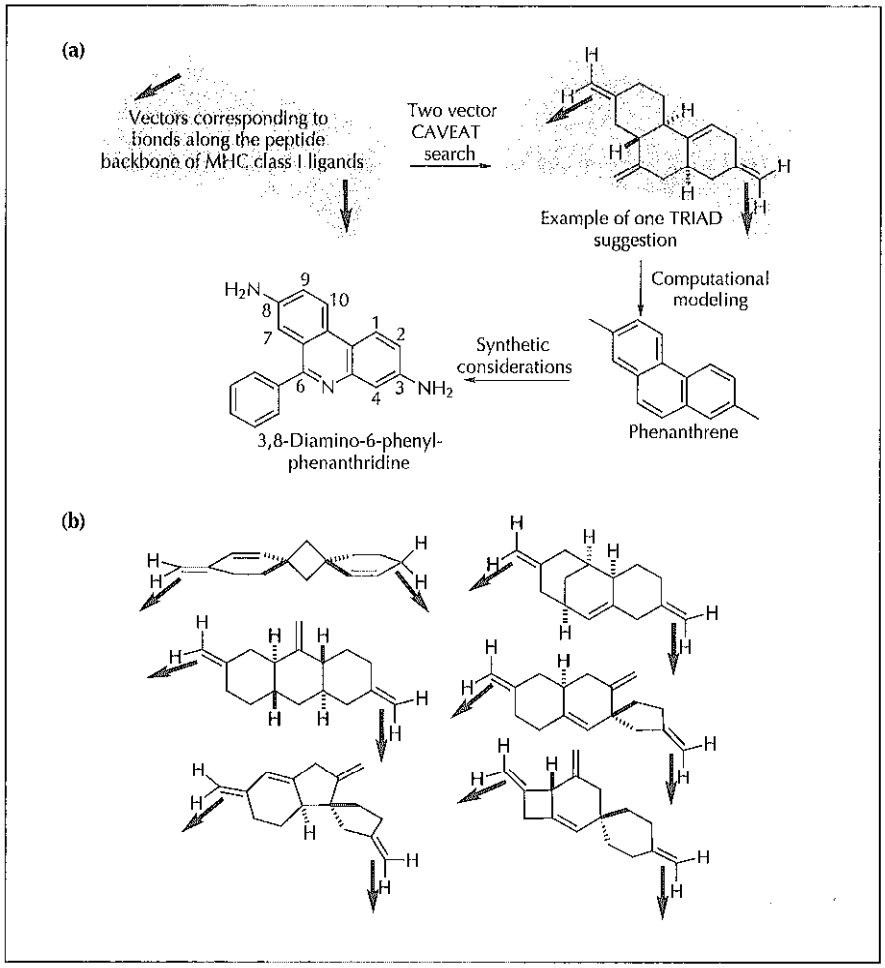


Fig. 2. The design of a tricyclic linker. The initial search criteria consisted of two vectors, from two bonds along the peptide backbone (the C_α–N bond of P4 and the C_α–carbonyl carbon bond of P7). A computational search through the TRIAD database of vectors from 403 000 three-ring molecules revealed the 3,8-disubstituted tricyclic pattern. Although the suggestions were not planar, it was recognized that the chosen bonds could be joined by a planar 3,8-disubstituted ring system. This was confirmed by computational modeling. Synthetic considerations guided the choice of starting material. **(b)** The sample structures shown illustrate the structural diversity of the tricyclic molecules found by CAVEAT.

characteristic 'anchor' residues (Fig. 1), often at their second (P2) and last positions, and are generally eight to ten residues long [3–6]. These anchor residues fit into specificity pockets in the binding groove that are found in class I proteins from both human [7–9] and mouse [10,11]. With their second and last residues fitted into pockets, peptides tend to arch away from the MHC molecule from about residues P4 to P7; these same residues have the greatest sequence variation (reviewed in [12]). Presumably, this central region is the most important element in the interaction with the TCR. Peptidic ligands to single subtypes of MHC molecules have been designed [13,14], but suffer from poor bio-availability in human sera [15]. We sought to replace the variable domains of MHC-binding peptides with a non-peptidic moiety, while retaining the ability of the terminal residues to bind to the ends of the peptide-binding groove in specific MHC class I proteins.

Results and discussion

From the refined X-ray structure of a peptide presented by the MHC class I molecule HLA-Aw68 [16], two bonds, along the peptide backbone and flanking the variable region, were identified as candidate parameters for ligand design (Fig. 1). The two bonds, represented as two vectors (Fig. 2a), were used in a computational search [17,18] through a comprehensive database of 403 000 three-ring molecules (TRIcyclics for Automated Design, TRIAD) ([19] and P.A. Bartlett, G.A.W. & G. Lauri, unpublished work). Seventy-six tricyclics were identified that contain bonds with a vectorial relationship similar to that used in the search. After computational modeling of possible ligands (using the program Macromodel [20]), we turned our attention to the 3,8-disubstituted phenanthracene ring system, in part due to its rigid conformation. An aza analog (phenanthridine) of this molecule was selected since a starting material for ligands incorporating this element, 3,8-diamino-6-phenyl-phenanthridine, is commercially available. A rapid and modular synthesis of the phenanthridine linker was accomplished by using an isocyanate of the penultimate residue [21], which reacted with phenanthridine site-specifically to yield a urea. Based upon NMR spectroscopic analysis, the reaction appears to occur at the more nucleophilic amino group at the 3-position, although we note that a highly similar spacer element would result from isocyanate coupling at the 8-position. Solid-phase methods were used to complete the syntheses of the ligands (Fig. 3).

The phenanthridine-based ligands bound to MHC molecules and promoted their *in vitro* folding, despite the reduced conformational flexibility of the phenanthridine spacer. The yield of MHC complex was about 25 % based on the MHC heavy chain, comparable to that seen

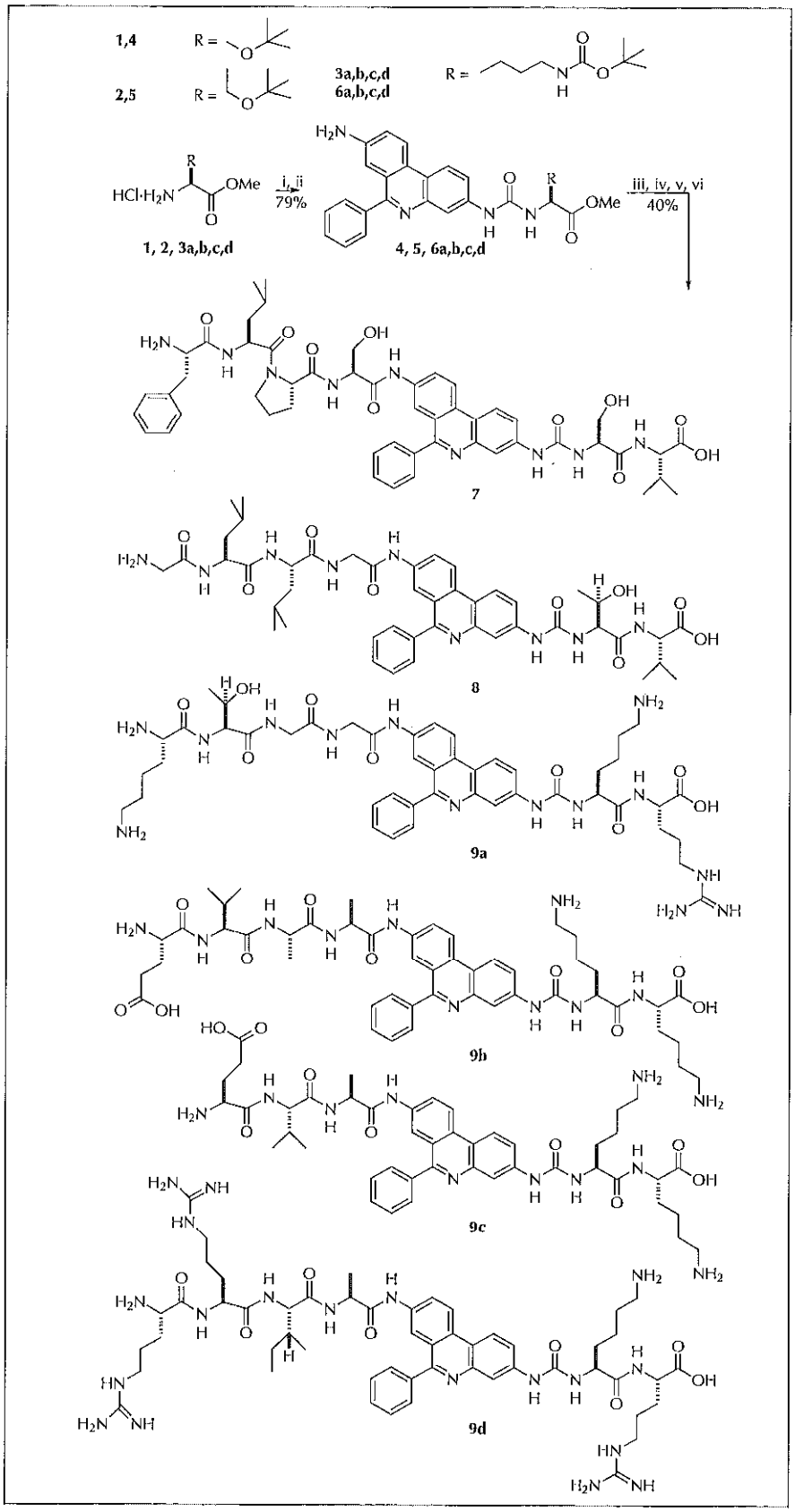
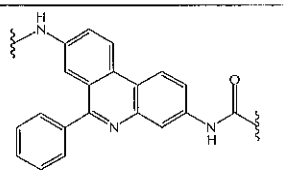


Fig. 3. Synthesis of the phenanthridine-based ligands. Starting from a suitably protected version of the ligand's penultimate amino acid, the following conditions were used for subsequent transformations: (i) Triphosgene, *i*-Pr_2NEt (*N,N*-diisopropylethylamine), THF (tetrahydrofuran); (ii) 3,8-diamino-6-phenylphenanthridine, THF, 0 °C to room temperature; (iii) LiOH, THF:H_2O (3:1); (iv) Fmoc-OSu (*N*-(9-Fluorenylmethoxycarbonyloxy)succinimide), dimethylformamide; (v) amino acid linked to solid support (50 mol %), HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HOBT (1-hydroxybenzotriazole), *i*-Pr_2NEt, NMP (*N*-methylpyrrolidone); (vi) solid phase peptide synthesis.

with peptides, and refolding was found to be dependent on the addition of the phenanthridine-based ligands, as it is with peptides [22]. Retention times of the class I–ligand complexes in gel filtration high-pressure liquid chromatography (HPLC) are similar to those of class I–antigenic peptide complexes. SDS-PAGE analysis of

Table 1. Structures of MHC class I ligands and their corresponding MHC class I subtypes.

MHC	Parent peptide	Amino terminus	 Carboxyl terminus	Structure
A2.1	Hep-B	Phe**Leu**ProSer	Ser**Val**	7
	Flu-M	Gly**Leu**LeuGly	Thr**Val**	8
Aw68	Flu-NP	Lys**Thr**GlyGly	Lys**Arg**	9a
	Human	Glu**Val**AlaAla	Lys**Lys**	9b
	Human	Glu**Val**Ala	Lys**Lys**	9c
B27	Model	Arg**Arg**IleAla	Lys**Arg**	9d

Each MHC class I molecule–ligand complex was assayed for its ability to fold by gel filtration chromatography, as described previously [22]. Parent peptides are Hep-B: FLPSDFFPSV, from hepatitis B nucleocapsid [23]; Flu-M: GILGFVFTL, from influenza virus matrix, with modifications described previously [25]; Flu-NP: KTGGPIYKR, from influenza virus nucleoprotein [30]; Human: EVAPPEYHRK [16] with P4A and R9K non-anchor substitutions; and Model: RRIKAITLK, a model peptide [29] that is modified in the present study with the following changes: K4A, L8K and K9R. Anchor residues are in bold face.

the isolated complexes revealed the presence of both the heavy chain and the light chain of MHC class I molecules, β_2-microglobulin (β_2m), in the complex (data not shown). The class I molecule HLA-A2.1 complexed with the phenanthridine-based ligand corresponding to a peptide from hepatitis B virus (Table 1, entry 1, structure **7**) forms crystals nearly isomorphous to HLA-A2.1–peptide complexes [23]. X-ray diffraction studies

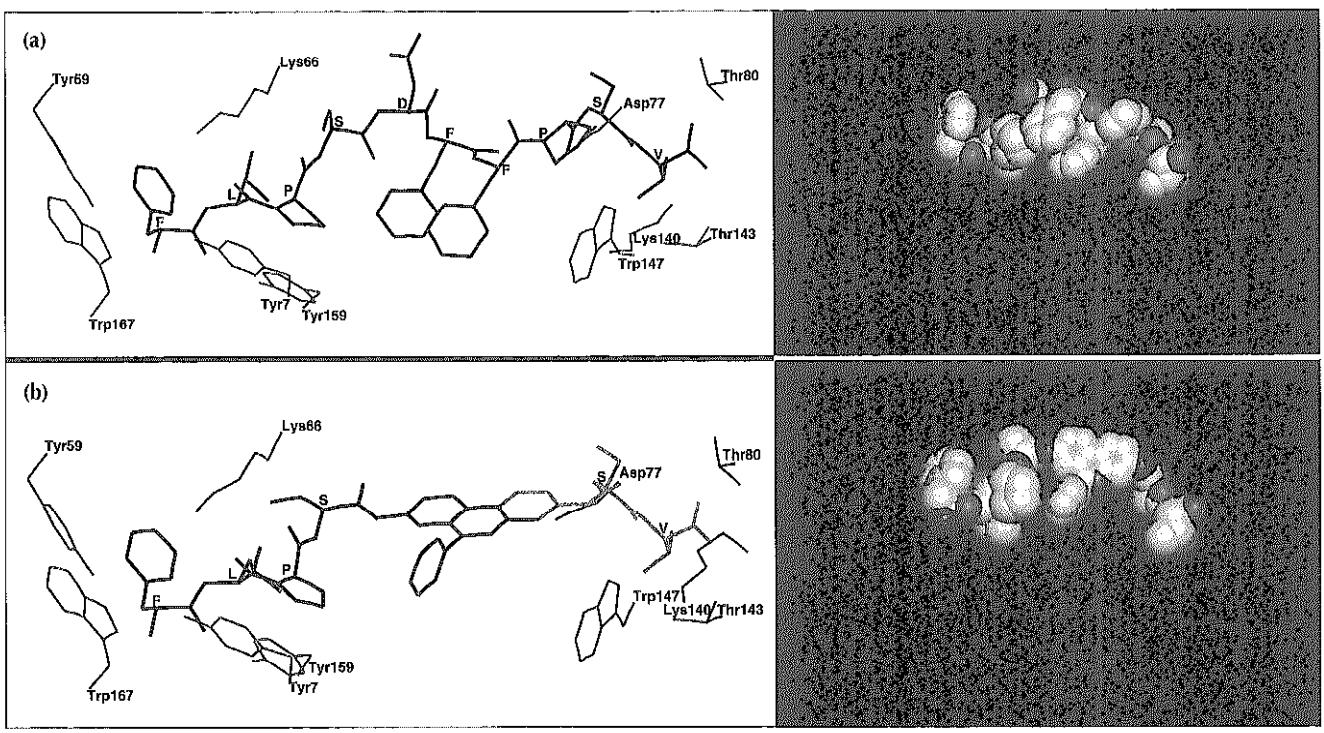


Fig. 4. Modified and unmodified MHC class I A2.1 ligands. **(a)** The X-ray crystal structure of the hepatitis B virus peptide (FLPSDFFPSV) bound to HLA-A2.1 [23]. On the left, HLA residues are shown in gray, with the peptide in black; the right panel shows a space-filling model of the same structure. **(b)** Left, structure of the corresponding HLA-A2.1–phenanthridine-based ligand complex. The atoms drawn in black could be modeled with confidence into clear, continuous electron density. At the carboxyl terminus of the ligand the positions of the atoms drawn in gray are less certain because of breaks in the electron density of the ligand. Right, space-filling model of the same structure. The poor electron density at the carboxyl terminus of the ligand in this structure may indicate some disorder of these ligand atoms. A large mosaic spread of the crystal (half width of 0.6 degrees) and the possibility of conformational heterogeneity of the ligand may contribute to this apparent disorder (E.J.C. & G.A.W., unpublished data). Experiments to grow and collect data from better crystals are ongoing.

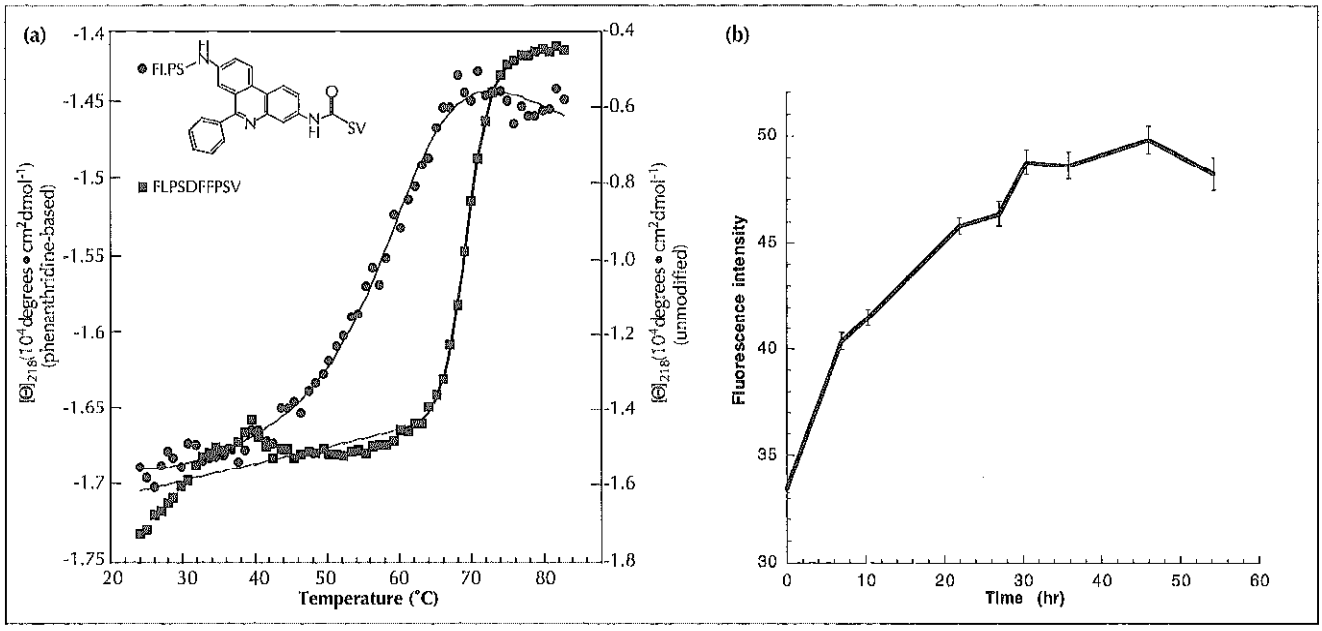


Fig. 5. Dissociation of the hepatitis B peptide-based phenanthridine ligand from HLA-A2.1 measured thermodynamically and kinetically. **(a)** Thermodynamic stability was measured as described previously [24–26], by monitoring the circular dichroism signal at 218 nm. The complex of HLA-A2.1 and phenanthridine-based ligand has a lower melting temperature (T_m = 56 °C) than does the complex with the unmodified peptide (T_m = 68 °C) (FLPSDFFPSV). **(b)** Ligand dissociation measured by fluorescence. Error bars represent average error.

show that the phenanthridine-based ligand bound in an orientation that is essentially identical to the parent peptide, with the central residues of the peptide replaced by the flat phenanthridine ring (Fig. 4).

Thermodynamic stability, based upon circular dichroism-monitored denaturation temperatures (T_m), has been measured for a number of complexes of peptides and MHC class I molecules [24–26]. In general, higher melting temperatures correlate with higher binding affinity. Various peptide modifications, for example extending the length of the ligand to 11 amino acids, decrease the thermal stability of the MHC class I complex [27]. A phenanthridine ligand (7) that is based on a hepatitis B peptide known to bind to the MHC class I molecule HLA-A2.1, also forms complexes with HLA-A2.1 that are stable at physiological temperatures but which exhibit somewhat decreased thermal stability (T_m = 56 °C) in comparison to the parent peptide complex (T_m = 68 °C) (Fig. 5a). This T_m of 56 °C is within the normal range of class I MHC–peptide complexes.

The fluorescence of the phenanthridine moiety was exploited to measure half lives for binding of phenanthridine-based ligands to MHC molecules. When these ligands dissociate from the protein complex, the fluorescence intensity of the phenanthridine chromophore increases (Fig. 5b). The hepatitis B peptide-based, phenanthridine ligand (7) complex with HLA-A2.1 showed a half life of 15 h at 37 °C (versus 25 h for the unmodified peptide complex, as measured indirectly by β_2m dissociation [28]). This shorter half life of the phenanthridine-based ligand correlates with the lower melting temperature observed for HLA complexed with that ligand.

The strategy of replacing variable regions was also successful in all other subtypes of MHC class I molecules examined (Table 1). The anchor residues of the ligand must match the requirements of the targeted MHC class I protein; mismatched anchor residues and MHC class I molecules did not result in folded complexes. Six phenanthridine-based ligands derived from five parent peptides were synthesized; as anticipated from the specificities of the parent peptides, two were specific for HLA-A2.1, three for HLA-Aw68 and one for HLA-B27. For both HLA-A2.1 and HLA-Aw68, ligands based on decamer and nonamer peptides were synthesized; one ligand based on a nonamer was synthesized for HLA-B27. In each case, subtype-specific binding of ligand to MHC molecule was observed. The phenanthridine spacer was used to replace three amino acids in three different nonameric peptides, four amino acids in two decameric peptides, and five amino acids in a decameric peptide (Table 1). The length of the planar spacer is ~11.5 Å. The X-ray crystal structures of all of the parent peptides complexed with their respective MHC molecules have been determined previously ([23,29] and E.J.C., unpublished data). The distance that the spacer replaces, measured in the complexes of the parent peptides with MHC molecules ([23,29,30] and E.J.C., unpublished data), was between 8.1 Å and 12.9 Å. This variation indicates that there is some accommodation in the position of the amino acids at the joints with the spacer, suggesting that the phenanthridine spacer element can be made to bind in a

subtype-specific manner to many HLA molecules by equipping it with suitable terminal peptide elements.

Significance

The recognition step regulating CTL function involves a trimolecular complex of TCR, MHC and the antigenic peptide. Since the antigenic peptide is relatively small, it is amenable to investigation using synthetic chemistry. The phenanthridine ligands based upon antigenic peptides reported here are a first step in this direction.

A pharmacological antagonist that blocks CTL responses could be used to counter graft rejection, and to treat autoimmune disorders [31]. Conversely, an agonist to enhance CTL response may be useful for treatment of tumors [32] or viral infections. The phenanthridine-based ligands are the first non-peptidic ligands reported to form structurally characterized complexes with MHC class I molecules. These complexes are stable at physiological temperatures and above, a prerequisite for effective competition with natural peptides. This study shows that the information gained from X-ray structures of MHC class I–peptide complexes, together with computer-based design techniques, can be used to design replacement elements that remove the requirement for long peptides as ligands to MHC class I molecules. In addition, the fluorescence of the phenanthridine spacer may prove valuable in biophysical studies of MHC–ligand and MHC–ligand–TCR complexes.

Materials and methods

Preparation of phenanthridine-based ligands

Ligands were prepared by chemical synthesis as outlined in Fig. 2 using standard laboratory procedures. All compounds were characterized by infrared spectroscopy, ^{1}H NMR, and fast atom bombardment (FAB) mass spectroscopy or high resolution mass spectroscopy (HRMS). The first amino acid coupled to compounds **4**, **5**, **6a,b,c,d** and all arginines were double coupled. Reverse phase HPLC (ligand dependent solvent gradient of 100:0 to 70:30 or 50:50 0.1 % trifluoroacetic acid in H_2O:acetonitrile) was used to purify the ligands.

*Synthesis of **6a***

To a 0.1 M THF solution of a hydrochloride salt **3a** (1.158 g, 3.5 mmol, 1 equiv), triphosgene (0.343 g, 1.16 mmol, 0.33 equiv) in THF (5 ml) was added. *i*-Pr_2NEt (3.1 ml, 17.5 mmol, 5 equiv) was added slowly, and the solution was stirred for one hour at room temperature. Strong infrared absorbance at 2253 cm^{-1} verified the presence of an isocyanate. This solution was cooled to 0 °C and added slowly via syringe to an ice-cold THF (20 ml) solution of 3,8-diamino-6-phenyl-phenanthridine (1.0 g, 3.5 mmol, 1 equiv), which was allowed to warm gradually to room temperature. Following overnight stirring, the solvent was rotary evaporated, and the oily residue redissolved in CH_2Cl_2. The solution was washed once with saturated $NaHCO_3$ and once with saturated NaCl and dried on anhydrous sodium sulfate. Flash chromatography (95:5 CH_2Cl_2:MeOH) was used to purify the compound (1.5861 g, 79.3 %).

*Characterization of ureo-phenanthridine **6a***

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.30 (d, J = 14.0 Hz, 1H, Ar), 8.22 (d, J = 13.9, 1H, Ar), 7.65 (m, 2H, Ar), 7.48 (m, 3H, Ar), 7.37 (m, 1H, Ar), 7.13 (m, 2H, Ar), 7.01 (m, 1H, Ar), 5.61 (br s, 1H, NH(CO)N*H*-Ar), 4.76 (br m, 1H, N*H*(CO)NH-Ar), 4.38 (m, 1H, C*H*CO_2CH_3), 3.86 (br s, 2H, NH_2), 3.67 (s, 3H, CH_3), 3.02 (m, 2H, NHCH_2), 1.83 (m, 2H, CHCH_2), 1.42 (s, 9H, C(CH_3)$_3$), 1.25 (m, 4H, CH_2);HRMS: m/z calc'd for $C_{32}H_{37}N_5O_5$ (M+H)+ 572.2891, found 572.2873.

Folding and purification of complexes of MHC class I molecules with ligands

Denatured heavy chain and β_2m were purified from an *Escherichia coli* expression system, as described previously [22]. After being individually diluted ten-fold in 8 M urea, heavy chain (final concentration of 1 μM) and β_2m (final concentration of 2 μM) were added to 45 ml of 100 mM Tris (pH = 8), 400 mM L-arginine, 0.5 mM Na_2EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, containing 50 μM phenanthridine-based ligand. After incubating for 36 h at 10 °C, the solution was concentrated with a Centriprep-10 followed by a Centricon-10, to a volume of 45 μl. The concentrated protein was purified using gel filtration HPLC with an eluent of 20 mM Tris (pH = 7.5) containing 150 mM NaCl and had a retention time of 18 minutes (44 kD) in a Phenomenex Biosep SEC-S3000 column. The 1.5 ml fraction containing the complex was concentrated to 45 μl using a Centricon-10.

Thermal stability measurements

Circular dichroism experiments were conducted in 10 mM MOPS (pH = 7.5), in a 1 mm cell. Concentrations (from 3 to 10 μM) of the complexes were determined spectrophotometrically (at 280 nm, ε = 94 240 cm^{-1} M^{-1}). The Aviv 62DS spectropolarimeter was equipped with a thermoelectric temperature controller.

Fluorescence kinetics

Folded MHC class I–phenanthridine ligand complex (50 nM to 750 nM) was incubated at 37 °C and cooled to 10 °C before being excited at 310 nm and monitored for emission at 452 nm. Cuvettes remained inside the Hitachi F-2000 fluorimeter during the course of each experiment. The buffer contained 20 mM TRIS, 0.02 % NaN_3, at pH = 7.5.

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