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Cytotoxic T Lymphocyte Epitope Analogues Containing *cis*- or *trans*-4-Aminocyclohexanecarboxylic Acid Residues

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Abstract—In order to improve the immunotherapeutical potential of H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-OH (CLG) peptide, an Epstein–Barr virus (EBV) subdominant epitope derived from the membrane protein LMP2, we have synthesized and tested CLG analogues containing *cis*- and/or *trans*-4-aminocyclohexanecarboxylic acid (ACCA) replacing Gly-Gly and/or Thr-Met dipeptide units. All pseudopeptides were tested for metabolic stability and for their capacity to bind HLA-A2 molecules and to sensitize target cells to lysis. All new compounds exhibited higher enzymatic resistance compared to the original CLG and some *trans*-ACCA-derivatives were able to associate HLA-A2 and to efficiently stimulate CTL responses directed against the CLG natural epitope.

Introduction

Major histocompatibility complex (MHC) class I molecules bind and display oligopeptides deriving from viral proteins in infected cells.[1] MHC–peptide complexes form the antigens that can be recognised by specific T cell receptors (TCRs) expressed on cytotoxic T lymphocytes (CTLs). In this way, CTL can identify and kill infected cells selectively, sparing healthy cells.[2] This is a critical stage in the initiation of all immune responses, leading finally to the effective elimination of intracellular parasites. MHC class I molecules bind short peptides, 8–10 amino acids long and sequences of naturally presented peptides eluted from class I molecules has revealed some of the rules governing the interaction between peptides and MHC.[3,4] The main anchor residues, usually at positions 2 and 9, interact with pockets B and F localized in the binding site of MHC.[5–7] All these interactions contribute to the formation of stable MHC/peptide complexes and at present, the general consensus is that stability of HLA/peptide complexes is important for determination of peptide immunogenicity. Crystallographic studies performed on purified MHC class I/peptide and TCR/MHC/peptide complexes have elucidated that the majority of peptide residues are buried in the MHC groove and that the TCR binds MHC/peptide complexes and contacts peptide residues by the CDR3 domain.[8–10] We focused our analysis on the H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-OH (CLG) peptide, an Epstein–Barr virus (EBV) epitope derived from the membrane protein LMP2 which represents the target of HLA-A2.01, HLA-A2.06 and HLA-A2.07 restricted EBV-specific cytotoxic T lymphocytes responses.[11] EBV is a lymphotropic virus associated with a number of human malignancies. The CLG nonamer represents a good target for the immunotherapy of EBV-associated malignancies since it is expressed and conserved in nasopharyngeal carcinoma and Hodgkin's disease biopsies.[12–15] However, the CLG peptide has low affinity for HLA-A2, does not produce stable complexes, and then produces weak CTL responses to this epitope.[16–18] Furthermore, this epitope showed low enzymatic stability being rapidly hydrolysed by plasma enzymes at the Met-Val bond.[19,20] In order to improve the immunotherapeutical potential of this epitope, we have synthesized and tested CLG analogues containing *cis*- and/or *trans*-4-aminocyclohexanecarboxylic acid (ACCA) replacing the dipeptide Thr-Met, next to scissile amide bond, and/or the flexible Gly-Gly (Table 1).

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Table 1. Sequence, physico-chemical, analytical data and metabolic degradation of CLG analogues

Compound		Purity[a]	$[\alpha]_D^{21}$	HPLC	MS	Amino acid analysis							Half-life (min)	
No.	Sequence	%	($c=1$, MeOH)	(t_R)	(MH^+)	Cys	Leu	Gly	Thr	Met	Val	ACCA	Medium	Plasma
CLG	H-Cys-Leu-Gly-Gly-Gly-Leu-Leu-Thr-Met-Val-OH												82.3±0.9	12.7±0.6
1	H-Cys-Leu-Gly-Gly-Gly-Leu-Leu-*cis*-ACCA-Val-OH	96	−14.8	12.7	800.5	0.89	3.05	2.09			1.00	1.08	> 360	229.4±4.6
2	H-Cys-Leu-*cis*-ACCA-Leu-Leu-Thr-Met-Val-OH	97	−22.3	11.9	918.7	0.91	3.11		0.89	0.82	1.00	1.11	190±2.1	49.3±1.7
3	H-Cys-Leu-*cis*-ACCA-Leu-Leu-*cis*-ACCA-Val-OH	95	−25.1	11.2	811.5	0.85	2.89				1.00	1.95	> 360	214.1±5.7
4	H-Cys-Leu-Gly-Gly-Gly-Leu-Leu-*trans*-ACCA-Val-OH	98	−11.9	13.6	800.5	0.87	3.21	2.13			1.00	0.97	> 360	195.8±4.3
5	H-Cys-Leu-*trans*-ACCA-Leu-Leu-Thr-Met-Val-OH	98	−19.5	12.5	918.7	0.90	3.23		0.86	0.79	1.00	1.04	157±2.0	60.9±1.4
6	H-Cys-Leu-*trans*-ACCA-Leu-Leu-*trans*-ACCA-Val-OH	96	−10.9	12.1	811.5	0.84	3.17				1.00	2.14	> 360	230.8±5.4
7	H-Cys-Leu-*trans*-ACCA-Leu-Leu-*cis*-ACCA-Val-OH	97	−9.2	11.6	811.5	0.87	2.93				1.00	1.87	> 360	289.1±7.2
8	H-Cys-Leu-*cis*-ACCA-Leu-Leu-*trans*-ACCA-Val-OH	96	−8.7	11.7	811.5	0.89	3.15				1.00	2.09	> 360	198.6±5.2

[a]Homogenity of purified peptides was determined by analytical HPLC detected at 220 and 254 nm.

Conformationally restrained ACCA peptides have been extensively used to examine biologically 'active conformations' or to obtain more resistant compounds towards enzymolysis.[21] All CLG ACCA analogues were tested for metabolic stability and for their capacity to bind HLA-A2 molecules and to induce specific CTL activation.

Results and Discussion

Chemistry

cis- and *trans*-ACCA were prepared according to procedures reported in literature by hydrogenation of the appropriate aminobenzoic acid with PtO_2 as a catalyst (Scheme 1).[22] The resulting mixture of *cis* and *trans* isomers was separated by fractional recrystallization from H_2O/EtOH and the structure of pure isomers was confirmed by ^1H NMR. Both *cis* and *trans* isomers were converted separately to corresponding Fmoc (9-fluorenylmethoxycarbonyl-) derivatives.[23] CLG analogues **1**–**8** (Table 1) were synthesized by solid-phase methods, using an automated continuous-flow peptide synthesizer.[24] The stepwise synthesis were carried out by Fmoc/*t*Bu-chemistry. The Nα-Fmoc amino acids (4 equiv), were condensed using DIPCDI (*N*,*N*-diisopropyl carbodiimide) (4 equiv) and HOBt (1-hydroxybenzo triazole) (4 equiv) as coupling agents for 1 h starting to functionalised Wang resin.[25] Each Fmoc-*cis* or *trans*-ACCA (8 equiv) was coupled for 4 h by HATU [2-(1H-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] and DIPEA (diisopropylamine) (8 equiv). Pseudopeptides were deprotected and cleaved from the resin by treatment with TFA/H_2O/Et_3SiH (88:5:7, v/v) at room temperature for 1 h. After purification by preparative HPLC, structure verification

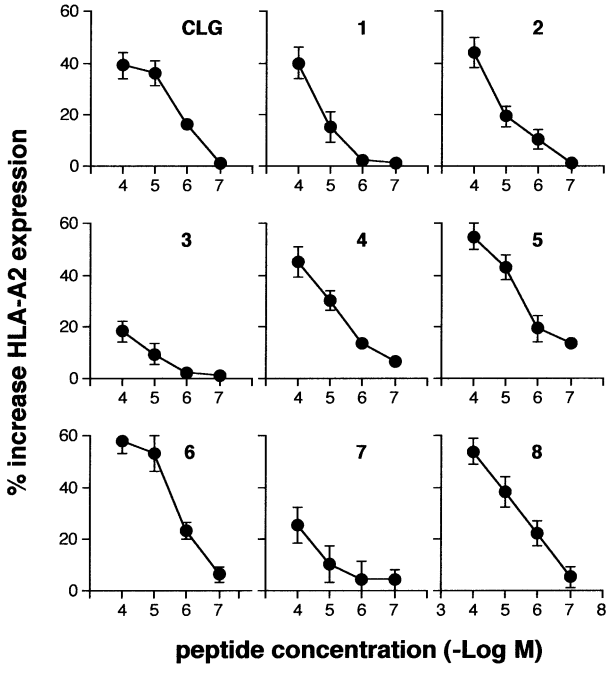


Figure 1. Induction of cell surface HLA-A2 molecules by the indicated peptides. Results represent a mean ±SD of three different experiments.

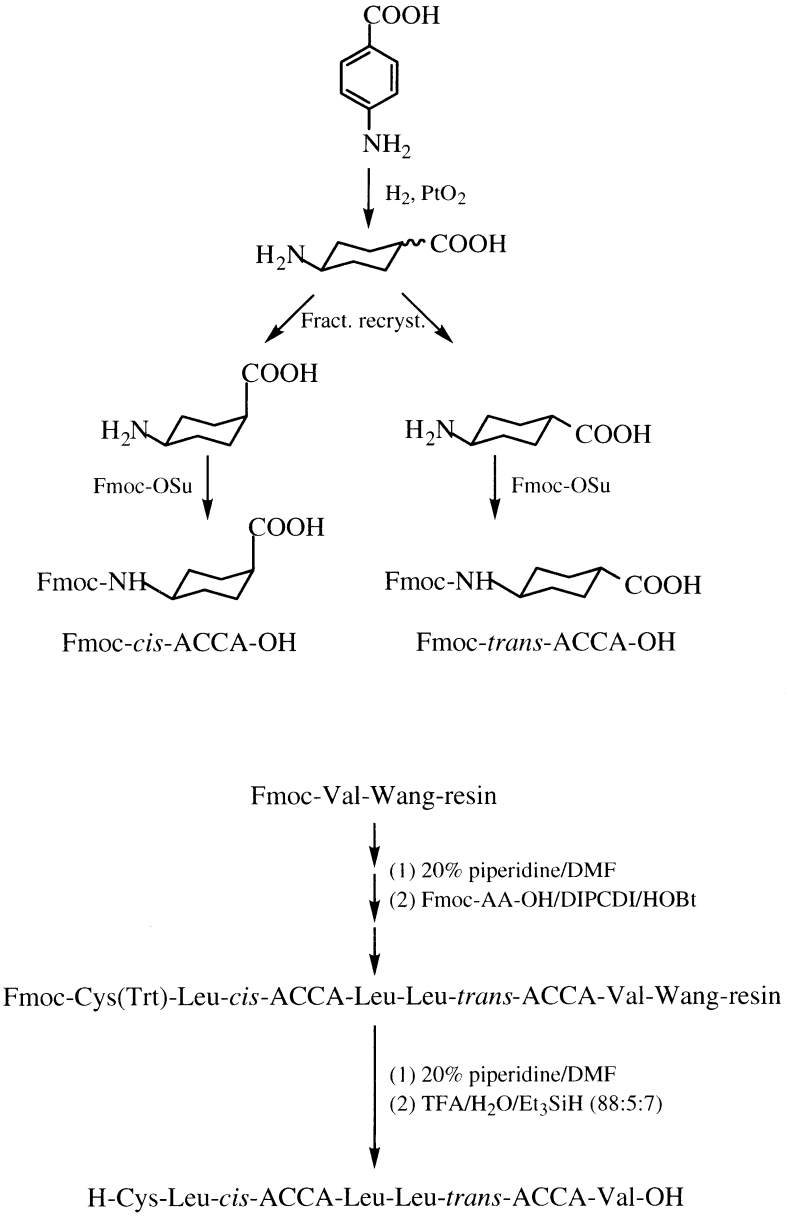
was achieved by amino acid analysis, mass spectrometry and NMR spectroscopy (Table 1).

Biological results

In order to evaluate the susceptibility to enzymatic hydrolysis, the analogues **1**–**8** were incubated at 37 °C in culture medium (RPMI) in the presence of 10% fetal calf serum (RPMI + 10% FCS) and in human plasma. The time course of ACCA analogues degradation was followed by HPLC analysis at varying periods of incubation, and half-lives of pseudopeptides are reported in Table 1 in comparison with CLG. As expected, C-terminal modifications of the original sequence considerably increased the stability of peptides in cell culture medium and in human plasma ($T_{1/2} > 200$ min).

We then evaluated the ability of all peptides to bind to HLA-A2. Peptide association was assessed by the induction of surface HLA-A2 expression in the T2 mutant cell line. As shown in Figure 1, all peptides, except ACCA disubstituted analogues **3** and **7**, bound to HLA-A2. Pseudopeptides **4**, **5**, **6** and **8** induced higher levels of HLA-A2 molecules if compared to the parent CLG peptide. Apparently, the presence of *trans*-ACCA unit in the C-terminal part of original nonameric sequence induces a favourable conformational arrangement for activity, while the *cis*-ACCA residue (**1**, **3** and **7**) in the same position results deleterious for the HLA binding.

The analogues **4**, **5**, **6** and **8** that increased the surface levels of HLA-A2 molecules were also evaluated for their capacity to stimulate CTL responses directed against the wild-type epitope. PBL from EBV-seropositive donors were stimulated, in parallel experiments, with T2 cells pulsed with 10^{-6} or 10^{-8} M of the synthetic ACCA peptides, and CTL cultures were tested



Scheme 1. Preparation of Fmoc-ACCA-OH isomors and solid-phase synthesis of [*cis*-ACCA,[3,4] *trans*-ACCA[7,8]] CLG analogue.

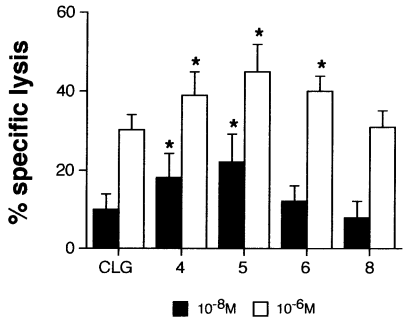


Figure 2. Activation of CLG-specific responses by ACCA analogues. Results represent a mean ±SD of three different experiments. $^{*}P<0.01$.

after three consecutive stimulations against HLA-A2 single-matched PHA blasts treated or not with 10^{-7} CLG peptide. CTL reactivation by CLG analogues is reported in Figure 2. Mono *trans*-ACCA derivatives **4** and **5** and bis *trans*-ACCA pseudopeptide **6** reactivated CLG-specific responses with an increased efficiency in comparison with CLG. The structural rearrangement of the molecules due to the presence of *trans*-ACCA seems to induce a favourable spatial disposition of the side chains, in the central part of the sequence, for the TCR recognition and for the consequent CTL responses.

Conclusion

The LMP2-derived CLG epitope may be regarded as a target of specific immunotherapies for the treatment of EBV-associated malignancies. However, the feasibility of specific CTL therapy may be limited by the poor immunogenicity of this antigen. We sought to increase its immunogenicity by defining CLG analogues forming stable comlexes with HLA-A2 that are able to induce efficient CLG-specific CTL responses. Now, to increase HLA-A2/peptide complex stability and immunogenicity and to improve enzymatic stability, we prepared and tested *cis*- and/or *trans*-ACCA containing CLG derivatives. ACCA octamers **4** and **5** and the heptamer **6** retain affinity for HLA-A2 and induce significant CTL responses directed against the subdominant CLG natural epitope. In addition, these variants are more resistant to proteolytic cleavage and thus may represent good candidates for EBV-specific immunotherapies in the treatment of human malignancies such as NPC and HD.

Experimental

General

Amino acids, amino acid derivatives, resins and chemicals were purchased from Bachem, Novabiochem, or Fluka (Switzerland).

Crude pseudopeptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30×4 cm, 300 A, 15 μm spherical particle size column). The column was perfused at a flow rate of 40 mL/min with a mobile phase containing solvent A (10%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0 to 100% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) in 25 min was adopted for the elution of compounds. HPLC analysis was performed by a Beckman System Gold with a Beckman ultrasphere ODS column (5 μm; 4.6×250 mm). Retention time (t_R) of the peptides were determined using HPLC conditions in the above solvent system (solvents A and B) programmed at flow rates of 1 mL/min using the following linear gradients: from 0 to 100% B in 25 min. All pseudopeptides showed less than 1% impurities when monitored at 220 and 254 nm.

Molecular weight of compounds were determined by a MALDI-TOF analysis using a Hewlett Packard G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as a matrix. The values are expressed as MH^+. TLC was performed in precoated plates of silica gel F254 (Merck, Darmstadt, Germany) using the following solvent systems: (c) AcOEt/n-hexane (1:1, v/v), (d) CH_2Cl_2/methanol (9.5:0.5, v/v), (e) CH_2Cl_2/methanol (9:1, v/v), (f) CH_2CL_2/methanol/toluene (17:2:1, v/v/v). Ninhydrin (1%) or chlorine iodine spray reagents were employed to detect the peptides. Melting points were determined by a Kofler apparatus and are uncorrected. Optical rotations were determined by a Perkin–Elmer 141 polarimeter with a 10-cm water-jacketed cell. ^{1}H NMR spectroscopy was obtained on a Bruker spectrometer (Bruker WM 500 MHz).

Solid-phase peptide synthesis

Pseudopeptides **1**–**8** (Table 1) were prepared by solid-phase method with a continuous-flow instrument with on-line UV monitoring (Milligen/Biosearch 9050). The stepwise syntheses were carried out by Fmoc chemistry using Wang resin. For each peptide, were used 0.2 g (0.1 mequiv) of Wang resin. The functionalized resin was swelled in DMF for 1 h and packed in the reaction column. *tert*-Butyl was used as a side-chain protecting group for threonine and trityl for the N-terminal cysteine. Each Nα-Fmoc amino acid was coupled in a 4-fold excess using DIPCDI in the presence of HOBt always in a 4-fold excess for 1 h. Fmoc-*cis*- or *trans*-ACCA were coupled by HATU/DIPEA as coupling agents in a 8-fold excess for 4 h. Each peptide was simultaneously cleaved from the resin and deprotected by treatment with modified reagent B (88% TFA, 5% H_2O, 7% Et_3SiH; 7 mL) for 1 h at room temperature. The resin was removed by filtration and washed with TFA (2×1 mL), the filtrate and washing were combined and evaporated at 25 °C, and the oily residue was triturated with ethyl ether (10 mL). The resulting solid was collected by centrifugation and purified by preparative HPLC.

Physico-chemical, analytical and amino acid analysis were reported in Table 1. As an example we report the chemical shift values of H-Cys-Leu-Gly-Gly-Leu-Leu-*trans*-ACCA-Val-OH (**4**): ^{1}H NMR (DMSO) δ 1.03 (δ, Leu), 1.14 (γ, Val), 1.71 (β, Leu), 1.80 (γ, Leu), 1.81–

2.28 (–CH_2–CH_2–, *trans*-ACCA), 2.07 (SH, Cys), 2.83 (CH–CO–, *trans*-ACCA), 3.04 (β, Cys), 3.49 (–CH–NH–, *trans*-ACCA), 3.91 (α, Cys), 4.09–4.25 (α, Gly), 4.46 (α, Val), 4.57–4.69 (α, Leu), 7.78 (NH, Cys), 7.97–8.08 (NH, Leu), 8.21 (NH, Val), 8.63–8.74 (NH, Gly).

Metabolic stability assay

The kinetics of CLG analogues degradation were studied in culture medium (RPMI) and human plasma.[26,27] A solution (0.1 mL) of each compound (10 mg/mL in acetonitrile/H_2O 1:1) was added to 1 mL of RPMI containing 20% fetal calf serum. Alternatively, test compound were incubated with plasma (0.6 mL) in a total volume of 1.5 mL of 10 mM Tris–HCl buffer, pH 7.5. Incubations were performed at 37 °C for different time: up to 360 min in the case of human plasma and up to 8 h in the case of RPMI containing 20% FCS. The incubation was terminated by addition of ethanol (0.2 mL), the mixture poured at 21 °C and after centrifugation (5000 rpm for 10 min), aliquots (20 μL) of the clear supernatant were injected into RP-HPLC column. HPLC was performed as described above (see Experimental procedures, general).

Cell cultures

The .174/T2 cell line (T2) was obtained by fusion of the peptide transporter mutant .174 LCL with the T-cell line CEM.[28] Cell lines were mantained in RPMI-1640 supplemented with 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10% heat-inactivated fetal calf serum and 200 μg/mL hygromycin B. PHA-activated blast were obtained by stimulation of peripheral blood lymphocytes (PBLs) with 1 μg/mL of purified PHA for 3 days and expanded in medium supplemented with interleukin-2 (IL-2), as described.[29]

Preparation of peptide-pulsed cells

Aliquots of 1×10^6 T2 cells were cultured overnight at 26 °C in 1 mL serum-free AIM-V medium. Cells were then washed, treated with mitomycin C to avoid cell proliferation and pulsed with the indicated concentrations of peptides for 3 h at 37 °C in AIM-V medium. After extensive washing the cells were used as stimulators for the reactivation of memory CTL responses.[30]

Detection of peptide binding to HLA-A2 molecules by immunofluorescence

T2 cells were treated at 26 °C for 18 h in serum-free medium in the presence or not of the indicated concentrations of peptides. Cells were then kept at 37 °C for 4 h and extensively washed to remove unbound peptides. The surface expression of HLA class I complexes was evaluated by immunofluorescence using the monoclonal antibody MA2.1 that recognizes HLA-A2 molecules. Mean fluorescence intensity was determined by fluorescence-activated cell sorting (FACS) analysis.[17] Data are expressed as the % increase in HLA-A2 expression calculated with respect to that of untreated T2 cells.

Cytotoxicity tests

Cytotoxic activity was assayed in standard 5-h ^{51}Cr-release assay.[31] LCL and PHA blasts were labeled with 0.1 μCi/10^6 cells of $Na_2^{51}CrO_4$ (NEN, Brussels, Belgium) for 90 min at 37 °C. For the peptide sensitization assays, 4×10^3 PHA blasts were placed in triplicate to V-shaped 96-well plates. Peptides were added to each well, and the plates were incubated for 1 h at 37 °C before addition of the effectors.[29] Peptide toxicities were checked in each assay, and were always $\leq$3%. Percent specific lysis was calculated as 100×(cpm sample−cpm medium)/(cpm Triton X-100−cpm medium).

Statistical analysis

Results are shown as mean ±SD. Thepaired Student's *t*-test was used for comparison of paired conditions.

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