# Peptide Analogues of a Subdominant Epitope Expressed in EBV-Associated Tumors: Synthesis and Immunological Activity

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H-Cys-Leu-Gly-Gly-Leu-Leu-Thr-Met-Val-OH (CLG) peptide is an EBV subdominant epitope that represents the target of HLA-A2 restricted CTL responses. The CLG peptide has low affinity for HLA-A2 and does not produce stable complexes, both factors that determine weak CTL responses. In contrast, the [Tyr$^1$, Ala$^3$]CLG (YLA) analogue showed high affinity for HLA-A2 molecules and efficiently stimulated CLG-specific CTL precursors. Nevertheless, this modified epitope showed low enzymatic stability. To further improve the immunotherapeutical potential of this "improved epitope", we have synthesized and tested YLA analogues containing different modifications next to the scissile peptide bond. Among the analogues we found three peptides, with higher enzymatic resistance, that efficiently stimulate CTL responses. These peptides may be used for EBV-specific immunotherapies.

## Introduction

Major histocompatibility complex (MHC) class I molecules bind and display oligopeptides deriving from processed proteins in infected cells.[1] MHC-peptide complexes form the antigens that can be recognized by specific T cell receptors (TCR) expressed on cytotoxic T lymphocytes (CTL). In this way, CTL can identify and kill infected cells selectively, sparing healthy cells. MHC class I molecules bind short peptides (8−10 amino acids long), and sequencing of naturally presented peptides eluted from class I molecules has revealed some of the rules governing the interaction between peptides and MHC. 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.[2] EBV is a lymphotropic virus associated with a number of human malignancies including endemic Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD), and immunoblastic B cell lymphomas of immunosuppressed patients.[3−5] 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.[6] Recently we have shown that the subdominant CLG epitope does not produce stable complexes with HLA-A2, and therefore we have prepared and tested CLG analogues carrying amino acid substitutions at nonanchor positions.[7] Among the analogues tested, [Tyr$^1$, Ala$^3$]CLG (YLA) showed high affinity for HLA-A2 molecules, potent stimulatory capacity, but poor metabolic stability, being rapidly hydrolyzed by plasma enzymes at the Met-Val bond. To enhance pharmacokinetic properties of MHC class I binding peptides,[8] we synthesized a series of YLA-derived analogues containing modifications next to the scissile peptide bond.[9−16] In peptides **1** and **2** (Table 1) the terminal carboxylic group is transformed in alcohol or amide function(s). Nonamers **3** and **4** contain D-Val$^9$ or D-Met$^8$, respectively. The Met-Val peptide bond is replaced by a $CH_2$-NH link in pseudopeptide **5**, while the Nα-methyl Val$^9$ is present in the compound **6**. The peptoids[17] **7** and **8** contain achiral N-substituted glycines in positions 9 and 8, respectively: in these peptides the side chains of Val or Met are shifted from the α-carbon to the adjacent nitrogen. The nonapeptide **9** bears a Met$^8$-sulfoxide, and compound **10** is a glycopeptide in which a D-glucopyranosyl unit is β-O-glycosidically linked to the Thr$^7$ side chain. Compounds **1**−**10** were tested for metabolic stability in cell culture medium and in human plasma for their ability to bind HLA-A2 molecules, to produce stable HLA/peptide complexes, and for their capacity to stimulate CTL responses.

## Results and Discussion

**Synthesis.** YLA analogues **1**−**10** were synthesized by solid-phase methods, using an automated continuous-flow peptide synthesizer. The stepwise syntheses were carried out by Fmoc/tBu-chemistry. The Nα-Fmoc amino acids (4 equiv) were condensed using DIPCDI (4 equiv) and HOBt (4 equiv) as coupling agents for 1 h. Amide **1** was assembled on a Rink amide resin. Alcohol **2** was prepared by condensation of the N-terminal octapeptide with valinol which, in turn, was obtained by reduction of Nα-protected valine anhydride.[18] Nonamers **3**, **4**, **8**, **9**, and **10** were prepared using functionalized Wang resin with the C-terminal Nα-Fmoc protected amino acid. Met$\psi$[$CH_2$NH]Val containing pseudopeptide **5** was obtained by a mixed solution−solid-phase synthesis approach (Scheme 1). C-Terminal pseudodipeptide was obtained by reductive alkylation of the valine methyl ester with Boc-Met-CHO.[19] Nα-deprotected pseudopep-

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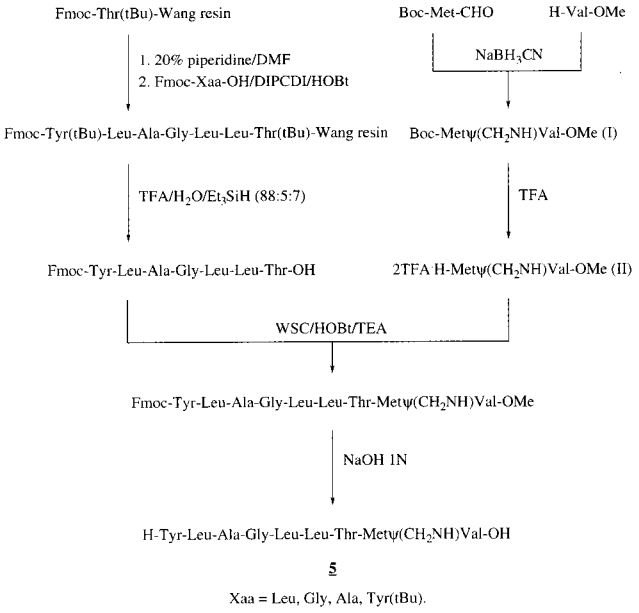
**Scheme 1.** Synthesis of Pseudononamer **5**




tide was then acylated with N-terminal eptapeptide. Sodium hydroxide treatment of partially protected pseudononapeptide gave compound **5**. Peptides **6** and **7** were prepared starting from Fmoc-MeVal-[20] or Fmoc-Nval-Wang resin obtained by the Sieber method known to proceed with low enantiomerization and dipeptide formation.[21] Peptoid monomers Fmoc-Nval-OH and Fmoc-Nmet-OH were prepared following the procedure described by Liskamp et al.[22] Peptide **9** was obtained as a diastereomeric mixture due to presence of methionine(*R,S*)-sulfoxide. Finally, glycopeptide **10** was prepared using the building block Fmoc-Thr[$\beta$-D-Glc(OAc)$_4$]-OH.[23] The $\beta$-glucosylated peptide was cleaved from the resin, and acetyl groups were removed from the carbohydrate unit with hydrazine in methanol. Protected peptides were cleaved from the resin by treatment with TFA/$H_2O$/$Et_3SiH$ (88:5:7, v/v) at room temperature for 1 h. Purification of YLA analogues **1**−**10** was achieved by preparative HPLC. Negligible epimerization was detected by HPLC analysis after peptide hydrolysis and amino acidic chiral derivatization by (+)-1-(9-fluorenyl)-ethyl chloroformate on the purified title nonamer **2** and on the purified Fmoc-nonapeptide ester that leads to compound **5** after basic treatment.

Structure verification was achieved by amino acid analysis, mass spectrometry, and NMR spectroscopy. Materials, the general procedure for solid-phase synthesis, peptide purification, and analytical determinations of all new compounds are given in the Supporting Information.

**Biological Results**. To evaluate the susceptibility to enzymatic hydrolysis, the analogues **1**−**10** were incubated at 37 °C in culture medium (RPMI) in the presence of 10% fetal calf serum (RPMI + 10% FCS) and in human plasma. Half-lives of peptides are reported in Table 1 in comparison with YLA. As expected all C-terminal modifications considerably increased the stability of peptides in cell culture medium and in human plasma.

We then evaluated the ability of all peptides to associate with HLA-A2. Peptide association was assessed by the induction of surface HLA-A2 expression in the T2 mutant cell line.[24] As shown in Figure 1, all peptides, except variants **2**, **3**, and **4**, bound to HLA-A2. The amide **1**, reduced peptide bond pseudopeptide **5**, and Nval peptoid **7** induced high levels of HLA-A2 molecules comparable to the parent YLA peptide. Me-Val[9] peptide **6**, Nmet peptoid **8**, sulfoxide **9**, and glycopeptide **10** showed slightly lower affinity than YLA.

All peptides were tested, in parallel experiments, for their ability to stably associate with HLA-A2 molecules expressed at the surface of T2 cells. The results of HLA-A2/peptide complexes' stability generally match the binding data: in fact, the low number of complexes induced by analogues **2**, **3**, and **4** were virtually disrupted after 24 h, whereas the other peptides induced complexes that were still detectable after 24 h. The trend of latter peptides is comparable to that obtained using the reference compound YLA. This demonstrates that modifications apported in peptide **1** and peptides **5**−**10** do not affect the binding characteristics of the reference peptide (Figure 2). Indeed, all binding peptides mantained the correct C-terminal primary anchor required for HLA-A2 association; moreover our results confirm that residues in postions 7 and 8 only partially interact with the HLA-A2 binding groove.[25]

The peptides that stably associated with HLA-A2 were 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 YLA derivatives, and CTL cultures were tested 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 3. Amide **1** reactivated CLG-specific responses with an increased efficiency in comparison with YLA. Analogues **5** and **10** induced responses as did the reference YLA peptide, variants **7** and **9** reactivated CTL responses as the natural CLG epitope, while peptides **6** and **8** did not stimulate CLG-specific CTL responses.

Peptides were then titrated on PHA blasts across a $10^{-4}$ to $10^{-12}$ M range of concentrations to determine the level of recognition by CLG-specific CTL cultures. None of the CLG analogues, except peptides **1** and **7**, were recognized by CLG-specific CTL (Supporting Information), confirming that the majority of variations apported at the CLG antigenic sequence can induce conformational changes in the peptide affecting target cell recognition and then abolish CTL-mediated lysis.[26]

## Conclusion

The LMP2-derived CLG epitope may be regarded as a target of specific immunotherapies for the treatment of EBV-associated tumors.[6] However, the feasibility of specific CTL therapy may be limited by the poor immunogenicity of this antigen. We previously found that YLA analogue showed satisfactory affinity for HLA-A2 molecules, potent stimulatory capacity,[26] but poor enzymatic stability in human plasma. Now, to maintain HLA-A2/peptide complex stability and immunogenicity and to improve enzymatic stability, we prepared and tested C-terminal modified YLA analogues. Peptide amide **1**, $CH_2$-NH containing pseudopeptide **5**, and

**Table 1.** Sequence, Analytical Data, and Metabolic Degradation in Cell Culture Medium and Human Plasma of CLG Analogues

| | compound | HPLC $K'$ | MS $(M + H)^+$ | half-life (min) culture medium | half-life (min) human plasma |
|---|---|---|---|---|---|
| YLA | H-Tyr-Leu-Ala-Gly-Leu-Leu-Thr-Met-Val-OH | 6.97 | 981.5 | 82.3 | 12.7 |
| **1** | H-Tyr-Leu-Ala-Gly-Leu-Leu-Thr-Met-Val-$NH_2$ | 7.09 | 980.5 | >360 | 39.4 |
| **2** | H-Tyr-Leu-Ala-Gly-Leu-Leu-Thr-Met-Val-ol | 6.43 | 953.4 | >360 | 99.3 |
| **3** | H-Tyr-Leu-Ala-Gly-Leu-Leu-Thr-Met-D-Val-OH | 7.21 | 981.5 | >360 | 272 |
| **4** | H-Tyr-Leu-Ala-Gly-Leu-Leu-Thr-D-Met-Val-OH | 7.17 | 981.5 | >360 | 35.8 |
| **5** | H-Tyr-Leu-Ala-Gly-Leu-Leu-Thr-Met$\psi$($CH_2NH$)Val-OH | 6.73 | 967.4 | >360 | 304 |
| **6** | H-Tyr-Leu-Ala-Gly-Leu-Leu-Thr-Met-MeVal-OH | 7.41 | 995.6 | >360 | >360 |
| **7** | H-Tyr-Leu-Ala-Gly-Leu-Leu-Thr-Met-Nval-OH | 6.99 | 981.5 | >360 | >360 |
| **8** | H-Tyr-Leu-Ala-Gly-Leu-Leu-Thr-Nmet-Val-OH | 6.96 | 981.5 | >360 | 51.6 |
| **9** | H-Tyr-Leu-Ala-Gly-Leu-Leu-Thr-MetO-Val-OH | 5.23 | 997.5 | 93.8 | 31.3 |
| **10** | H-Tyr-Leu-Ala-Gly-Leu-Leu-Thr($\beta$-D-Glc)-Met-Val-OH | 3.89 | 1144.7 | >360 | 60.8 |

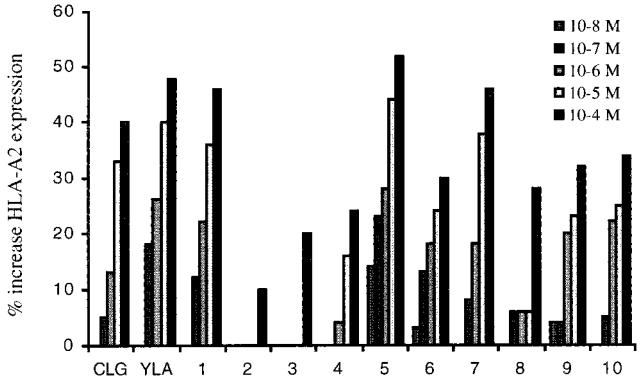



**Figure 1.** Induction of cell surface HLA-A2 molecules by peptides. T2 cells were treated at 26 °C for 18 h in serum-free medium in the presence of the indicated concentrations of peptides. Cells were then kept at 37 °C for 4 h and extensively washed to remove unbound peptides. After the cells were washed, surface expression of HLA-A2 molecules was detected by indirect immunofluorescence using the MA2.1 mAb. Data are expressed as the percent increase in HLA-A2 expression calculated with respect to that of untreated T2 cells. Results are the mean of four separate experiments.

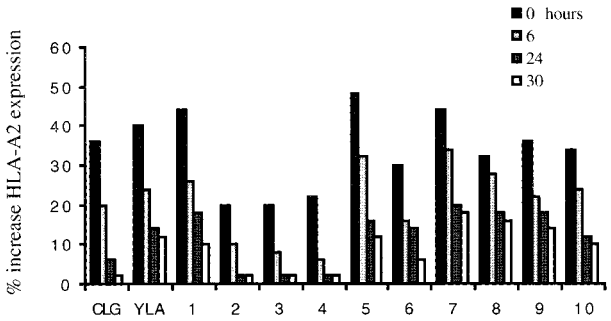



**Figure 2.** HLA-A2/peptide complex stability. T2 cells were treated as described in Experimental Section. At the indicated time points, the surface expression of HLA-A2 molecules was detected by indirect immunofluorescence using the MA2.1 mAb. Data are expressed as the percent increase in HLA-A2 expression calculated with respect to that of untreated T2 cells. Results are the mean of four separate experiments.

glycopeptide **10** retain affinity for HLA-A2, produce stable complexes, and induce strong 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. Interestingly, peptides **5** and **10** failed to sensitize PHA blasts to lysis by CLG-specific CTLs, indicating that these analogues cannot sufficiently mimic the natural

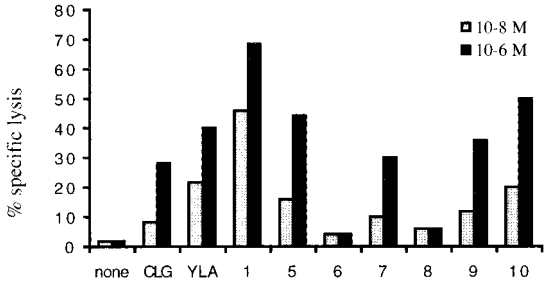



**Figure 3.** Activation of CLG-specific CTL responses by YLA analogues. Freshly isolated PBL derived from the EBV-seropositive donor FR (HLA-A2,24-B7,35) were stimulated with T2 cells pulsed with $10^{-6}$ M and $10^{-8}$ M of the indicated peptides. CTL cultures obtained after three consecutive stimulations were tested in cytotoxicity assays against HLA-A2 single-matched PHA blasts (none) or PHA blasts pulsed with $10^{-7}$ M CLG peptide for 1 h before the assay. The percent specific lysis recorded at an E:T ratio of 20:1 in one representative experiment is shown.

epitope on target cells to the point of inducing CLG-specific killing. Concerning the triggering of cytotoxicity, we have shown that CTL killing and reactivation of memory CTL precursors have different requirements.[7] The possibility of employing partial agonist peptides that selectively induce memory CTL reactivation without sensitizing cells to lysis may be relevant for the design of wild-type-derived synthetic epitopes suitable for specific immunotherapies, since it may increase the safety and efficacy of peptide-based immunotherapies.

## Experimental Section

**Metabolic Stability Assays**. The kinetics of peptide degradation were studied in culture medium (RPMI) or in heparinized human plasma. To 0.5 mL of culture medium containing 10% fetal calf serum or 0.5 mL of human plasma was added a solution (50 $\mu$L) of each peptide (5 mg in acetonitrile/$H_2O$ 1:1) and mixed in a vortex mixer. The solution was incubated at 37 °C for varying periods of time up to 360 min, and the incubation was terminated by addition of ethanol (1.0 mL). After centrifugation (5000 rpm for 5 min), an aliquot (20 $\mu$L) of the clear supernatant was analyzed by RP-HPLC. The degradation half-life ($T_{1/2}$) was obtained by a least-squares linear regression analysis of a plot of the logarithmic non-apeptide concentration versus time, using a minimum of five points.

**Cell Cultures.** Cell lines, peptide pulsed cells, and CTL cultures were prepared as previously described.[26,27]

**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 absence 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. Data are expressed as the percent increase in HLA-A2 expression calculated with respect to that of untreated T2 cells.

**Detection HLA-A2/Peptide Complex Stability**. Aliquots of $5 \times 10^6$ T2 cells were cultured overnight in 5 mL of serum-free AIM-V medium containing $10^{-4}$ M of the indicated peptide. Cells were then extensively washed, treated with mitomycin C (Sigma-Aldrich, Milan, Italy) to avoid cell proliferation, divided in aliquots in serum-free AIM-V medium (Life Technologies, Milan, Italy) containing 1 $\mu$g/mL brefeldin A (Sigma-Aldrich) to block the egress of new MHC class I molecules, and maintained at 37 °C for kinetic experiments. Surface expression of HLA class I molecules was detected at 0, 6, 24, and 30 h by indirect immunofluorescence using the mouse mAb MA2.1, which recognizes HLA-A2.[24] Mean logarithm fluorescence intensity was measured with a FACS analyzer (Becton Dickinson). Data are expressed as the percent increase in HLA-A2 expression calculated with respect to that of untreated T2 cells.

**Cytotoxicity Tests**. Cytotoxic activity was assayed in satandard 5 h $^{51}$Cr-release assay. LCL and PHA blasts were labeled with 0.1 $\mu$Ci/$10^6$ cells of $Na_2{}^{51}CrO_4$ (NEN, Brussels, Belgium) for 90 min at 37 °C. For the peptide sensitization assays, $4 \times 10^3$ PHA blasts were placed in triplicate in 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.[27] Peptide toxicities were checked in each assay and were always ≤3%. Percent specific lysis was calculated as 100 × (cpm sample − cpm medium)/(cpm Triton X-100 − cpm medium).

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**Supporting Information Available:** General experimental procedure for preparation, purification, and analysis of intermediates and CLG analogues and data of the peptide recognition by CLG-specific CTL cultures are available free of charge via the Internet at http://pubs.acs.org.

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