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Synthetic peptides derived from an N-terminal domain of the E2 protein of GB virus C in the study of GBV-C/HIV-1 co-infection

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Synthetic peptides derived from GB virus C (GBV-C) have previously been studied in our group for the development of new systems capable of diagnosing diseases caused by this humanotropic virus. We also recently described specific peptide domains of the E2 envelop protein of GBV-C that have the capacity to interfere with the HIV-1 fusion peptide, produce a notable decrease in cellular membrane fusion, and perturb HIV-1 infectivity in a dose-dependent manner.

The present work discloses the design and synthesis of both linear and cyclic branched peptides based on a previously reported N-terminal sequence of the GBV-C E2 protein. Immunoassays and cell–cell fusion assays were performed to evaluate their diagnostic value to detect anti-GBV-C antibodies in HIV-1 patients, as well as their putative anti-HIV-1 activity as entry inhibitors.

Our results showed that chemical modifications of the selected E2(7–26) linear peptide to afford cyclic architecture do not result in an enhanced inhibition of gp41 HIV-1-mediated cell–cell fusion nor improved sensitivity in the detection of GBV-C antibodies in HIV-1 co-infected patients. Thus, the ELISA data reinforce the potential utility of linear versions of the E2(7–26) region for the development of new peptide-based immunosensor devices for the detection of anti-GBV-C antibodies in HIV-1 co-infected patients. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: GB virus C; E2 protein; peptide synthesis; multiple antigenic peptide; immunoassays; cell–cell fusion assay

Introduction

The recent development of improved synthetic, purification, and analytical methodologies have led to the broad application of MAPs in biomedical research. Among others, Tam and colleagues pioneered the design of branched peptide constructs based on polylysine scaffolds, and the group has extensively reported various synthetic methodologies to MAPs [1].

Although different types of branched peptide macromolecules have been reported, the constrained peptide dendrimers have been particularly investigated in an effort to generate molecules that closely mimic native protein conformations. The structure-stabilized cyclic peptides, which are predicted to adopt conformational features of the original antigenic sites of the native protein, can be presented in a branched scaffold. In fact, various constrained cyclic peptide dendrimers have been synthesized and investigated for their biological activities. For example, solid-phase and solution synthetic strategies were utilized to prepare a multivalent lipopeptide dendrimer containing four copies of the disulfide-mediated cyclic epitopes of foot and mouth disease virus [2]. Furthermore, different chemical approaches were applied for the presentation of cyclic RGD peptides on dendrimeric scaffolds as potential tools for tumor targeting. Thus, cyclic RGD peptides were synthesized by solid-phase strategy followed by chemoselective oxime ligation [3] to afford multimeric constructs. Similar dendrimeric constructs in which the cyclic RGD peptides were appended by Cu(I)-catalyzed 'click' chemistry have also been reported [4,5].

As indicated in the preceding examples, the preparation of the cyclic peptide dendrimers is synthetically challenging because direct synthesis of cyclic peptide on a branched scaffold is complicated by competing interchain and intrachain cyclization. In a branched MAP, these competing reactions could not be minimized by using high dilution. Moreover, the convergent assembly of a preformed cyclic peptide to a MAP core has the limitation of the insolubility of the protected precursors. Consequently, alternative approaches have been developed that exploit the conjugation of unprotected precursors to appropriately functionalized dendrimeric scaffolds [6]. One of these approaches entailed the formation of cyclic peptides by a modified native chemical ligation strategy introduced by Kent [7], which is based on an intramolecular *S,N*-acyl transfer resulting in the formation of a peptide bond at the ligation site. The cyclic peptide with a Cys residue at the cyclo-ligation site was then used to functionalize a pre-prepared branched MAP sub-unit. Here, chemoselective functionalization was achieved

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using the unique chemistry of the Cys sulfhydryl side chain and an appropriately modified branched MAP sub-unit. Hence, fully functional MAPs were obtained upon the conjugation of pre-synthesized cyclic peptide monomers to functionalized branched sub-units [8].

Over the past two decades, many applications of MAPs in different research fields, such as molecular biology, immunology, biochemistry, and therapeutic research, have been described [9]. In particular, the usefulness of MAPs as immunoassay reagents for serodiagnosis [10], radiolabeled molecules for imaging diagnosis [11], immunostimulatory reagents for antibody production in vaccines [12], and therapeutic agents [13,14] has been extensively reported.

In our group, synthetic peptides derived from both the structural and non-structural proteins of GB virus C (GBV-C; also known as hepatitis G virus) have been studied for the development of new systems to diagnose infections caused by the virus. We have investigated the ability of the synthetic peptides to recognize anti-GBV-C antibodies in HIV-1 and HCV/HIV-1 co-infected patients in order to secure a better understanding of the effect of exposure to GBV-C on the progression of illness caused by HIV-1 infection, as well as the putative role as a prognostic marker in the context of other viral infections. The usefulness of synthetic tetrameric branched peptides containing sequences from both envelope and non-structural GBV-C proteins for the diagnosis of GBV-C infection has been reported, demonstrating the potential clinical value of these heterogeneous branched molecules to perform prevalence studies of the infections among the hemodialyzed HCV-infected and HIV-1-infected population [15,16]. The presence of antibodies against chimeric branched peptides formed by two domains of different GBV-C proteins could represent a good biomarker of exposure to GBV-C in HIV-1 patients.

On the other hand, dendrimers can act as polyvalent viral inhibitors by presenting multiple contact sites on a single molecule, which could efficiently prevent or disrupt the molecular interactions that mediate the entry of viruses into host cells [14]. In this context, the design of powerful HIV-1 entry inhibitors through the multimerization of D-peptides containing a disulfide bridge has been described [17].

We have also previously identified an N-terminal portion of the E2 protein of GBV-C that is most likely exposed on the virion surface by means of biophysical studies [18,19]. In addition, we have recently described specific domains of the envelop E2 GBV-C protein that interfered with the HIV-1 fusion peptide vesicle interaction, produced a notable decrease of the cellular membrane fusion, and interfered with the HIV-1 infectivity in a dose-dependent manner [20].

Bearing in mind the aforementioned results, we herein report the design and synthesis of both linear and cyclic branched peptides based on the previously reported N-terminal sequence of the GBV-C E2 protein. Immunoassays and cell–cell fusion assays were performed to evaluate their diagnostic value to detect anti-GBV-C antibodies in HIV-1 patients and their putative anti-HIV-1 activity as entry inhibitors, respectively.

Materials and Methods

Reagents

Fmoc-protected amino acids, NovaSyn®TGR, and 2-chlorotrityl resin were purchased from Novabiochem (Läufelfingen, Switzerland). Peptide-synthesis-grade *N*,*N*-DMF and TFA were obtained from Scharlau (Barcelona, Spain). HPLC-grade acetonitrile diethyl ether and DCM were from Merck (NJ, USA). HATU was from Genscript (Piscataway, USA). Other coupling activators such as HOBt, DIPCI, and DIEA, as well as the scavengers, triisopropylsilane (TIS), and 1,2-ethanedithiol, were from Fluka-Sigma-Aldrich (St. Louis, USA). Methyl 3-mercaptopropionate and tris-(carboxyethyl)phosphine (TCEP) were obtained from Calbiochem, EMD Chemicals Inc. (New Jersey, USA), and Alfa Aesar (Massachusetts, USA), respectively.

Synthesis of Linear Tetrameric MAPs

Linear tetrameric MAPs were synthesized manually as described by Tam [21]. Amino acid side-chain protection was effected by the following: Trt for glutamine and asparagine; *tert*-butyl for glutamic acid, serine, and threonine; Pmc for arginine; and Boc for tryptophan. The Cys residues were replaced by Abu residues.

The first amino acid coupled to the Rink amide resin (NovaSyn®TGR, 0.1 mmol) was β-Ala. The tetravalent lysine core was obtained by sequential coupling of 0.4 and 0.8 mmol of Fmoc-Lys(Fmoc)-OH activated using HATU and DIEA. The assembly of the peptide sequences E2(17–26), E2(11–26), and E2(7–26) was then accomplished at both Nα-lysine and Nε-lysine positions. An eightfold molar excess of Fmoc-amino acids, HATU/DIEA (1 : 2), was used throughout the synthesis. The efficiency of these reactions was evaluated by Kaiser's (ninhydrin) test, and repeated couplings were carried out when a positive ninhydrin test was observed.

The linear tetrameric MAPs were concomitantly side-chain-deprotected and cleaved from the resin by treatment with TFA in the presence of TIS and water as scavengers (TFA : TIS : H_2O, 9.5 : 2.5 : 2.5) for 3 h with occasional agitation at room temperature. The volatile reagents were removed *in vacuo*, and the crude peptides were precipitated with diethyl ether. The residual solids were dissolved in 30% acetic acid in water and lyophilized.

Linear MAPs were desalted using Oasis HLB Plus cartridge 225 mg/60 μg from Waters. Yields of post SPPS reactions were 58%, 68%, and 72% for MAP_{LP1}, MAP_{LP2}, and MAP_{LP3}, respectively.

Purification of the desalted crude molecules was achieved by semi-preparative HPLC and characterization by analytical-scale HPLC and MS (ES-MS or MALDI-TOF). Final yields after HPLC purification were 5%, 10%, and 14% for MAP_{LP1}, MAP_{LP2}, and MAP_{LP3}, respectively.

Synthesis of Cyclic Tetrameric MAPs

Synthesis of a tetra-chloroacetylated branched Lys core

A tetravalent (chloroacetyl)lysinyl core peptide was obtained on a Rink amide resin (NovaSyn®TGR) of functionalization 0.29 mmol/g, by sequential coupling of 0.4 mmol of Fmoc-Lys(Fmoc)-OH and 0.8 mmol of Fmoc-Lys(Fmoc)-OH, which were incorporated through HATU : DIEA-mediated (1 : 2) carboxyl activation. After Fmoc deprotection, the tetravalent chloroacetyl moieties were introduced to the core peptide by using a fourfold excess of chloroacetic acid *via* HATU : DIEA (1 : 2) activation. The chloroacetylation reaction was repeated. The tetravalent (chloroacetyl)lysyl core peptide was cleaved from the resin by treatment of TFA/TIS/H_2O (95/2.5/2.5) for 2 h. The core peptide was characterized by analytical HPLC and ES-MS (observed mass = 778.3, calculated mass = 778.5). The yield of post SPPS was 78%.

Synthesis and cyclization of E2(7–26) and shorter analogs

The protected peptides were synthesized on a 2-chlorotrityl resin (200 mg, 1.3 mmol/g), by solid phase and following a

9-fluorenylmethoxycarbonyl (Fmoc) strategy. The resin was left in 6 ml of dry DCM containing 0.1% DIEA for 15 min.

To obtain a lower functionalization of the resin, 0.13 mmol (0.5 eq relative to the resin) of Fmoc-Gly-OH and 0.52 mmol (4 eq relative to the carboxylic acid) of DIEA were dissolved in dry DCM and added to the above-described amount of resin. The mixture was stirred for 1 h. At the end of this time, the resin was washed with 10 ml 3 × DCM/MeOH/DIEA (17 : 2 : 1), 3 × 10 ml DCM, 2 × 10 ml DMF, and 2 × 10 ml DCM and dried *in vacuo* over KOH pellets for 24 h at room temperature. A Cys residue, protected with both Boc and Trt at Nα and thiol group, respectively, was incorporated at the N-terminus of the peptide sequence to enable the 'head-to-tail' cyclization of the E2(7–26) peptide.

The couplings were performed by activating the reagent with HATU and DIEA. The efficiency of these reactions was evaluated by the ninhydrin colorimetric reaction, trinitrobenzene sulfonate, or chloranil tests when required.

The cleavage of the protected peptides from the 2-chlorotrityl resin was carried out by treatment of 1% TFA in dry DCM for 20 min. The solution was filtered into a flask containing 10% pyridine in methanol (MeOH). The resin material was then washed with 3 × 3 ml DCM, 3 × 3 ml MeOH, 2 × 3 ml DCM, and 3 × 3 ml MeOH. The filtrate was evaporated under reduced pressure. Water was added to the residual material, and the mixture was cooled with ice to aid precipitation of the product, which was isolated by filtration through a sintered glass funnel. The product was dried in a desiccator under vacuum over KOH. All peptides were characterized by ultra-performance liquid chromatography (UPLC)–ES-MS. The yields of post-SPPS were 69%, 73%, and 76% for LP1, LP2, and LP3, respectively.

Subsequently, coupling of the protected peptides with methyl 3-mercaptopropionate (20 eq) was then conducted in DMF using a water-soluble carbodiimide (15 eq) and HOBt (15 eq) at 4 °C for 24 h [22]. Treatment of the protected thioester segment with 95% TFA, 2.5% TIS, and 2.5% H_2O at room temperature for 3 h and subsequent isolation by precipitation with cold diethyl ether, centrifugation, and lyophilization in 10% acetic acid afforded the desired thioester peptide. The yields of thioester peptides were between 46% and 48%.

The linear *N*-cysteinyl thioester peptides at a concentration of 1 mg/ml were dissolved in 0.2 M Na_2HPO_4, 0.1 M citric acid buffer (pH 7.5) containing TCEP (2 mg, 6 μmol, 2 eq), and $HSCH_2CH_2COOCH_3$ (2 μl, 15 μmol, 5 eq). The cyclization reactions were followed by HPLC and judged to be completed after 24 h. The yields of cyclizations were 72%, 88%, and 98% for CP1, CP2, and CP3, respectively.

Thioether ligation

To the chloroacetylated oligolysine core (0.4 mg, 0.5 μmol) dissolved in 0.1 M Tris–HCl buffer, pH 8.2, small portions in solid form of the cyclic peptide, CP1: cyc-HT-E2(7–26), CP2: cyc-HT-E2 (11–26), or CP3: cyc-HT-E2(17–26), were added to the solution from time to time in order to obtain the cyclic tetrameric MAPs by means of a thioether ligation. In all cases, the thioether ligation reactions were monitored by HPLC and allowed to proceed until no changes in the HPLC profile were observed. The reactions were terminated by acidification. Fractions representative of different degrees of peptide incorporation were isolated by semi-preparative HPLC and characterized by analytical HPLC and ES-MS and MALDI-TOF spectrometry.

Chromatography

Analytical HPLC was performed on Kromasil C_{18} or Kromasil C_8 (Teknokroma, 4.6 × 250 mm, 5 μm particle size) and Poroshell 300-SB C_{18} (Agilent Technologies, 2.1 × 75 mm, 5 μm particle size) reverse-phase columns on Perkin Elmer or Agilent 1260 Infinity systems. Linear gradients of 0.05% TFA in acetonitrile (solvent B) into 0.05% TFA in water (solvent A) over 20–30 min at 1 ml/min flow rate, with UV detection at 220 nm, were used for the separation.

Analytical UPLC was performed on ACQUITY UPLC BEH C_{18} (Waters, 2.1 × 100 mm, 1.7 μm particle size) or UHPLC Zorbax SB C_{18} (Agilent Technologies, 2.1 × 150 mm, 1.8 μm particle size) reverse-phase columns on a Waters ACQUITY UPLC system. Solvent A was 0.05% formic acid in water, and solvent B was 0.05% formic acid in acetonitrile. Elution was done with linear gradients of solvent B into A over 10 min at 0.3 ml/min. Both variable wavelength UV detector and ES-MS were connected to the UPLC for the peptide characterization.

Semi-preparative HPLC was performed on Kromasil C_{18} and Kromasil C_8 (Teknokroma, 5 μm, 22 × 250 mm, 5 μm) in a Waters Delta Prep 4000 system or XBridge BEH300 prep C_{18} (Waters, 10 × 250 mm, 5 μm particle size) in an Agilent 1260 Infinity system. Linear gradients were performed at the same conditions as that of analytical HPLC but at a flow rate of 3.5–4 ml/min. Semi-preparative fractions characterized by analytical HPLC were pooled and lyophilized.

Mass Spectrometry

Electrospray ionization mass spectrometry was performed with a liquid chromatograph-TOF detector, LCT Premier XE (Micromass Waters), coupled to the UPLC (Waters). Samples were dissolved in a mixture of acetonitrile/water (1 : 1, v/v) and analyzed previously at the UPLC with a flow rate of 0.3 ml/min. Mass spectra were recorded in positive ion mode in the *m/z* 500–2500 range.

MALDI-TOF mass spectra were recorded in an Autoflex III Smartbeam (Bruker Daltonics), using either sinapinic or α-hydroxycinnamic acid matrixes on an MTP 384 target plate (Bruker). MS spectra were in linear positive mode.

Enzyme Linked Immunosorbent Assay

Two different panels of serum samples were analyzed. The first panel consisted of 45 sera from HIV-infected patients from the Hospital of Bellvitge of Barcelona. The second panel corresponded to 45 control sera from volunteer blood donors from the Hospital Clinic of Barcelona.

Peptides were coupled to assay plates via their amine terminal groups and/or by means of the non-covalent adsorption of the molecules to the surface (preactivated *N*-oxysuccinimidyl active ester plates, Costar Corp., DNA Bind) at 1 μg per well. Nunc Immobilizer amino plates were also used to bind CP1, CP2, and CP3 cyclic peptides by their thiol group. Coupling was performed at 4 °C overnight in 0.05 M carbonate/bicarbonate buffer pH 9.6.

Each plate contained control wells that included all reagents except the serum sample in order to estimate the background reading and control wells that included all reagents except the peptide to evaluate non-specific reactions of sera. For blank controls, wells were coupled with 2 μg of BSA per well. After incubation, the plates were blocked with 2% BSA in 0.05 M carbonate/bicarbonate (pH 9.6) buffer for 1 h at room temperature. Sera were diluted 50-fold in RIA buffer (1% BSA, 350 mM NaCl, 10 mM Tris–HCl, pH 7.6, 1% Triton X-100, 0.5% sodium

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deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with 10% fetal bovine serum. An amount of 100 μl was added to each well, and the mixture was incubated for 1.5 h at room temperature. After the mixture was washed six times with phosphate-buffered saline/0.05% Tween-20, 100 μl per well of antihuman IgG conjugated to peroxidase diluted 1 : 1000 in RIA buffer was added. After incubation for 1 h at room temperature, the plates were washed six times with phosphate-buffered saline/0.05% Tween-20, and bound antibodies were detected with *o*-phenylenediamine dihydrochloride (Sigma Chemical Company) and 8 μl/10 ml 30% hydrogen peroxide. The plates were incubated at room temperature for 30 min. The reaction was stopped with 50 μl of 2 M aqueous H_2SO_4 per well, and absorbance values were measured at a wavelength of 492 nm. All sera were tested in duplicate. Control sera were also included to monitor inter-assay and intra-assay variations. The cutoff was calculated for each peptide from negative control sera (mean + 2 standard deviations). Statistical analysis was conducted using the GraphPad Prism 5 program.

This study was approved by the ethics committee of the hospitals.

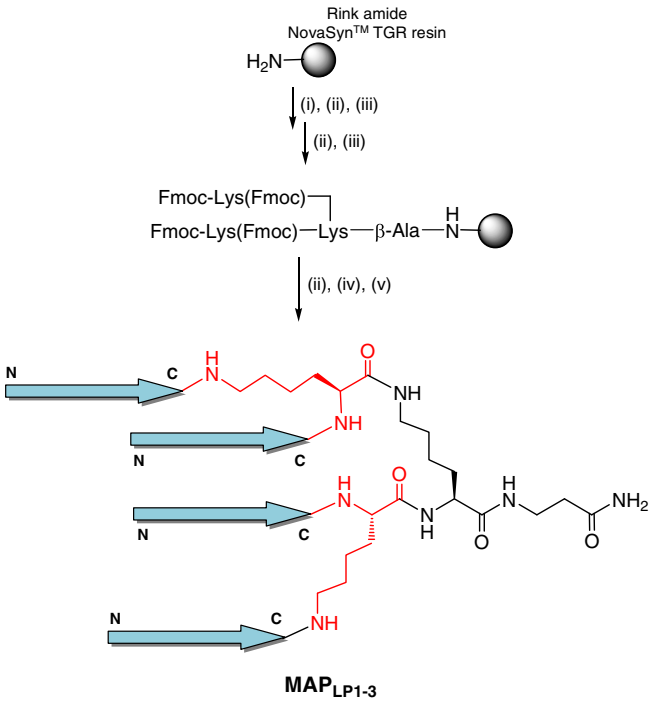

Figure 1. Synthesis of linear MAPs. Reagents: (i) Fmoc-*β*Ala-OH/HATU/DIEA, DMF; (ii) 20% piperidine/DMF; (iii) Fmoc-Lys(Fmoc)-OH/HATU/DIEA, DMF; (iv) SPPS; (v) TFA/H_2O/iPr_3SiH (95/2.5/2.5).

Inhibition of Cell Binding Assay

Two cell lines were used: HeLa-env (donated by Dr Blanco from Fundació IRSI Caixa), which indicates the protein from the HIV-1 envelope and includes the HIV-1 LTR promoter in its genome, and TZM-bl (AIDS reagents Cat. No 8129), which expresses the membrane receptor from CD4 lymphocytes and co-receptors CCR5 and CXCR4 and includes the luciferase and *β*-galactosidase genes in its genome.

In short, the trial on the inhibition of cell binding induced by GBV-C peptides consists of the incubation of ~2500 HeLa-env cells per well (Cultek cat. plates no. 15–3596) for 1 h with increasing concentrations (up to 200 μM) of the peptides to be tested, followed by the addition of around ten times (~25 000 cells) TZM-bl and incubation for 24 h.

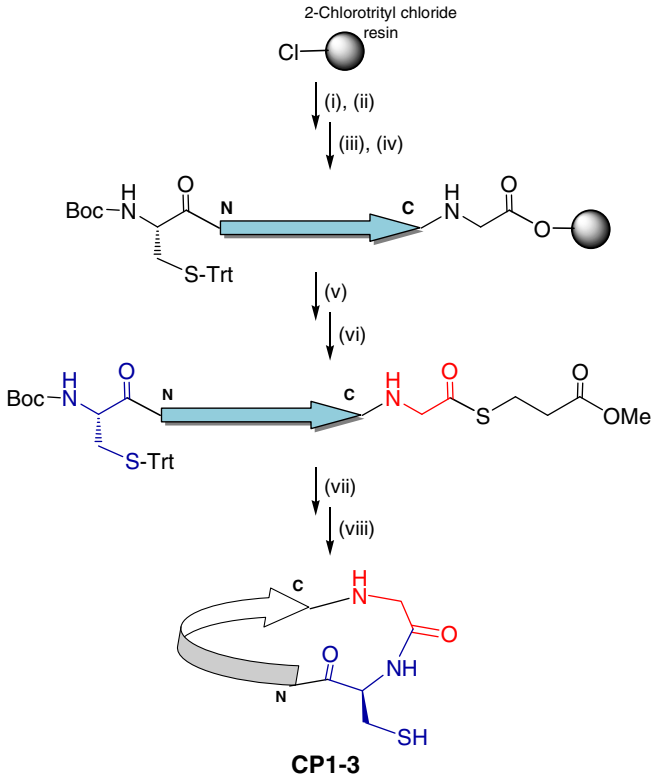

Figure 2. Synthesis and cyclization of linear peptides. Reagents and conditions: (i) Fmoc-Gly-OH/DIEA, CH_2Cl_2; (ii) 20% piperidine/DMF; (iii) SPPS; (iv) Boc-Cys(Trt)-OH,/HATU/DIEA, DMF; (v) 1% TFA/ CH_2Cl_2; (vi) $HS(CH_2)_2CO_2CH_3$, water-soluble carbodiimide/HCl, HOBt, 4 °C, 24 h; (vii) TFA/H_2O/iPr_3SiH (95/2.5/2.5); (viii) 0.2 M aq. phosphate buffer pH 7.5, TCEP, $HS(CH_2)_2CO_2CH_3$.

Table 1. Characterization of linear tetrameric MAPs

Name	Primary sequence	HPLC (*k'*)[a]	Calc. mass	Exp. mass[b]
MAP_{LP1}	GSRPFEPGLTWQS[Abu]S[Abu]RANG	2.3	8869.7	8869.9
MAP_{LP2}	FEPGLTWQS[Abu]S[Abu]RANG	2.4	7280.0	7280.2
MAP_{LP3}	WQS[Abu]S[Abu]RANG	1.8	4701.1	4701.1

[a]Kromasil C_{18} column (5 μm, 25 × 0.46 cm). Eluents: (A) 0.05% TFA in water; (B) 0.05% TFA in acetonitrile. Flow rate: 1 ml/min, detection at 215 and 280 nm. Gradient elution: 95% (A) to 5% (A) in 30 min.
[b]Experimental mass obtained by ES-MS

To control cell binding, wells without peptides were reserved, and a known cell binding inhibitor used, C-34 (AIDS reagents cat. no. 9824), as a positive control. The level of inhibition of cell binding was qualitatively assessed by observing the formation of syncytia under the microscope. The quantitative values were obtained by measuring the β-galactosidase activity. In the fluorimetric assay performed, 20% 5 µl Igepal in a solution of 100 mM phosphate buffer pH 7.2 and 0.1 mM $MgCl_2$ were added to each well, and the contents were mixed. β-Galactosidase activity was quantified at 37 °C in a 96-well plate by mixing 50 µl of each lysate and 50 µl of 4-methylumbellipheryl-β-D-galactopiranoside (1 mM in phosphate buffer 100 mM, pH 7.2, 0.1 mM $MgCl_2$) and incubating for 30 min. Enzymatic reaction was stopped by the addition of 150 µl glycine-NaOH buffer (100 mM, pH 10.6). The amount of 4-methylumbelliferone formed was determined using a SpectraMax M5 (Molecular devices) fluorimeter at 355 nm (excitation) and 460 nm (emission).

Results and Discussion

Different forms of presentation (linear, cyclic, and branched peptides) of an N-terminal peptide domain of the envelop E2 protein of GBV-C have been synthesized by solid-phase methodologies and evaluated regarding its diagnostic value to detect anti-GBV-C antibodies in HIV patients, as well as for their putative anti-HIV-1 activity as entry inhibitors.

Three antigenic overlapping peptides belonging to the E2 region, namely LP1: E2(7–26), LP2: E2(11–26), and LP3: E2(17–26), have been selected regarding the length of their primary sequence. The Cys residues in positions 20 and 22 were replaced by Abu residues because this amino acid has the same hydrophobic and steric properties as Cys but avoids the reactivity of the Cys thiol group without modifying the immunochemical properties of the parent peptide [23].

As described earlier, peptide dendrimers have many applications in different research fields and are potential new

Table 2. Characterization of linear and cyclic peptides

Name	Primary sequence	HPLC (k')[b]	Calc. mass	Exp. mass[c]
LP1	(C)GSRPFEPGLTWQS[Abu]S[Abu]RANG	3.5	2116.0	2116.3
CP1[a]		3.6	2201.0	2201.1
LP2	(C)FEPGLTWQS[Abu]S[Abu]RANG	3.2	1719.8	1719.6
CP2[a]		3.3	1805.0	1804.8
LP3	(C)WQS[Abu]S[Abu]RANG	2.9	1074.5	1074.8
CP3[a]		3.1	1159.5	1159.6

[a]cyc-HT: cyclic head-to-tail *N*-cysteinyl peptide. The N-terminal Cys residue is only present in CP peptides.
[b]Kromasil C_{18} column (5 µm, 25 × 0.46 cm). Eluents: (A) 0.05% TFA in water; (B) 0.05% TFA in acetonitrile. Flow rate: 1 ml/min, detection at 215 and 280 nm. Gradient elution: 95% (A) to 5% (A) in 30 min.
[c]Experimental mass obtained by ES-MS.

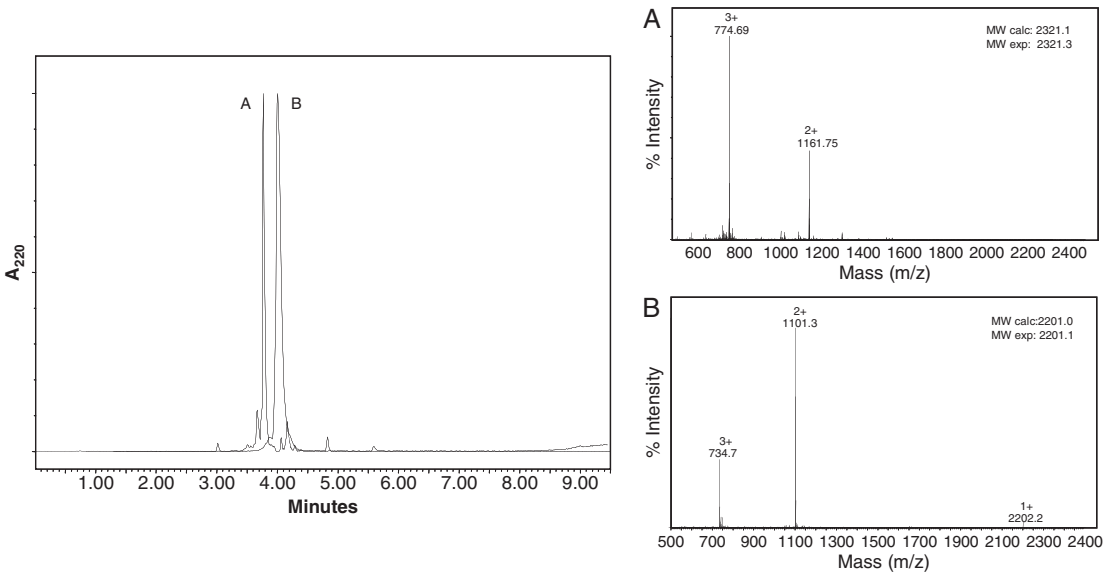

Figure 3. Progress of the cyclization reaction of LP1 monitored by UPLC/ES-MS at 0 and 24 h reaction time. UPLC conditions: 5–95% linear gradient of eluent B into A over 10 min at a flow rate of 0.3 ml/min. UPLC C_{18} column. Solvent A was 0.05% HCOOH in water, and solvent B was 0.05% HCOOH in acetonitrile. (A) *N*-cysteinyl LP1 thioester, calculated mass: 2321.1, experimental mass: 2321.3. (B) CP1, calculated mass: 2201.0, experimental mass: 2201.1.

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therapeutics. Among the several advantages of dendrimers, their ability to display multiple copies of epitopes required for biological recognition processes should be highlighted. In an attempt to improve both the antibody recognition and the anti-HIV-1 fusion activity of the selected E2 GBV-C protein N-terminal domain, different peptide dendrimers based on a polylysine scaffold were designed for the presentation of both linear and cyclic forms of this peptide region.

Firstly, a linear homogeneous tetrameric MAP of each overlapped peptide (MAP_{LP1}, MAP_{LP2}, and MAP_{LP3}) has been successfully synthesized. Their synthetic scheme is illustrated in Figure 1. Although some minor synthetic and analytical difficulties were encountered (e.g., repeated couplings were necessary and complex HPLC profiles of crude samples), the three tetrameric MAPs were obtained. Purification of the crude molecules was achieved by semi-preparative HPLC. HPLC chromatograms and MALDI-TOF and ES-MS spectra of purified tetrameric MAPs are shown in Figure S1 of the Supporting Information. Characterization of the final products by MALDI-TOF and ES-MS spectrometry confirmed the expected molecular mass of the linear tetrameric MAPs (Table 1).

In order to obtain cyclic tetrameric MAPs comprising the same N-terminal domain of E2 GBV-C protein, the synthesis of a tetra-chloroacetylated branched Lys core was firstly accomplished by solid-phase peptide chemistry. The core was appropriately characterized by analytical-scale HPLC and ES-MS (Figure S2 of Supporting Information).

The synthesis of cyclic E2 peptides bearing a free thiol for subsequent conjugation onto the chloroacetylated core was pursued. To this end, the three different linear E2 peptides were again synthesized in solid phase, and peptide cyclization was carried out by intramolecular native chemical ligation. As described earlier, this regiospecific method is based on an intramolecular transesterification reaction between a thioester group and the sulfhydryl group of a Cys residue specifically introduced in the N-terminal position of the peptide sequence. The synthetic strategy is outlined in Figure 2. As illustrated, a modular approach for the chemical macrocyclization of the E2 linear peptides, which would subsequently deliver scaffolds bearing reactive sulfhydryl functionality, was followed. This approach was based on a chemoselective head-to-tail cyclization using unprotected (native) *N*-cysteinyl peptide thioester *via* a proximity-driven S-to-N acyl transfer pathway.

The synthesis of the thioester at C-terminal position using Fmoc chemistry is considered to be troublesome because of their poor stability to strong nucleophiles such as piperidine. To overcome this limitation, an approach usually described in the literature consists of the synthesis in solution using partially protected peptides [24]. Because the major drawback of this method is the likely epimerization of a C-terminal residue during the thioesterification reaction, a Gly residue was incorporated in the C-terminus to overcome this limitation [25]. Thus, the desired linear peptides (listed in Table 2) were assembled on *o*-chlorotrityl resin using standard Fmoc/t-Bu chemistry, and for analytical purposes, samples of totally deprotected peptides were obtained and characterized by UPLC and ES-MS (Table 2).

Following cleavage of the partially protected peptides using mild conditions, thioester formation at the C-terminus was accomplished in organic solution. Upon removal of all protecting groups, the crude cysteinyl peptide thioesters were found to be sufficiently pure and were therefore used directly for intramolecular macrocyclization.

Efficient macrocyclization was ensured by the presence of TCEP to inhibit disulfide bond formation and a thiol auxiliary reagent [8]. As an example, the progress of the LP1 cyclization process, after 24 h, monitored by analytical HPLC is shown in Figure 3. The mass identification of each chromatographic peak is also shown. It has been reported by others that this cyclization occurs cleanly and efficiently in yields ranging from 75% to 82% for peptides of 14 and 16 residues [8]. In our hands, the cyclization reaction of the three E2 peptides proceeded also cleanly because oligomerization of the thioester peptide was not detected in any case; the yields of CP1: cyc-HT-E2(7–26), CP2: cyc-HT-E2(11–26), and CP3: cyc-HT-E2(17–26) were almost quantitative by HPLC. Characterization of purified cyclic peptides by analytical HPLC and ES-MS is shown in Figure S3 of the Supporting Information.

Finally, the assembly of each cyclic E2 peptide to the previously obtained tetra-chloroacetylated polylysine MAP subunit was performed in solution as illustrated in Figure 4.

The ligation reactions of the largest CP1 and medium-size CP2 cyclic E2 peptides, 21-mer and 17-mer, respectively, to the

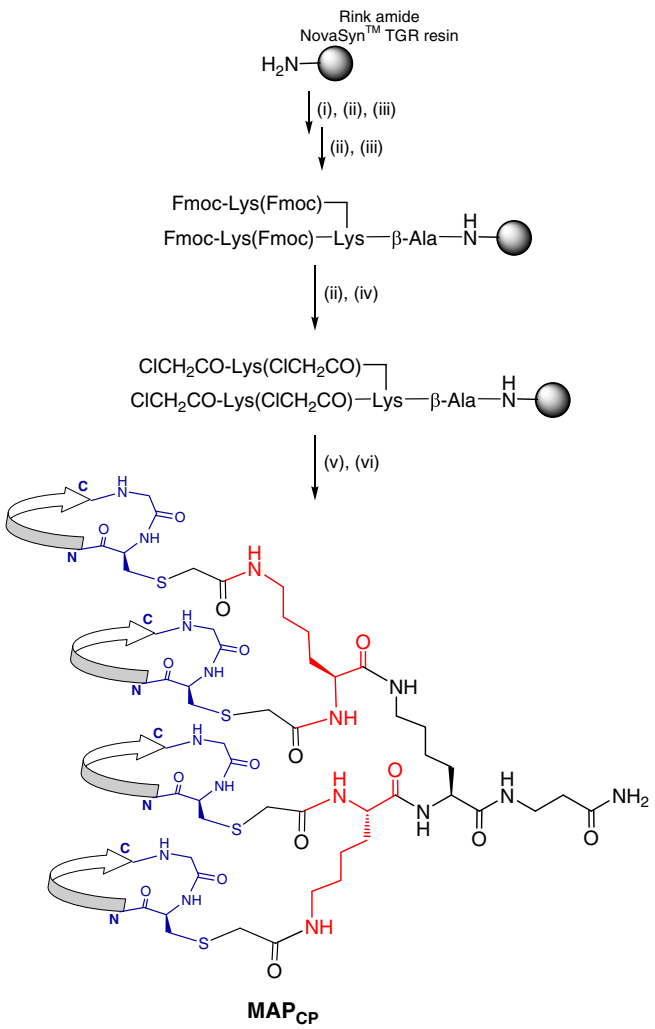

Figure 4. Synthesis of cyclic MAPs. Reagents and conditions: (i) Fmoc-βAla-OH/HATU/DIEA, DMF; (ii) 20% piperidine/DMF; (iii) Fmoc-Lys(Fmoc)-OH/HATU/DIEA, DMF; (iv) $ClCH_2CO_2H$/DIPCI/HOBt, DMF; (v) TFA/H_2O/iPr_3SiH (95/2.5/2.5); (vi) cyclic peptide added portionwise, 0.1 M Tris–HCl buffer pH 8.2.

tetra-chloroacetylated core were allowed to proceed for 48 h. In both cases, UPLC analysis showed that the expected final products were not obtained. MALDI-TOF or ES-MS analysis of the different fractions collected by UPLC did not show the presence of the expected cyclic tetrameric MAPs (Table 3). Nevertheless, we conclusively identified products showing the incorporation of one or two copies of each cycle (CP1 or CP2) into the derivatized oligolysine sub-unit by MALDI-TOF (Figure S4, Supporting Information). MALDI-TOF analysis showed some differences between theoretical $[M+H^+]$ and observed masses. Because spectra were acquired in the linear TOF mode, we considered such differences to be within expectations.

Table 3. MALDI-TOF MS characterization of cyclic MAPs

Name		Mass calc.	Mass found
MAP_{CP1}	MAP_{CP1-1}	2944.50	2947.64
	MAP_{CP1-2}	5110.45	5118.59
	MAP_{CP1-3}	7276.40	—
	MAP_{CP1-4}	9442.34	—
MAP_{CP2}	MAP_{CP2-1}	2547.07	2547.94
	MAP_{CP2-2}	4315.58	4307.07
	MAP_{CP2-3}	6084.11	—
	MAP_{CP2-4}	7852.62	—
MAP_{CP3}	MAP_{CP3-1}	1903.34	1903.12
	MAP_{CP3-2}	3027.14	3027.93
	MAP_{CP3-3}	4150.94	4150.97
	MAP_{CP3-4}	5274.42	5276.69

In addition, the thioether ligation of CP3, the smaller cyclic E2 peptide comprised of 11 residues, was also sluggish, and a complex crude product was detected by UPLC. As shown in Figure 5, we could identify a set of peaks that were assigned to the MAPs containing different copies of CP3. It is worth nothing that, in this case, we were able to identify by MALDI-TOF and ES-MS the tetrameric MAP composed of four copies of the cyc-HT-Cys E2(17–26). Unfortunately, the complex crude product obtained did not enable a good isolation of the target product with four copies of CP3 in a desirable amount and purity to be used in biological assays. The difficulties found could be related with steric effects and have previously been reported by others [2,26,27].

Diagnostic value of antibodies against synthetic peptides derived from the N-terminal domain of E2 GBV-C. We investigated the ability of the E2(7–26)-derived peptides, as described earlier, to interact with anti-GBV-C antibodies in HIV-infected patients. This work follows our previous research using chimeric molecules formed by two domains of different GBV-C proteins, in which

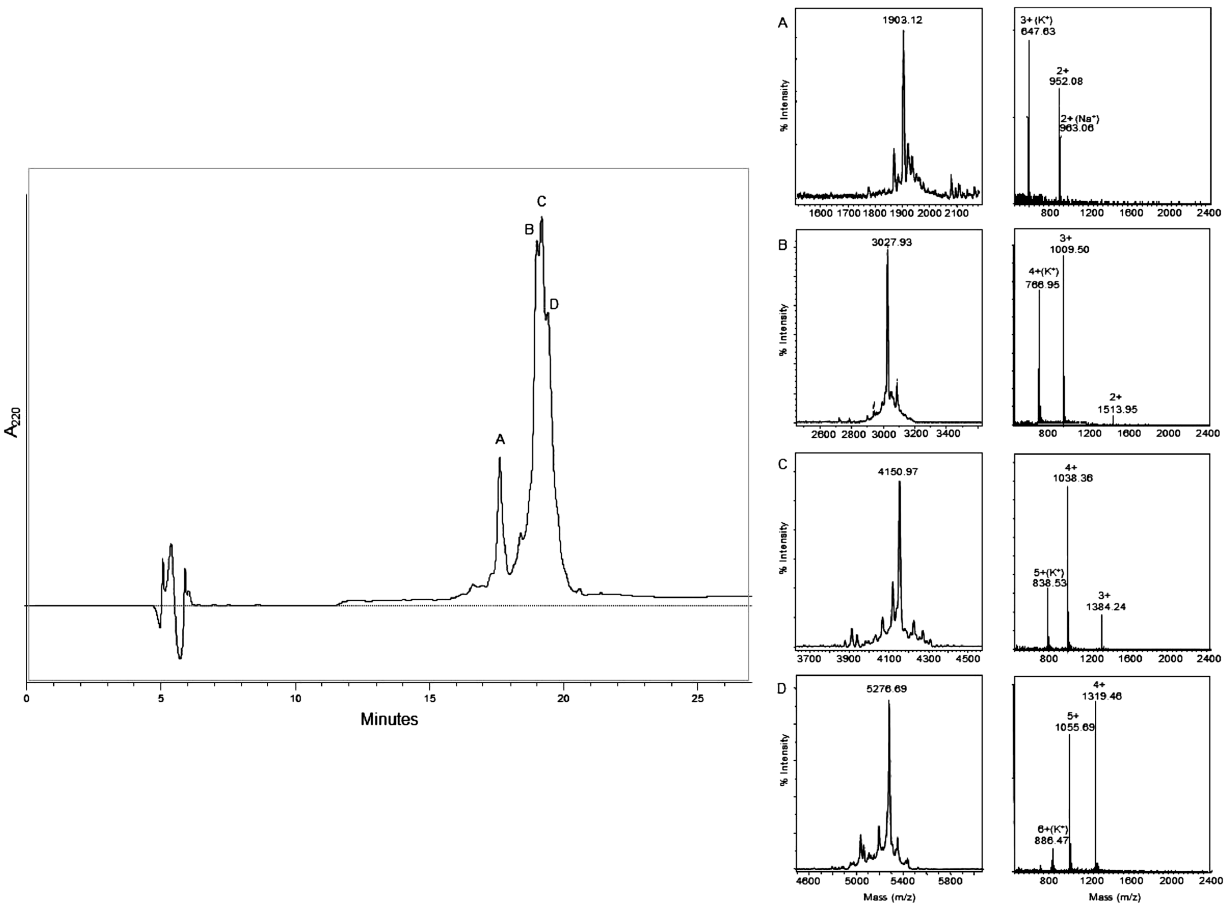

Figure 5. UPLC of the crude MAP_{CP3} and assignation of peaks by MS (MALDI-TOF and ES-MS). HPLC conditions: 10–50% linear gradient of eluent B into A over 27 min at a flow rate of 1 ml/min. HPLC C_{18} column. Solvent A was 0.05% TFA in water, and solvent B was 0.05%TFA in acetonitrile. (A) MAP_{CP3-1} calculated mass: 1903.34, experimental mass (ES-MS): 1902.06; (B) MAP_{CP3-2} calculated mass: 3027.14, experimental mass (ES-MS): 3025.80; (C) MAP_{CP3-3} calculated mass: 4150.94, experimental mass (ES-MS): 4149.65; (D) MAP_{CP3-4} calculated mass: 5274.42, experimental mass (ES-MS): 5274.33.

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we observed good sensitivity/specificity for the detection of anti-GBV-C antibodies in hemodialyzed chronic hepatitis and HIV-infected patient samples.

ELISA was performed as previously described [15] . Using the LP1 linear peptide, we initially assessed the serologic reactivity of the 95 sera panel from HIV-infected patients that previously tested positive for GBV-C anti-E2 protein antibodies by the Abbott test. Also, a panel of 97 sera from blood donors was used as negative controls. The results obtained when performing the comparative ROC curve analysis rendered a cutoff value of 0.357 ODU for LP1, which corresponded to 94.8% specificity [16]. Considering this cutoff value, seven sera from a panel of 45 HIV patients with unknown GBV-C/HIV-1 co-infection reported positive and were therefore selected for further evaluation. Hence, these seven samples were tested for their reactivity against the remaining E2(7–26)-derived peptides and multimer constructs, that is, LP2, LP3, and the corresponding cyclic (CP1, CP2, and CP3) and multimeric (MAP_{LP1}, MAP_{LP2}, and MAP_{LP3}) analogs. In Table 4, the obtained results are shown. As observed, only the CP3 cyclic peptide derived from the shorter E2 peptide reported positive for all the assayed sera.

For comparative purposes, the cutoff values for all peptides derived from the E2(7–26) region were calculated in this work by using the media + 2 standard deviation of the OD values obtained with a panel of 45 sera of blood donors that were considered as negative controls. With these cutoff values taken into account, when the LP1 is compared with their corresponding cyclic or multimeric versions, the linear LP1 peptide was significantly more reactive when probed using HIV-infected patients' sera (Figure 6). However, cyclization of the shorter linear versions of the E2 domain afforded relatively more reactive compounds, when compared with the parent linear versions. For example, the CP3 (Table 4) was the most reactive of the three cyclic compounds either using NOS-activated Costar (23 positive sera from the 45 assayed samples) or Nunc Immobilizer amino plates (15 positive sera from the 45 assayed samples) (Figure S5 of Supporting Information). Hence, we performed a further comparative analysis using the CP3 cyclic peptide and its corresponding MAP against the whole panel of HIV sera. Although the mean of the absorbance values was slightly lower for the MAP, no significant differences were observed between the monomeric and multimeric versions of the CP3 peptide (Figure 7). In both cases, 23 of the 45 sera of HIV-infected patients with unknown GBV-C/HIV-1 co-infection reported positive outcomes, which corresponded to a reactivity of 51%. These results are considered not to be significantly different to the ones previously reported for LP1 [16]. In this case, the presence of antibodies anti-LP1 was detected in 79 of 164 HIV-infected patients (48% sensitivity). These values agree well with the reported prevalence of GBV-C viremia ranging from 14% to 43% among people with HIV infection [28]. Consequently, in our hands, under the same ELISA conditions, neither the shorter versions of LP1 nor their head-to-tail cyclization or multimerization achieved diagnostic properties that are superior to the LP1 peptide.

Other ELISA plates could also be used in order to test if better antibody recognition is achieved with cyclic peptides. The secondary amino (CovaLink NH, Nunc) grafted ELISA plates could

Table 4. Reactivity of E2 peptides against sera of HIV-infected patients

Serum	LP1	LP2	LP3	CP1	CP2	CP3	MAP_{LP1}	MAP_{LP2}	MAP_{LP3}
S1	+	–	–	+++	–	+	++	+	–
S12	++	+++	++	+	–	+	–	+	–
S16	+	+	–	–	–	+	–	–	–
S30	+	++	+	+	+	+	++	+++	+
S39	+	+	+	+	–	+	+	+	+
S40	+	++	–	+	+	+	+	+	+
S41	+	–	+	–	+	+	+	+	–
	7/7	5/7	4/7	5/7	3/7	7/7	5/7	6/7	3/7

–, 1 > DO/CO; +, 2.5 > DO/CO ≥ 1; ++, 4 > DO/CO ≥ 2.5; +++, 4 ≥ DO/CO.

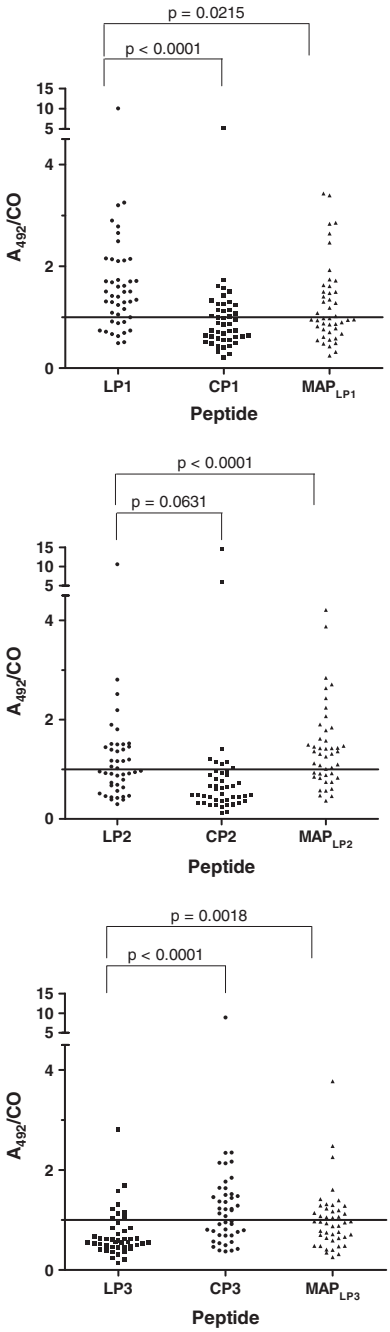

Figure 6. Reactivity of linear, cyclic, and MAP analogs of LP1, LP2, and LP3 peptides with a panel of 45 HIV-infected patient sera. The samples were considered anti-GBV-C positive when $A_{492}/CO \geq 1$.

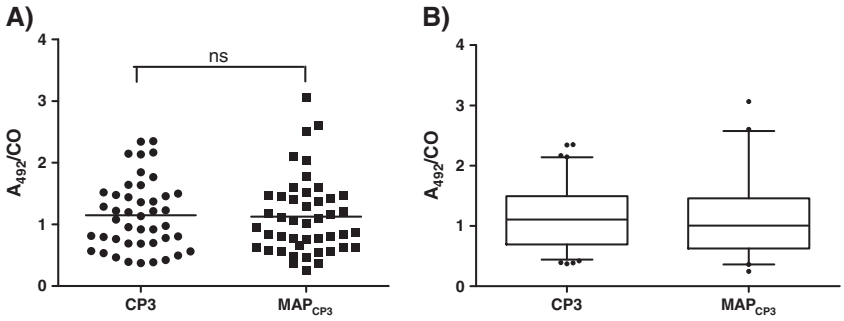

Figure 7. (A) Reactivity of CP3 and MAP_{CP3} with a panel of 45 HIV-infected patient sera. The samples were considered anti-GBV-C antibodies positive when $A_{492}/CO \geq 1$. (B) Distribution of test's values by populations. Boxes represent median and interquartile range (distance between the 25th and 75th percentiles). Whiskers represent values outside interquartile range. Single points represent outliers and extreme values. Serum levels of anti-GBV-C antibodies in 45 HIV-infected patients, CP3: mean = 1.148, *SD* = 0.562, MAP_{CP3}: mean = 1.125; *SD* = 0.624.

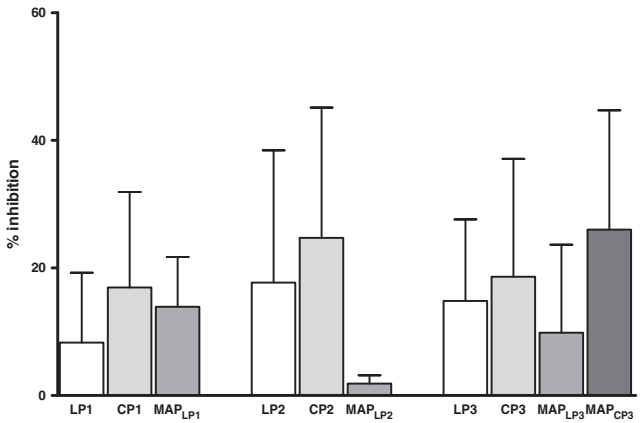

Figure 8. Inhibitory activity of linear, cyclic, and MAP E2 peptides on gp41-mediated cell–cell fusion assay at a concentration of 40 μg/ml. LP1: E2(7–26) linear; CP1: E2(7–26) cyclic; LP2: E2(11–26) linear; CP2: E2 (11–26) cyclic; LP3: E2(17–26) linear; CP3: E2(17–26) cyclic, MAP_{LP1}: MAP_4E2(7–26); MAP_{LP2}: MAP_4E2(11–26); MAP_{LP3}: MAP_4E2(17–26); MAP_{CP3}: MAPE2(17–26) cyclic. This assay was performed in triplicate.

be used in future work to further analyze the potential utility of cyclic peptides for antibody recognition. In this context, these cyclic peptides would be conjugated *via* the Glu residues to the surfaces of the grafted ELISA plates.

Anti-HIV-1 activity of synthetic peptides derived from the N-terminal domain of E2 GBV-C. We have also investigated the potential anti-HIV-1 activity of different forms of presentation of the N-terminal sequence of GBV-C E2 protein as entry inhibitors, by examining their effect on cell–cell fusion assays and analyzing their capacity to block syncytium [20].

Before using the peptides on cells, we evaluated their cytotoxicity. Thus, both families of linear and cyclic peptides did not exhibit any observable cytotoxicity as evaluated by the MTT assay, at the maximum assayed concentration (300 μM). However, the MAPs (MAP_{LP1}, MAP_{LP2}, MAP_{LP3}, and MAP_{CP3}) precipitated at the highest concentrations; thus, the maximum non-cytotoxic concentration tested was 40 μg/ml.

Next, we examined the effect of the E2 synthetic constructs on cell–cell fusion assays, analyzing their capacity to block syncytium formation between HeLa cells expressing the envelope protein of HIV-1 and TZM-bl cells expressing the human CD4 receptor CXCR4 or CCR5 HIV-1 co-receptors in the presence of various amounts of each peptide.

Values of cell–cell fusion inhibition lower than 50% for all the assayed peptides concentrations were obtained, and they were not produced in a dose-dependent manner (data not shown). In Figure 8, the comparative results obtained at 40 μg/ml peptides concentration is shown. As observed, a clear but non-significant trend was obtained for the three overlapping peptides ($p > 0.05$), the cyclization being the modification that rendered higher inhibition values.

Because of the non-dose-dependent inhibition results obtained, we were not able to calculate the IC_{50} values. However, the potencies of the reported GBV-C E2 analogs were quite modest (close to millimolar range), the concentration needed to inhibit gp41-mediated cell–cell fusion being considerably higher compared with well-known entry inhibitors [29]. Consequently, it appeared that the chemical modifications carried out in the selected E2(7–26) peptide did not result in a substantial reduction of the concentration needed to inhibit virus entry.

Conclusions

Our results showed that chemical modifications of the selected E2(7–26) linear peptide to afford cyclic architecture do not result in an enhanced inhibition of gp41 HIV-1-mediated cell–cell fusion nor improved sensitivity in the detection of GBV-C antibodies in HIV co-infected patients. Thus, the ELISA data reinforce the potential utility of linear versions of the E2(7–26) region for the development of new peptide-based immunosensor devices.

Nevertheless, we envisaged that our overall construct design and synthetic strategy, especially to the unique cyclic peptide MAPs, could be exploited for exploring the effect of structural constraint and avidity in other biological systems.

Acknowledgement

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