Towards peptide based therapeutics-applications in celiac disease and infectious diseases

Kapoerchan, V.V.

Chapter 7

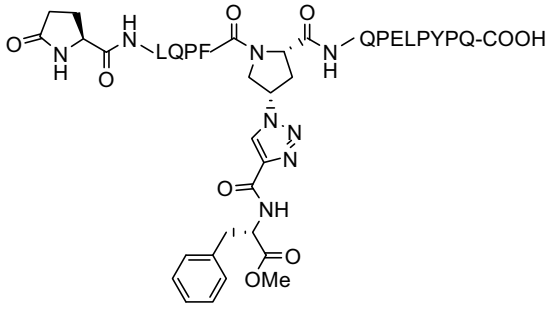
Summary and Future Prospects

HLA-DQ2 blockers

Part of the research described in this Thesis deals with the design, synthesis and evaluation of peptides possibly suitable as HLA-DQ2 blockers. HLA-DQ2 is critically involved in the pathogenesis of Celiac Disease (CD) as it presents gluten peptides, resulting from inefficient breakdown of gluten proteins present in food, to gluten-specific $CD4^+$ T cells. The resulting immune response leads to inflammation and ultimately to severe small-intestinal damage and the symptoms seen in CD. **Chapter 1** describes the pathogenesis of CD and the key factors involved herein: the gluten proteins, HLA-DQ2 and the enzyme tissue transglutaminase (tTG). The only CD treatment available today is adherance to a strict gluten-free diet (GFD). Such a diet effectively restores the small intestinal surface and disappearance of symptoms is seen. However, as the GFD is burdensome and has a large impact on the quality of life, alternative therapies have been the subject of extensive research. These are discussed in Chapter 1 as well and comprise the following strategies: 1. The complete breakdown of gluten *in vivo* by enzyme digestion. 2. Inhibiting tissue transglutaminase, by either competitive inhibition, reversible inhibition or irreversible inhibition. 3. The blocking of the HLA-DQ2 protein.

In **Chapter 2**, the HLA-DQ2 blocker approach is studied further. The symptom-causing immune response in CD can potentially be prevented by the use of HLA-DQ2 blockers: peptides which have higher affinity for HLA-DQ2 than the gluten peptides and, when in complex with HLA-DQ2, are not recognized by gluten-specific T cells. The latter can be achieved by either steric or electronic factors. In this Chapter, the known gluten epitope α-9 (QLQ**PFPQPELPY**, epitope shown in bold) was taken as a starting point. The N-terminal glutamine was considered unsuitable for peptide synthesis and purification, as it is known that this residue readily cyclizes to the pyroglutamate moiety, especially under acidic conditions,[1] leading to a mixture of products. It was found that introducing the pyroglutamic acid residue during peptide synthesis gave rise to one single product, and that the corresponding pyroglutamate peptide showed equal affinity for HLA-DQ2 as α-9. Subsequently, the peptides were decorated with different azido-proline residues at positions 3 and 5 of the epitope, as these residues have limited interaction with the HLA-DQ2 binding pocket and are solvent exposed.[2] The azido functionality was then used to introduce several side chains *via* the Cu(I) catalyzed Huisgen cycloaddition between an alkyne and an azide.[3] In this way, a series of 12 peptides was prepared, all of which were evaluated for their binding affinity for HLA-DQ2 and their ability to elicit a $CD4^+$ T cell-induced immune response. It was found that peptide **1** (Figure 1), containing a (4*S*)-4-azido-L-proline (*cis*-azidoproline) at the p3 position of the epitope and modified with an L-phenylalanine derived ynone showed the highest binding affinity of the series and was able to almost completely inhibit T cell response. A competition experiment performed in the presence of α-9 showed that **1** was indeed able to block the T cell response in the presence of the natural peptide, although at fairly high concentrations (200-400 μM).

Figure 1 A gluten-based HLA-DQ2 blocker identified in Chapter 2.



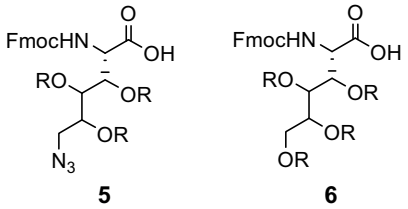
1

As the blocking approach by attachment of large side chains to HLA-DQ2 binding peptides has been shown to be effective, an attempt was made in **Chapter 3** to design peptides with a higher affinity for HLA-DQ2. By the means of molecular modeling studies and further manual optimization by systematic substitution of amino acid residues, high affinity sequence ADA**YDYESEELF**AA (**2**, epitope in bold) was identified. This sequence was found to show a binding affinity for HLA-DQ2 of IC_{50} = 1.2 μM. While this peptide has been optimized using natural amino acids, further optimization of the binding affinity was carried out using unnatural amino acids. For this purpose, peptide **2** was modified by the incorporation of several unnatural amino acids: adamantyl amino acids, lysine analogs and fluorescent amino acids. As the absence of multiple proline residues in the 'superbinder' sequence renders the peptide more susceptible to proteolytic breakdown,[4] peptide Pyr-ADAYDYESEELFAA (**3**), protected at the N-terminus with an N-terminal pyroglutamate, was modified as well. As a control, the same amino acids were incorporated in the α-9 derived peptide Pyr-QLQ**PFPQPELPY**PQ (**4**). The residues were incorporated at the p1 position, which is an 'anchor' position of the epitope and as such important for peptide binding to HLA-DQ2. Furthermore, the p1 pocket in HLA-DQ2 is large and shows room for residues larger than the proline present in the α-9 peptide.[2] The peptide series thus generated was evaluated for HLA-DQ2 binding affinity and it was found that incorporation of lysine analogs in peptide **2** did indeed improve affinity for HLA-DQ2. The same was the case for a small fluorescent amino acid.

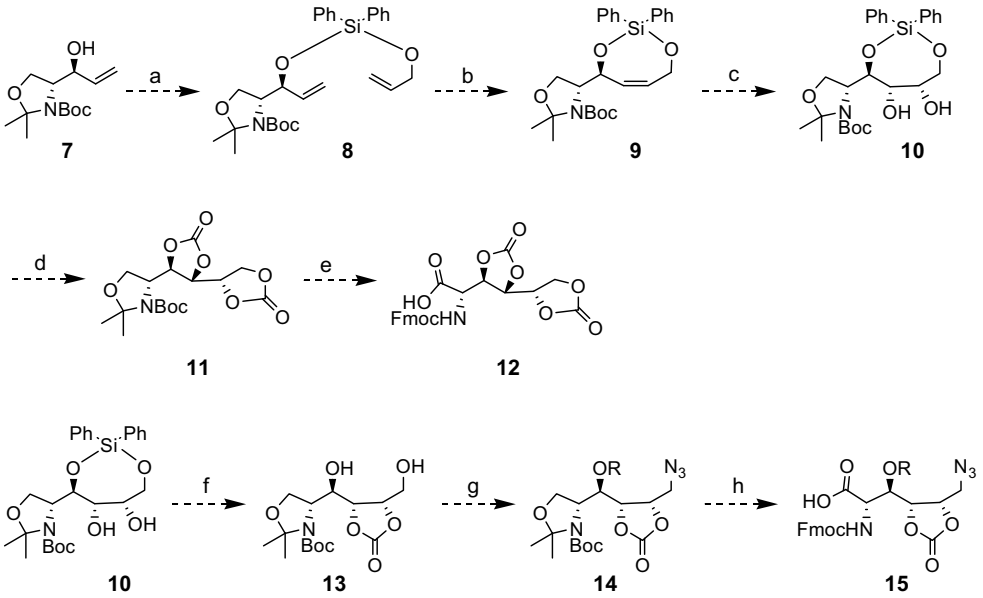
Upon examination of the crystal structure of the QLQ**PFPQPELPY**/HLA-DQ2 complex,[2] and specifically looking at the p1 pocket, it becomes clear that not only there is ample space for larger amino acids, but that the space underneath the proline side chain is filled with three water molecules, which form a hydrogen bond network throughout the pocket, together with a Glu and a His residue. A peptide containing a side chain that can participate in the hydrogen bond network, and thus can replace the water molecules, possibly shows further improved binding affinity. A lysine analog containing multiple hydroxyl groups in the side chain, such as amino acids **5** and **6** (Figure 2) might fit this requirement. These amino acids are accessible from carbohydrate sources, such as D-mannosamine or D-ribose, or can be synthesized *de novo* starting from Garner's aldehyde **7** (a possible synthesis route is given in Scheme 1). After formation of the silyl tethered compound **8**, the seven-membered ring can be constructed by RCM, followed by dihydroxylation of the double bond. Removal of the tether and protection of the hydroxyl functionalities with a cyclic carbonate would yield protected **11**. Subsequent removal of the isopropylidene, oxidation of the primary alcohol and deprotection and reprotection of the amino group, would yield building

block **12** suitable for peptide synthesis. In an analogous manner, building block **15** may be obtained.

Figure 2 Lysine analogs expected to further improve HLA-DQ2 binding affinity.



Scheme 1 Possible synthesis of polyhydroxylated lysine analogs.

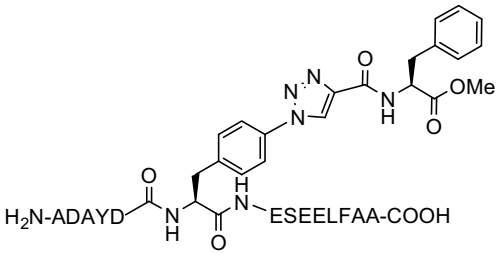


Reagents and conditions: a) Allyl alcohol, Ph_2SiCl_2. b) Grubbs 2nd generation. c) OsO_4, N-methylmorpholine N-oxide. d) 1. TBAF. 2. CDI. e) 1. TsOH. 2. TEMPO/BAIB. 3. TFA/DCM. 4. Fmoc-OSu, $NaHCO_3$. f) 1. CDI. 2. TBAF. g) 1. Mitsunobu reaction. 2. Protect free hydroxyl group. h) 1. TsOH. 2. TEMPO/BAIB. 3. TFA/DCM. 4. Fmoc-OSu, $NaHCO_3$.

Chapter 4 describes the application of the modification strategy as outlined in Chapter 2 to three high affinity sequences. Peptide **2**, two other high affinity peptide sequences ADAFVAEYEEPVLAA (**16**) and ADAFRAEYEEWLAA (**17**) and gluten-derived peptide **4** were subjected to the incorporation of an azido-amino acid and subsequent attachment of acetylene-functionalized side chains using the Cu(I) catalyzed Huisgen cycloaddition. As it followed from Chapter 2, modification of the p3 position gave the best results. Therefore, the amino acids at the p3 position of the

peptides were replaced with the corresponding azido-amino acids. Subsequently, the peptides were modified with two different ynones. A series of 12 peptides was generated. After biological evaluation of all peptides, it was found that the adamantane-modified peptides showed lower affinity than the phenylalanine-modified peptides. Peptide **18** (Figure 3) showed the highest binding affinity of the peptides tested.

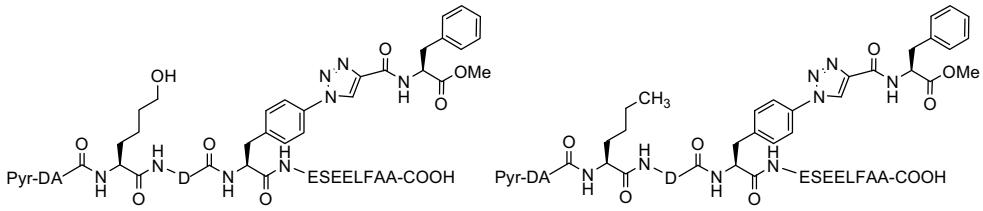
Figure 3 A p1-modified peptide with a high binding affinity for HLA-DQ2.



18

To arrive at a highly efficient blocker, the 'lead' peptides identified in Chapters 3 and 4 can be combined. This would yield peptides **19** and **20** (Figure 4) which are expected to bind efficiently to HLA-DQ2 while not recognized by gluten-specific $CD4^+$ T cells when bound to HLA-DQ2. These peptides contain lysine analogs at the p1 position, and are modified with a phenylalanine moiety at the p3 position.

Figure 4 Efficient blockers by combining lead compounds found in Chapters 3 and 4.

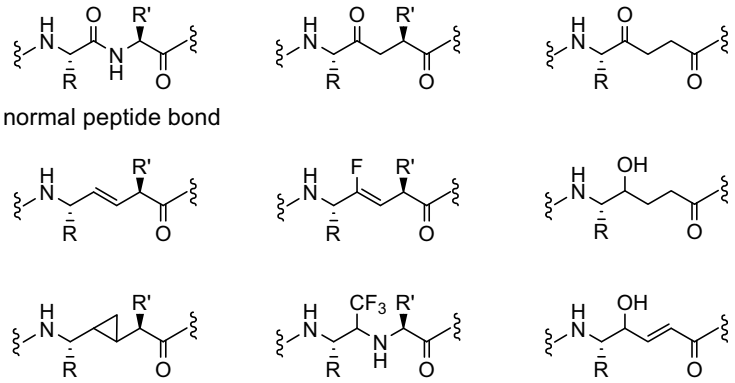


19 **20**

The peptide sequences depicted in Figure 4 differ from the α-9 peptide sequence as in the latter multiple prolines are present. This feature, unique for the gluten proteins, makes this peptide (and other gluten peptides) highly resistant against breakdown by proteases and other proteolytic enzymes[4] and this is an important factor in the pathogenesis of CD. From a therapeutic viewpoint, proteolytic stability is highly favorable, as this increases the stability of the peptide drug *in vivo*, and thus results in better bioavailability. The lack of proline residues in peptides **19** and **20** could make

these compounds labile *in vivo*. Therefore, incorporation of D-amino acids at non-anchor positions or using peptide bond isosteres such as E-alkenes and cyclopropanes (Figure 5) might improve the therapeutic utility of the peptides.

Figure 5 Several examples of peptide bond isosteres.



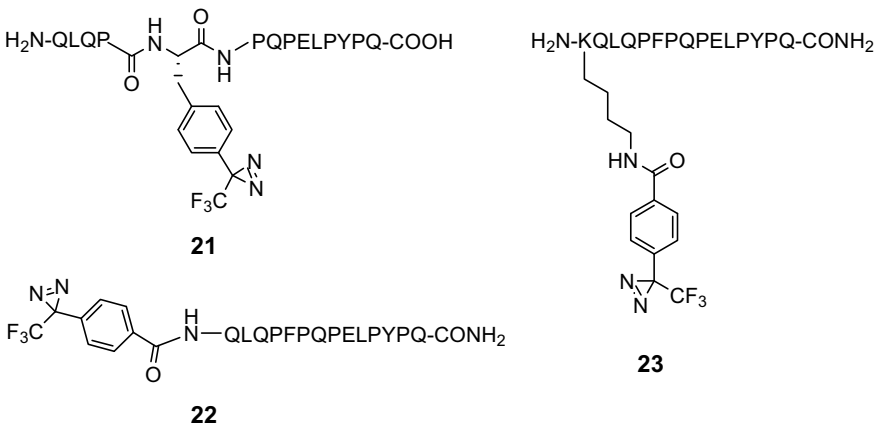
As it has been shown that high affinity peptides can be developed, and that attachment of a large side chain to specific amino acid residues is indeed capable of inhibiting T cell response, the HLA-DQ2 blocker approach seems viable as a treatment for the symptoms of CD. However, as only a few MHC-class bound peptides are necessary to induce T cell response[5] and subsequent small intestinal damage, a truly efficient blocker should *completely* inhibit gluten peptides from binding to HLA-DQ2. Furthermore, there seems to be a limit to the extent in which the binding affinity can be improved, as the HLA-DQ2 binding affinity of natural peptides can be improved 60-fold by natural amino acid substitutions but only an additional 4-5-fold with unnatural amino acids. The question remains if the peptides identified in this Thesis have a sufficient high affinity to be an efficient HLA-DQ2 blocker, and if not, it is unclear if the binding affinity can be much more improved by simple amino acid substitutions. Based on the work performed by Khosla and co-workers,[6] a peptide with multiple copies of high affinity peptide epitopes might display a higher binding affinity than the epitopes itself and such an approach might be a valuable addition to the approach taken in the current Thesis.

In comparison with the TG2 inhibitors, much less research has been dedicated to HLA-DQ2 blockers, as described in Chapter 1. This might be because the generation of toxic gluten peptides by TG2 is a more 'upstream' process in the pathogenesis of CD, as compared to binding to HLA-DQ2 binding. However, it has been suggested that the *initial* small intestinal damage due to which TG2 is released is caused by the

low-affinity binding of non-deamidated peptides to HLA-DQ2.[7] Therefore, the development of efficient HLA-DQ2 blockers might be more effective in preventing intestinal damage and the resulting release of TG2, and thus the HLA-DQ2 blocking strategy may still be viable.

While the abovementioned strategies describe peptides that are not recognized by T cell receptors (TCRs), peptides that are recognized by T cells may also be useful in preventing the symptom-causing immune response in CD. Usually, a T cell circulates through the lymphoid tissues and associates to every antigen-presenting cell (APC) it comes across. In this way, it is able to 'scan' MHC Class II molecules on the surface of APCs for the presence of antigens. When an antigen is recognized, the association between the APC and the T cell is further stabilized and the T cell proliferates, ultimately leading to the immune response.[8] The interaction between the T cell and the MHC-Class II/peptide complex is of a non-covalent nature. When a covalent bond is formed between these two entities, the complex might be disrupted, possibly leading to T cell death. Peptides containing a photocrosslinker moiety could be suitable candidates to investigate this approach. As photoreactive group, the trifluoromethyldiazirine (Tmd) moiety was chosen, due to its high reactivity. Upon UV irradiation, a carbene which is stabilized by the adjacent trifluoromethyl group is formed. The carbene can then react with any electrophile in close proximity. Therefore, a series of α-9 derived peptides was prepared (Figure 6) using standard Fmoc SPPS methods. In the case of peptide **21**, the Tmd functionality was introduced as a modified phenylalanine, and peptides **22** and **23** were obtained by coupling Tmd-benzoic acid either to the N-terminus of the peptide, or to the side chain of a lysine residue.

Figure 6 α-9 derived peptides containing a photocrosslinker moiety.

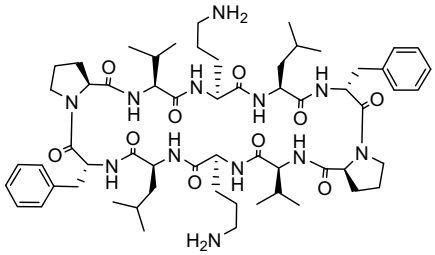


The peptides were first evaluated for their binding affinity for HLA-DQ2. While all three peptides showed good binding affinity (**21**: 4.6 μM, **22**: 4.1 μM, **23**: 1.9 μM), peptide **21** was shown not to induce T cell proliferation when bound to HLA-DQ2. Compounds **22** and **23** were recognized by T cells and therefore, peptide **22** was used to evaluate the crosslinking properties of the Tmd-containing peptides. For this purpose, a mixture of **22** and either lysine or a lysine-containing peptide in water was irradiated for 2 min with UV light. After irradiation, the samples were analyzed by mass spectrometry. However, only the product resulting from reaction of the carbene with water was observed. Given the fact that the experiment is carried out in water, this seems to make sense as the water concentration is much higher than the lysine concentration in the sample (55 M vs. 60 μM), and apparently the probability of a TCR being in close proximity to the HLA-DQ2-peptide complex is low. Furthermore, the therapeutic utility of this specific method is limited, as UV light is necessary for this treatment and this light does not penetrate the human skin. However, when more data concerning the interaction of the HLA-DQ2 bound peptide with the TCR become available, it should be possible to design a peptide which can covalently attach to the TCR, if in the TCR a cysteine, serine or threonine is present near the peptide in the HLA-DQ2 binding pocket. By incorporating an amino acid in the HLA-DQ2-binding peptide containing a reactive group specific for these nucleophilic side chains, it may be possible to covalently attach the peptide to the TCR.

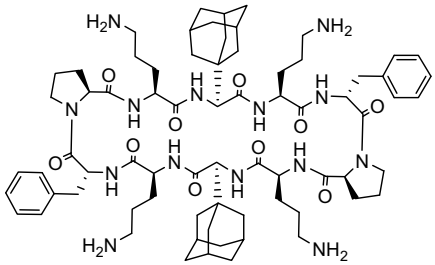
GS analogs

The second part of this Thesis deals with the cyclic peptide antibiotic Gramicidin S (GS, **24**, Figure 7). This cationic peptide shows efficient antibacterial activity against both Gram-positive and Gram-negative bacteria,[9] but human red blood cells are targeted as well. This hemolytic activity limits the therapeutic utility of GS to topical applications. Modification of GS to tune its antibacterial and hemolytic properties could lead to analogs with reduced hemolytic activity that still show antibacterial activity. In **Chapter 5**, a series of GS analogs containing adamantyl amino acids was prepared and evaluated for their biological properties. It was found that 'inverted' GS analogs **25** and **26** (Figure 7) exhibited lower hemolytic activity than GS, with the antibacterial activity being in the same range as GS. Furthermore, it was shown that the biological activity can be correlated in a qualitative manner to the LC-MS retention time of the peptide. According to this empirical method, there seems to be an optimal 'hydrophobicity window' for GS analogs in which antibacterial activity is high, and hemolytic activity is low. Additionally, the presence of four charged side chains, compared to two in GS itself, seems to be important.

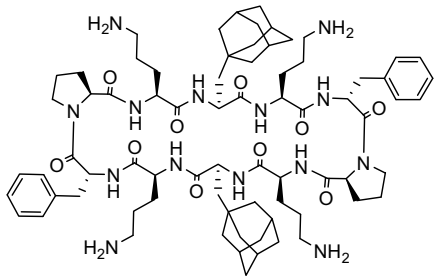
Figure 7 GS and 'inverted' GS analogs with reduced hemolytic activity.



24



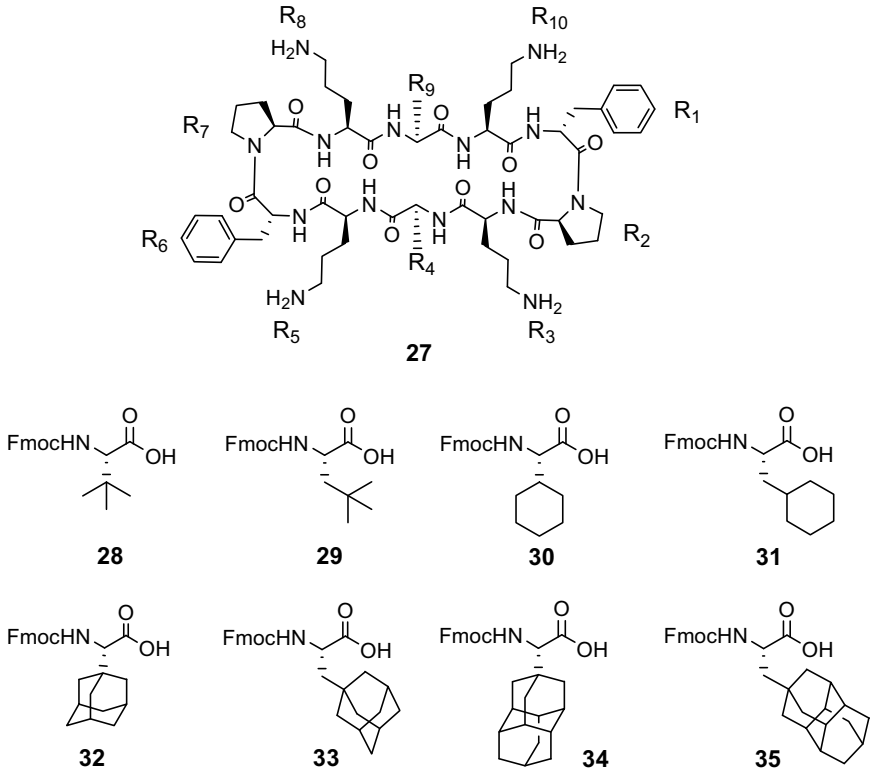
25



26

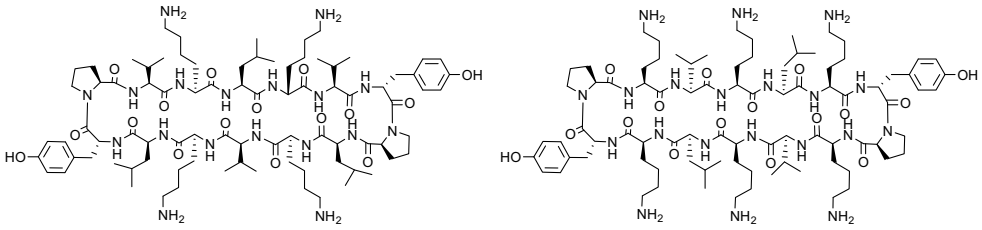
Keeping the 'hydrophobicity window' in mind, GS analogs can be designed which have a different LC-MS retention time under the same conditions than lead compounds **25** and **26**. These compounds might show improved biological properties. Given the fact that four charged residues are important, new analogs should be based on the 'inverted' GS scaffold (**27**, Figure 8). By systematically introducing hydrophobic residues, such as *t*-butyl- and cyclohexyl amino acids (**28**-**31**, Figure 8), and the adamantyl- and diamantyl amino acids (**32**-**35**) at one or both the R_4 and R_9 positions, in different combinations, a series of peptides can be prepared with different hydrophobicities. If the empirical rule proposed in Chapter 5 is valid, then peptides with a lower LC-MS retention time than **25** should be less hemolytic, and compounds which have a higher retention time than **26** should be more hemolytic, while retaining their antibacterial activity.

Figure 8 Unnatural amino acids which can be used to generate new 'inverted' GS analogs based on peptides **25** and **26**.



Recently, it was found that extended GS analog GS14K4 (**36**, Figure 9) displayed less hemolytic activity combined with efficient antibacterial activity.[10] The GS14K4 molecule contains 14 amino acid residues, instead of 10 residues for GS, the Orn residues are replaced with Lys, and the DPhe residues are replaced with DTyr. It possesses four charges and six hydrophobic residues. Furthermore, one of the L-lysine residues is substituted with the enantiomeric DLys. The low hemolytic activity of GS14K4 confirms the observation made in Chapter 5 that the presence of a high number of charges is important in decreasing the hemolytic activity, but not the antibacterial activity. To improve the biological properties of GS14, the molecule can be inverted, while leaving all lysines in the L-configuration, to **37** which contains six positive charges. In the inverted GS14, one or more Val or Leu residues can be replaced with the corresponding adamantyl amino acid, as in Chapter 5, and these modified GS14 peptides might display a further improved biological profile. Additionally, it would be interesting to see whether in this case the LC-MS retention times correlate to antibacterial and hemolytic activity as well, as observed in Chapter 5.

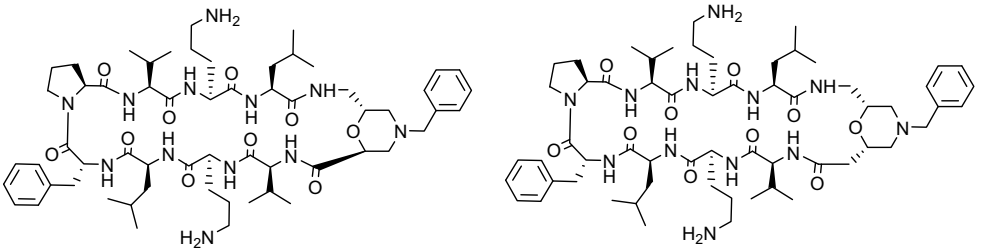
Figure 9 GS14K4 and inverted GS14.



36 **37**

In **Chapter 6**, morpholine amino acids (MAA) are presented as replacements for the DPhe-Pro motif in the β-turn of GS. Four different MAAs were incorporated in GS, and the biological properties of the analogs evaluated. It was found that subtle differences in the β-turn architecture lead to pronounced effects on both antibacterial and hemolytic activity. GS analog **38** (Figure 10), showed the highest similarity to GS, both structurally and biologically. Interestingly, the crystal structure of this compound showed the presence of a supramolecular channel structure. The other GS analogs displayed both reduced antibacterial activity and reduced hemolytic activity. Moreover, X-ray analysis of peptide **39** incorporated revealed the presence of an 'amide flip': instead of the usual four hydrogen bonds, only three were observed within the peptide, due to the 'flip' of the amide bond connecting the MAA and the neighboring Leu residue.

Figure 10 GS analogs containing morpholine amino acids.



38 **39**

As **38** is slightly less hemolytic than GS itself, changing the morpholine side chain from a benzyl moiety to other hydrophobic functionalities might decrease the toxicity of the compound.

Experimental Section

General

Peptides were synthesized on solid support (Tentagel S PHB resin, Rapp Polymere) and amino acids were coupled manually using Fmoc based peptide synthesis methods and commercially available Fmoc amino acids. 4-(1-Azi-2,2,2-trifluoroethyl)benzoic acid (Tmd-benzoic acid) was purchased from Bachem. LC-MS analysis was performed on a Jasco HPLC-system (detection simultaneously at 214 and 254 nm) coupled to a Perkin Elmer Sciex API 165 mass instrument with a custom-made Electrospray Interface (ESI). An analytical Gemini C_{18} column (Phenomenex, 50 x 4.60 mm, 3 micron) was used in combination with buffers A: H_2O, B: MeCN and C: 1.0% aq. TFA. For RP-HPLC purification of the peptides, a BioCAD 'Vision' automated HPLC system (PerSeptiveBiosystems, inc.), supplied with a semi-preparative Gemini C_{18} column (Phenomenex, 150 x 10 mm, 5 micron) were used. The applied buffers were A: H_2O, B: MeCN and C: 1.0% aq. TFA. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in H_2O/MeCN; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylpthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

Peptide binding assay

A binding assay was performed essentially as described previously applying minor modifications.[11] 96-well FluoroNunc™ plates were coated with the HLA-DQ-specific monoclonal antibody SPV-L3, 1 μg/well in 100 μl of carbonate buffer (50 mM Na_2CO_3, 50 mM $NaHCO_3$, pH 9.6) for 2 h at 37°C and subsequently blocked for 2 h at 37 °C with 1% BSA in PBS. HLA-DR3/DQ2 positive EBV-transformed B-cells were lysed in 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10 mM iodoacetamide, 0.5% NP-40 and protease inhibitor mix (Complete™, Roche) at 4 °C, at a concentration of 4 x 10^6 cells per 1 ml lysis buffer. Subsequently, nuclei and cell debris were removed by centrifugation (4°C, 2000G, 15 min). Such prepared lysates were mixed with an equal volume of ice-cold 1% solution of BSA in PBS and 100 μl aliquots were pipetted into the SPV-L3 coated wells. After overnight incubation at 4 °C the plates were washed and 50 μl binding buffer (0.1% NP-40, 0.1% Tween, 33.6 mM citric acid, 72 mM Na_2HPO_4, pH 5.5 and Complete™ protease inhibitor mix) was added to each well. A titration range of peptides to be tested (concentration range 600 – 0.04 μM) was prepared in 10% DMSO containing a fixed amount of the biotin-labeled indicator peptide (EEPRAPWIEQEGPEYWDQE (EPRAP)) at a concentration of 1.2 μM. Subsequently, 50 μl of the samples was applied to the SPV-L3/HLA-DQ2 coated plates. Following a 48 h incubation at 37 °C each well was washed extensively. Subsequently, 100 μl of 100 nM streptavidin-europium in assay buffer (both Wallac) was added and incubated for 45 minutes at RT. After extensive washing, 150 μl/well of enhancement solution (Wallac) was applied and the plates were read in a time resolved fluorimeter (1234, Wallac) 15-30 minutes thereafter. IC_{50} values were calculated based on the observed competition between the test peptides and biotin-labeled indicator peptides and indicate the concentration of the tested peptide required for half maximal inhibition of the binding of the indicator peptide. Experiments were carried out in triplicates.

T cell proliferation assays

Proliferation assays were performed in triplicate in 150 μl RPMI supplemented with 10% human serum in 96 well, flat-bottom plates using 10,000 α-9-specific T cells stimulated with 100,000 irradiated HLA-DQ2-matched allogenic PBMCs, in the presence of 33 μM modified peptide or 33 μM α-9-gluten peptide (α-9 = QLQPFPQPELPY) respectively. After 48 h at 37 ºC, cultures were pulsed with 0.5 μCi of 3H-thymidine and harvested 18 h later.[12]

Crosslinking experiments
To evaluate the crosslinking properties of peptide **22**, three different solutions were made:

1. 60 μM **22** in D_2O
2. 1 M lysine and 60 μM **22** in D_2O
3. 20 μM Biotin-AKAKAKFRPQQPYPQPQ and 60 μM **22** in D_2O

These solutions were split in two parts; one part was not irradiated, the other was irradiated for 2 min with UV light of 366 nm. After irradiation, all samples were analyzed by mass spectrometry.

Peptide Synthesis

a) First residue attachment for peptides 21-23, general procedure
Tentagel S PHB resin (2.5 g, 0.5 mmol) was swollen in DCM. After removal of the solvent, a solution of Fmoc-Gln(Trt)-OH (3.05 g, 5 mmol) and DMAP (61.1 mg, 0.5 mmol) in 30 ml of 1:1 (v/v) NMP/DCM was added to the resin. DIC (0.77 ml, 5 mmol) was added and the mixture shaken for 16 h. Solvents were removed, the resin was washed (NMP and DCM) and air-dried. The loading was determined by Fmoc-test to be 0.21 mmol g^{-1}.

b) Elongation of peptides
Peptides were elongated using standard Fmoc SPPS methods using repeating cycles of i) Fmoc deprotection (20% piperidine/NMP, 15 min). ii) Peptide coupling (4 eq Fmoc-AA-OH, 4 eq BOP, 8 eq DIPEA, NMP, 3h).

c) Cleavage of peptides from resin and purification
Peptides were cleaved from the resin by shaking for 2 h in 95:2.5:2.5 (v/v/v) TFA/H_2O/TIS and precipitated in a cold 1:1 (v/v) pentane/diethyl ether mixture. After centrifugation, the supernatant was removed, the peptide pellet was resuspended in fresh diethyl ether and spinned down again. This was repeated two times to remove final traces of TFA. The final pellet was air-dried, analyzed by LC-MS and purified by RP-HPLC. After purification, the fractions containing the pure product were pooled, concentrated and freeze-dried to yield the pure peptides.

Analytical data of peptides

Peptide 21
Following the general procedures for peptide synthesis and cleavage, **21** was obtained from Fmoc-Gln(Trt)-Tentagel S PHB resin (loading 0.21 mmol g^{-1}, 30.5 mg, 6.4 μmol) as a white powder (1.98 mg, 1,1 μmol, 17%) using a HPLC gradient of 80:10:10 → 20:70:10 of H_2O/MeCN/1.0% aq. TFA for purification. The Fmoc-Tmd-Phe residue was attached using 2 eq Fmoc-Tmd-Phe, 2 eq BOP, 2 eq HOBt and 2 eq DIPEA in NMP, using double coupling and coupling times of 16 h.
LC-MS retention time: 10.61 min (10 → 90% MeCN, 25 min run). Mass (ESI): m/z 896.0 $[M + 2H]^{2+}$; 1791.2 $[M + H]^{+}$. Exact mass: calculated for $[C_{82}H_{117}F_3N_{20}O_{22}]^{2+}$: 895.42962; $[C_{82}H_{116}F_3N_{20}O_{22}]^{+}$: 1789.85197. Found: 886.91719 [M(pyroglutamate peptide) + $2H]^{2+}$; 1772.82443 [M(pyroglutamate peptide) + $H]^{+}$.

Peptide 22
Following the general procedures for peptide synthesis and cleavage, **22** was obtained from Fmoc-QLQPFPQPELPYPQ-Tentagel-RAM resin (loading 0.19 mmol g^{-1}, 53.5 mg, 10 μmol) as a white powder which was pure enough to be tested. The Tmd-benzoic acid was coupled using 2 eq of benzoic acid (4.6 mg), 2 eq of PyBOP (11.2 mg) and 4 eq of DIPEA (7.1 μl) in NMP, with a coupling time of 3 h.
LC-MS retention time: 8.81 min (10 → 50% MeCN, 13.5 min run). Mass (ESI): m/z 842.4 $[M + 2H]^{2+}$; 1681.2 $[M + H]^{+}$; 1703.4 $[M + Na]^{+}$. Exact mass: calculated for $[C_{89}H_{122}F_3N_{21}O_{22}]^{2+}$: 946.95072; $[C_{89}H_{121}F_3N_{21}O_{22}Na]^{2+}$: 957.94169. Found: 946.95183 $[M + 2H]^{2+}$; 957.94291 $[M + Na + H]^{2+}$.

Peptide 23
Following the general procedures for peptide synthesis and cleavage, **23** was obtained from Fmoc-QLQPFPQPELPYPQ-Tentagel-RAM resin (loading 0.19 mmol g^{-1}, 53.6 mg, 10.2 μmol). After attachment of the Fmoc-Lys(Mmt)-OH residue, the side chain Mmt protecting group was removed by repeated treatment (6 times) of the resin with 1% TFA/DCM, until the yellow color of the washings disappeared. After washing of the resin with DCM and NMP, the Tmd-benzoic acid was attached using 2 eq of Tmd-benzoic acid (5.1 mg), 2 eq of PyBOP (11 mg) and 4 eq of DIPEA (7.1 μl) in NMP, with a coupling time of 16 h. After removal of the N-terminal Fmoc-group, the peptide was cleaved from the resin. Purification of the crude product yielded the title compound as a white powder (7.9 mg, 3.9 μmol, 38%) which was pure enough to be tested.
LC-MS retention time: 8.97 min (10 → 50% MeCN, 13.5 min run). Mass (ESI): m/z 1011.6 $[M + 2H]^{2+}$; 2022.6 $[M + H]^{+}$. Exact mass: calculated for $[C_{95}H_{133}F_3N_{23}O_{23}]^{+}$: 1010.99820. Found: 1010.99933 $[M + 2H]^{2+}$.

References

1. Bodanszky, M.; Martinez, J. *Synthesis* **1981**, *5*, 333-356.
2. Kim, C. Y.; Quarsten, H.; Bergseng, E.; Khosla, C.; Sollid, L. M. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 4175-4179.
3. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2002**, *41*, 2596-2598.
4. Kagnoff, M. F. *Gastroenterology* **2005**, *128*, S10-S18.
5. a) Krogsgaard, M.; Juang, J.; Davis, M. M. *Semin. Immunol.* **2007**, *19*, 236-244 and references cited therein. b) Ma, Z.; Sharp, K. A.; Janmey, P. A.; Finkel, T. H. *PLoS Biol.* **2008**, *6*, 328-342 and references cited therein. c) Stevanović, S.; Schild, H.; *Semin. Immunol.* **1999**, *11*, 375-384.
6. Xia, J.; Siegel, M.; Bergseng, E.; Sollid, L. M.; Khosla, C. *J. Am. Chem. Soc.* **2006**, *128*, 1859-1867.
7. Van de Wal, Y.; Kooy, Y. M. C.; Van Veelen, P.; Peña, A. S.; Mearin, M. L.; Papadopoulos, G. K.; Koning, F. *J. Immunol.* **1998**, *161*, 1585-1588.
8. Janeway, C. A.; Travers, P.; Walport, M.; Shlomchik, M. in *Immunobiology*; ed. Garland Science (New York); **2001**, sections 8.2 and 8.4.
9. Kondejewski, L. H.; Farmer, S. W.; Wishart, D. S.; Hancock, R. E. W.; Hodges, R. S. *Int. J. Peptide Protein Res.* **1996**, *47*, 460-466.
10. a) Lee, D. L.; Hodges, R. S. *Biopolymers* **2003**, *71*, 28-48. b) Lee, D. L.; Powers, J.-P. S.; Pflegerl, K.; Vasil, M. L.; Hancock, R. E. W.; Hodges, R. S. *J. Peptide Res.* **2004**, *63*, 69-84.
11. Vader, W.; Stepniak, D.; Kooy, Y.; Mearin, M. L.; Thompson, A.; Spaenij, L.; Koning, F. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 12390-12395.
12. Van de Wal, Y.; Kooy, Y.; Van Veelen, P.; Peña, S.; Mearin, L.; Molberg, Ø.; Lundin, L.; Mutis. T.; Benckhuijsen, W.; Drijfhout, J. W.; Koning, F. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 10050-10054.