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Synthesis and antibody recognition of synthetic antigens from MUC1

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Abstract: In the altered form of MUC1 mucin associated with breast cancer, the highly immunogenic sequence PDTRPAP is exposed, and may be an immunologically relevant target for the development of diagnostics or cancer immunotherapy. In this study, we report the preparation and antibody binding properties of monomeric and dimeric MUC1 peptides containing the epitope region recognized by monoclonal antibody (mAb) C595. Peptides contained a single or two copies of the whole 20-mer repeat unit (VTSAPDTRPAPGSTAPPAHG) of MUC1 protein. MUC1 40-mer peptides were prepared by the condensation of semi-protected fragments of the repeat unit, in solution or by chemical ligation. In the first case, cyclohexyl-type protecting groups were used for the synthesis of semi-protected fragments by the Boc/Bzl strategy. Unprotected fragments were used in the chemical ligation to produce thioether linkages. In one of the fragments, a Gly residue was replaced by Cys at the *C*-terminus and the other fragment was chloroacetylated at the *N*-terminus. In addition, the short peptide APDTRPAPG, and its disulfide dimer, $(APDTRPAPGC)_2$ were produced. The antibody binding properties of these MUC1 peptide constructs were tested by competition enzyme-linked immunosorbent assay (ELISA). The short epitope region peptide, APDTRPAPG and its dimer $(APDTRPAPGC)_2$ showed higher IC_{50} values ($IC_{50} = 56.3$ and 53.2 μmol/l, respectively). While the 20-mer peptide ($IC_{50} = 25.9$ μmol/l) and more markedly its 40-mer dimers ($IC_{50} = 0.62$ and 0.78 μmol/l) were recognized better. CD data obtained in water or in TFE indicated no significant conformational differences between the 20-mer and 40-mer peptides. We found a high level of similarity between the binding properties of the 40-mer peptides with amide or thioether links, providing a new possibility to build up oligomeric MUC1 peptides by thioether bond formation. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: mucin 1 epitope region; antibody recognition of mucin 1 tandem repeat; solution conformation; chemical ligation; antibody binding; synthetic antigen

INTRODUCTION

The MUC1 protein is a member of the mucin glycoprotein family. This protein is a transmembrane, often secreted protein which is coded by the *MUC-1* gene. It is normally present on the apical surface of polarized epithelial cells of respiratory tract, genitourinary tract and digestive system. MUC1 is a macromolecular glycoprotein, which is rich in Ser, Thr and Pro residues. The many carbohydrate chains, which account for about 50% of the molecular mass, are linked by *O*-glycoside bonds to the polypeptide core [1]. The polypeptide core contains a variable number (30–100) repeats of the 20-mer amino acid sequence APDTRPAPGSTAPPAHGVTS [2,3].

As an epithelial cell undergoes malignant transformation, it looses the normal apical-basolateral polarity and begins to express MUC1 on the entire cell surface. The MUC1 expressed by carcinoma cells is overexpressed and underglycosylated compared with the version produced by normal cells [4]. This difference results in a peptide core that can be recognized by immune system as epitope [5]. At the same time, IgM and IgG antibodies may be detected in the sera from patients with ovarian [6] and colorectal cancer [7]. Strong correlation between the presence of anti-MUC1 antibodies in sera from cancer patients and better prognosis as well as patient survival have been reported in pancreatic [8] and breast tumours [9]. The appearance of these epitope domains is mainly related to cancer cells; MUC1 is therefore an attractive target for immunodiagnosis and immunotherapy [10]. The majority, if not all, of epitopes recognized by different MUC1-specific monoclonal antibodies (mAbs) are located in a small region of the repeat unit. This is a highly hydrophilic region, APDTRPAP of the 20-mer repeat unit [11]. The C595 antibody used for the present study recognizes a tetramer (RPAP) sequence [12]. Hydropathicity and secondary structure prediction analysis on the 20-mer repeat unit indicated a hydrophilic region extending through residues PDTRPAP, and a high propensity to form β-turn structure located at the PDTR sequence [12]. Indeed, CD and NMR experiments, supported by independent computational non-restrained study on both synthetic PDTRPAPGSTAP and 20-mer peptides, have identified a type I β-turn in the PDTR region

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[13,14]. Fontenot *et al.* [15] reported that the NMR analysis of the $(VTSAPDTRPAPGSTAPPAHG)_3$ trimer indicates that the elongated MUC1 structure contains knobs connected by extended spacer sequences. This property of the structure may influence the binding ability, which depends on the number of 20-mer in a molecule [16]. In another study, isotope-edited NMR including ^{15}N- and ^{13}C-relaxation measurements were used to probe the recognition and binding of the PDTRPAP epitope sequence to Fab fragment of mAb B27.29. Two peptides were studied: an one-repeat MUC1 16-mer peptide of the sequence GVTSAPDTRPAPGSTA and a two-repeat MUC1 40-mer peptide of the sequence $(VTSAPDTRPAPGSTAPPAHG)_2$. The data demonstrated that the PDTRPAP sequence is immobilized in the antibody-combining site [17].

In this study, our aim was to prepare peptides containing one or two copies of the full repeat units of MUC1 protein as well as its epitope region. A synthesis of a large peptide, like 40-mer MUC1, on solid support is not easy, mainly because of the accumulation of side products. To overcome this drawback, convergent solid-phase peptide synthesis [18] is recommended. Short protected fragments are synthesized and are coupled to the starting fragment on the resin in order. The low solubility of the protected fragments, as well as the poor yield of the coupling reaction, requires the use of low capacity resin. In spite of the difficulties, 20-, 40-, 60-, 80-, and 100-mer MUC1 peptides were prepared using this method [19]. Another approach is to couple the fragments in solution. In this case, the 40-mer MUC1 peptide can be built up from fragments using semi-protected building blocks or unprotected precursor peptides. In the first case, the fragments are connected by amide bond formation, while in the second case application of chemical ligation (e.g. thioether bond) is necessary. MUC1 repeat unit contains only one carboxyl group in the sequence as the side chain of Asp residue. In accordance with our previously described procedure, Boc/Bzl strategy using cyclohexyl-type protection to block amino and carboxyl groups can be applied for the synthesis of semi-protected peptide fragments [20]. These groups, cyclohexyloxycarbonyl and cyclohexyl ester, are stable in 1 M TMSOTf-thioanisole/TFA cleavage mixture at 0 °C. After the amide bond formation, these protecting groups can be removed with hydrogen fluoride (HF). Chemical ligation by thioether bond formation is a simple and selective method, which can be carried out with unprotected fragments. The thioether bond forms rapidly under mild alkaline condition between a thiol group (e.g. side chain of Cys) and a haloacetylated amino group [21]. In most cases, the alkylation reaction is faster than the competing oxidation, which results in disulfide bridge; therefore, this side reaction is usually insignificant. However, the disulfide bond formation depends to some extent on the position of Cys in the peptide chain [22]. The chloroacetyl group is stable under the normal HF deprotection conditions, even in the presence of appropriate thiols as scavangers [23]. The thioether bond is especially used for the conjugation of peptide epitopes to proteins [24] or synthetic carriers [25].

Our goal was also to study the binding of these MUC1 peptide constructs to the mAb C595 and to develop synthetic antigens with appropriate binding properties to be utilized as effective diagnostic tools. In the competition enzyme-linked immunosorbent assay (ELISA), a target antigen is needed. The frequently used target antigen is a minimal epitope peptide conjugated to large protein (e.g. BSA) or polymeric peptide carrier molecules [26]. For our study, we prepared a new construct using a sequential oligopeptide carrier based on tuftsin derivative ($H–[Thr\text{-}Lys\text{-}Pro\text{-}Lys\text{-}Gly]_4–NH_2$, T20) [25,27].

MATERIALS

All amino acid derivatives were purchased from Bachem (Bubendorf, Switzerland) and Reanal (Budapest, Hungary) while *m*- and *p*-cresol, thioanisole, dithiothreitol (DTT), DIEA, HOBt, DCC, TFA, TMSOTf and HF were FLUKA (Buchs, Switzerland) products. EDCxHCl was ordered from Aldrich (Steinheim, Germany). The 4-methyl-benzhydrylamine (MBHA) and Merrifield resins were from Novabiochem (Läufelfingen, Switzerland). Solvents for syntheses and purification were obtained from Reanal. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) was from Aldrich (Gillingham, U.K.).

METHODS

Synthesis of Monomers and Fragments

Peptides H–**VTSAPDTRPAPGSTAPPAHG**–NH_2 (**1**), H–**VTSAPD**(*OcHex*)**TRPAPGSTAPPAHG**–NH_2 (**2**), and Cl–CH_2–CO–**VTSAPDTRPAPGSTAPPAHG**–NH_2 (**4**), were synthesized manually by solid-phase methodology on the same MBHA resin (0.5 g, 1 mmol/g) until the last step (see below). The amino acid side chain protecting groups were cyclohexyl ester (OcHex) for Asp, mesitylene-sulfonyl (Mts) for Arg. Side chains of Thr and Ser were protected with benzyl (Bzl) groups, while N^{π} of His was blocked by benzyloxymethyl (Bom) group.

Boc protection was removed with 33% TFA in DCM (2 + 20 min) followed by washing with DCM (5 × 0.5 min), neutralization with 10% DIEA in DCM (3 × 1 min) and DCM washing again (4 × 0.5 min). The amino acid derivatives and coupling reagents (DCC and HOBt) dissolved in DCM-DMF 4 : 1 (v/v) were used in 3 M excess for the resin capacity. The coupling reaction proceeded for 60 min at room temperature (RT). Then, the resin was washed [DMF (2 × 0.5 min), DCM (3 × 0.5 min)]. The efficiency of the coupling was checked by ninhydrin or isatin tests. After the removal of the last N^{α}–Boc group from peptide resin, the resin was split into three portions and in the case of the first part a chloroacetyl group was added at the *N*-terminus using 5 equiv chloroacetic acid pentachlorophenyl ester. The peptides from the resin were

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cleaved by 10 ml HF using 0.5 g *p*-cresol as scavenger. The crude product was precipitated by dry diethyl ether, dissolved in 10% acetic acid and freeze-dried (peptide **4**). The peptide **1** in the second case was cleaved from the resin directly as described above. The cleavage of peptide **2** from the third part of the resin was carried out with 1 M TMSOTf-thioanisole in TFA (10 ml total volume) in the presence of 0.2 ml *m*-cresol at 0 °C for 1 h. After cleavage, the reaction mixture was filtered into cold, dry diethyl ether and then it was centrifuged. The precipitate was washed with ether three times. The solid material was dissolved in 10% acetic acid and freeze-dried. The crude peptides were purified by RP-HPLC.

H–**VTSAPDTRPAPGSTAPPAHC**–NH_2 (**5**), H – **APDTRPAPGC**–NH_2 (**9**), H–**APDTRPAPG**–NH_2 (**8**) peptides were prepared manually by solid-phase methodology on MBHA resin (0.5 g or 0.3 g, 1 mmol/g). The side chain protecting groups of amino acids and the protocol of the synthesis were the same as described above. The thiol group of the additional Cys was protected by the 4-methylbenzyl group. All peptides were cleaved from the resin by 10 ml HF using 0.5 g *p*-cresol scavanger and 0.1 g DTT (in case of Cys containing peptides) at 0 °C for 1.5 h. Crude products were precipitated with dry diethyl ether, filtered and washed, dissolved in 10% acetic acid and freeze-dried. The crude peptides were purified by RP-HPLC.

The synthesis of Choc–**VTSAPD(***OcHex***)TRPAPGSTAPPAHG**–OH (**3**) was carried out manually by solid-phase methodology on Boc-Gly-Merrifield resin (0.5 g, 2 mmol/g) by Boc/Bzl strategy. The amino acid side chain protecting groups and the synthetic procedure, including activation, coupling and monitoring were the same as mentioned above. After the removal of the last N^{α}–Boc group cyclohexyloxycarbonyl (Choc) group was attached to the *N*-terminal amino group of the peptide resin by the application of 5 equiv Choc-OSu. Then the peptide was cleaved with 1 M TMSOTf-thioanisole in TFA (10 ml total volume) in the presence of 0.2 ml *m*-cresol at 0 °C for 1 h. The reaction mixture was worked up as describe above.

Synthesis of Dimers

The 40-mer oligopeptide with amide bond between the two fragments was prepared in solution (Figure 1). Peptide **3** (20 mmol), and **2** dissolved in 5 ml DMF were mixed with 24 µmol EDC, 24 µmol HOBt, 48 µmol DIEA in 2.5 ml DMF. The reaction mixture was stirred overnight at RT; the solvent was removed by vacuum evaporation. The remaining part was dissolved in acetic acid and freeze-dried. The product was dried over P_2O_5 for a day, then the cyclohexyl-type protecting groups were removed with anhydrous HF in the presence of scavenger (0.5 g *p*-cresol in 5 ml HF) at 0 °C for 90 min. The peptide was precipitated and washed with dry, cold diethyl ether, then the crude peptide was dissolved in 10% acetic acid and freeze-dried.

The synthesis of 40-mer oligopeptide prepared by thioether bond formation was performed as follows (Figure 2): peptide **5** was added in small portions into the buffered (0.1 M Tris, pH = 8.1) solution of peptide **4** ($C_{peptide}$ = 1 mg/ml) in every 15 min. The reaction was followed by RP-HPLC and the reaction was terminated when the chloroacetylated peptide was not present anymore in the reaction mixture. The solution was freeze-dried.

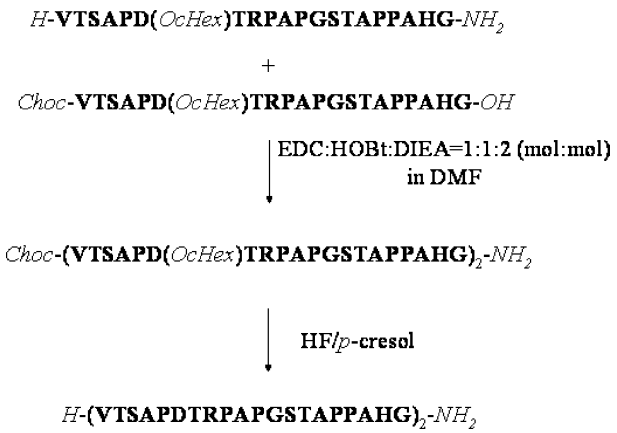

Figure 1 Outline of the synthesis of MUC1 40-mer peptide with amide bond.

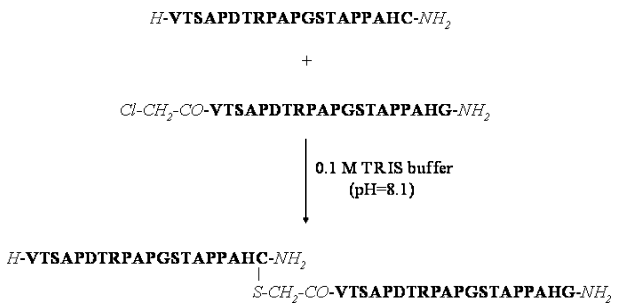

Figure 2 Outline of the synthesis of MUC1 40-mer peptide with thioether bond.

H–**APDTRPAPGC**(*H-APDTRPAPGC–*NH_2)–NH_2 dimer was prepared by air oxidation from peptide **9** in Tris buffer (0.1 M, pH = 8.1). 15 mg peptide was dissolved in 10 ml buffer and the solution was stirred for overnight and then freeze-dried.

All of the freeze-dried crude dimers were purified by RP-HPLC.

Synthesis of target antigen Ac–(TKPK(H-APDTRPAPGC{CH_2-CO}–NH_2)G)$_4$–NH_2

Twenty milligrams (8.3 µmol) of Ac–**[TKPK(**ClAc**)G]$_4$**–NH_2 was dissolved in 20 ml Tris buffer (0.1 M, pH = 8.1) and 5 mg of the peptide **9** was added into this solution for every 15 min (114 mg, 116 µmol altogether). The mixture was stirred overnight, and the side product [H-APDTRPAPGC–NH_2]$_2$ dimer was reduced by DTT. The crude product was purified by RP-HPLC. Besides the conjugate containing four copies of epitope, the excess of the epitope was recovered in this way.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)

Analytical HPLC was performed on a Waters (Nihon Waters Ltd, Tokyo, Japan) HPLC system using a Phenomenex Jupiter C18 column (250 × 4.6 mm I.D.) with 5 µm silica (300 Å pore size) (Torrance, CA) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile-water (80 : 20, v/v)) was used at a flow rate of 1 ml/min at

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ambient temperature. Peaks were detected at $\lambda = 214$, 254 and 280 nm. The samples were dissolved in eluent B. The crude products were purified on a semi-preparative Phenomenex Jupiter C18 column (250 × 10 mm I.D.) with 10 μm silica (300 Å pore size) (Torrance, CA). Flow rate was 4 ml/min. Linear gradient elution was applied. Gradient I: 0 min 5% B, 5 min 5% B, 50 min 50%B. Gradient II: 0 min 10% B, 5 min 10% B, 50 min 70%B.

Amino Acid Analysis

The amino acid composition of peptides was determined by amino acid analysis using a Beckman (Fullerton, CA) Model 6300 amino acid analyser. Prior to analysis, the samples were hydrolysed in 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h.

Mass Spectrometry

Positive ion electrospray ionization mass spectrometric analysis was performed on a PE API 2000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada). Samples were dissolved in methanol-water (1 : 1 v/v) mixture containing 0.1% acetic acid, and 5 μl of sample was injected with flow rate of 100 μl/min. The instrument was used in Q1 scan mode in the range of m/z 400–1700, with step size of 0.3 amu and a dwell time of 0.5 ms.

Binding Inhibition Assay

The MUC1 mono and dimer peptides were tested for their capacity to inhibit the binding of C595 antibody to Ac–[TKPK(H–APDTRPAPGC{CH_2–CO}–NH_2)G]$_4$–NH_2 target antigen. The target antigen at $c = 1$ μg/ml in 0.1 M PBS (pH 7.3) containing 0.02% NaN_3 was added to 96-well Falcon (Becton Dickinson and Co., Oxnard, CA., USA) at 100 μl/well and incubated at RT overnight. The wells were washed three times with distilled water. To block the non-specific adsorption sites the wells were incubated with PBS containing 1% BSA and 0.02% NaN_3 for 1 h. C595 antibody in $c = 2$ μg/ml or distilled water alone, 50 μl/well, was added to the wells in which 50 μl of the test peptide solution had been added (at concentration range of 2.7×10^{-6}–6.5×10^{-4} mol/l for the short peptides and 4×10^{-7}–2.17×10^{-4} mol/l for oligomer peptide). After incubation at RT for 1 h, the wells were aspirated and washed five times. Horse radish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, Glostrup, Denmark) was added, incubated for 1 h, aspirated and washed five times with distilled water. To each well, 100 μl of substrate solution [citrate-phosphate buffer (pH 4) containing 0.033% ABTS and 0.05% H_2O_2] was added. After 15 min incubation, the absorbance of the solutions was recorded on Labsystem iEMS Reader MF (Labsystem, Finland). In each case, the concentration of the added peptide required to inhibit antibody binding by 50% (IC_{50}) was calculated as a measurement of antigenic potency.

Circular Dichroism

The CD spectra were recorded by a Jasco Model J-810 dichrograph (Jasco International Co. Ltd, Tokyo, Japan) using a quarz cell with a path length of 0.2 mm and a peptide concentration of *ca* 0.5 mg/ml in TFE or water. The spectra were acquired between 300 and 185 nm at RT using a scan speed of 50 nm/min, a band width of 1 nm, and a response time of 1 s. Every spectrum was scanned five times to increase the signal-to-noise ratio. CD band intensities were expressed as mean residue molar ellipticity $[\Theta]_{MR}$ in deg cm^2/dmol.

RESULTS AND DISCUSSION

In this work, we report on the synthesis, solution conformation and antibody binding properties of peptides containing MUC1 epitope region (APDTRPAP) present in the repeat unit, (VTSAPDTRPAPGSTAPPAHG) of MUC1 protein.

Synthesis of Peptides Containing APDTRPAP MUC1 Epitope Region

Our aim was to synthesize first 40-mer peptides corresponding to the repeat sequence (VTSAPDTRPAPGSTAPPAHG) of MUC1 protein. For this, we prepared building fragments to avoid the formation of side products and difficulties during the purification of crude target compound. To prepare a native sequence with amide linkage (VTSAPDTRPAPGSTAPPAHG)$_2$, two fragments were synthesized first by Boc/Bzl strategy. In both the compounds, only the carboxyl group of Asp is reactive during the amide bond formation therefore it was protected as cyclohexyl ester. In the end of the synthesis, a cyclohexyloxycarbonyl group was coupled to the *N*-terminal amino group of peptide **3**. These two groups are stable under the cleavage condition of 1 M TMSOTf-thioanisole in TFA [20]. Peptide **2** was prepared as amide to block the *C*-terminal carboxyl group. The purified fragments were coupled in DMF by EDC : HOBt coupling reagents. Then, the cyclohexyl-type protecting groups were removed with HF to provide peptide **6**. The influence of the type of bond between the fragments on the antibody recognition was studied. For this, 40-mer peptide was also synthesized with thiother linkage by replacing the Gly residue at the *C*-terminus of peptide **5** by Cys, and introducing Cl–Ac group at the *N*-terminal amino group of peptide **4**. Reaction of the thiol and Cl–Ac group is rather selective, and in this way the protection of the other side chain functional groups in the peptide, is not necessary. Formation of thioether bond was carried out in 0.1 M Tris buffer (pH = 8.1). Two additional peptides were prepared in order to study their binding properties. One of the short peptides contained only the epitope region elongated by a *C*-terminal Gly residue (APDTRPAPG) of the repeat unit of the MUC1 protein. The dimer form of this peptide was produced after the modification of APDTRPAPG by the addition of a Cys residue at the *C*-terminus. The peptides were produced on MBHA resin by Boc/Bzl strategy and the dimer was prepared from the purified peptide H–APDTRPAPGC–NH_2 in Tris buffer by air oxidation.

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As target antigen for ELISA experiments, peptide H–APDTRPAPGC–NH_2 was conjugated with tetratuftsin derivative (T20) carrier [24]. First, we have chloroacetylated Lys residues in position 4 of each repeat unit. The chloroacetylated carrier molecule with four chloroacetyl functional groups was conjugated with the epitope peptide. Thus, four copies of peptide epitope, H–APDTRPAPGC–NH_2, were attached to this carrier through thiol groups. All peptides were characterized by amino acid analysis (data not shown), ESI-MS and RP-HPLC. Data are shown in Table 1.

MAb Recognition of the Peptides Containing MUC1 Epitope Region

Antibody recognition of peptides containing the APDTRPAP MUC1 epitope region were tested by competitive ELISA using mAb C595 that recognize RPAP core epitope [12]. In these experiments, the $(APDTRPAPGC)_4$-T20 conjugate as a target antigen was applied. The IC_{50} values determined on the basis of epitope concentration are shown in Table 1.

Peptide, H–APDTRPAPG–NH_2, exhibited the highest IC_{50} value, ($IC_{50} = 56.3$ μmol/l). Its dimer derivative, which contains two copies of the epitope region connected by disulfide bridge, has similar IC_{50} value, ($IC_{50} = 53.2$ μmol/l) (Figure 3). It is interesting to note that both the IC_{50} values are higher than that determined for the 20-mer MUC1 peptide presenting the whole repeat unit ($IC_{50} = 25.9$ μmol/l). These data indicate that the 20-mer peptide is somewhat better antigen than the short peptide containing only the epitope region of the repeat unit. It needs to be also emphasized that dimerization of peptide APDTRPAPGC with disulfide bond provided no improved antibody binding. This observation could be indicative of the need for the spatial accessibility of the epitope for antibody binding. This could be better attained in a longer peptide chain.

In the case of both the 40-mer peptides containing also two copies of the epitope regions, the IC_{50} values ($IC_{50} = 0.62$ μmol/l for amide linkage and $IC_{50} = 0.78$ μmol/l for thioether bond) were markedly lower than that of the 20-mer unit (Figure 4, and Table 1). The presence of two epitope regions in the 40-mer sequence resulted in much better antibody recognition ($IC_{50} = 0.62$ and 0.78 μmol/l for the 40-mer *vs* $IC_{50} = 25.9$ μmol/l for the 20-mer).

These results might indicate that the epitope (RPAP) could be much better recognized by mAb 595 in the dimeric form of the 20-mer repeat unit suggesting that the 3D appearance of epitope could be closer to the native conformation of the unglycolysated

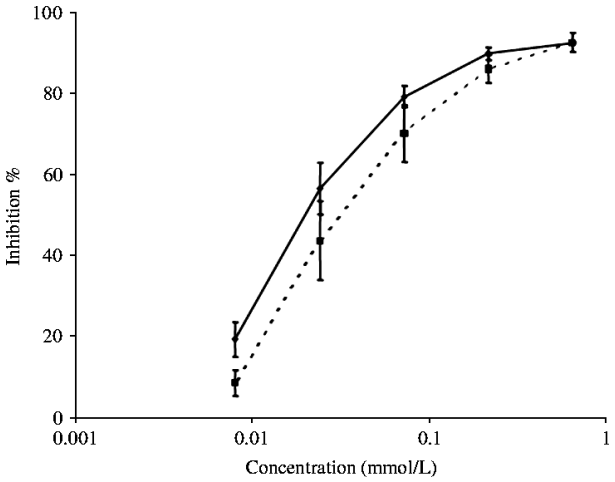

Figure 3 Inhibition of the binding of C595 mAb to Ac-[TKPK(H–APDTRPAPGC{CH_2–CO}–NH_2)G]$_4$–NH_2 target antigen in competition ELISA. H–APDTRPAPG–NH_2 (- - - -) and [H–APDTRPAPGC–NH_2]$_2$ (———).

Table 1 Characteristics and antibody binding of MUC1 peptide constructs

Code	Peptides	ESI-MS		R_t (min)[a]	IC_{50} (μmol/l)
		$M_{cal.}$	$M_{obsd.}$		
1	H–**VTSAPDTRPAPGSTAPPAHG**–NH_2	1884.9	1885.0	23.5	25.9
2	H—**VTSAPD**(*OcHex*)**TRPAPGSTAPPAHG**–NH_2	1966.9	1967.0	26.3	—
3	Choc–**VTSAPD**(*OcHex*)**TRPAPGSTAPPAHG**–OH	2093.9	2094.0	35.5	—
4	Cl–CH_2–CO–**VTSAPDTRPAPGSTAPPAHG**–NH_2	1962.4	1962.2	20.6	—
5	H–**VTSAPDTRPAPGSTAPPAHC**–NH_2	1932.1	1932.2	22.4	—
6	H–(**VTSAPDTRPAPGSTAPPAHG**)$_2$–NH_2	3755.0	3754.5	22.8	0.62
7	H–**VTSAPDTRPAPGSTAPPAHC**(–*CH_2*–CO–VTSAPDTRPAPGSTAPPAHG–*NH_2*)–NH_2	3859.2	3858.1	23.4	0.78
8	H–**APDTRPAPG**–NH_2	880.4	880.5	15.2	56.3
9	H–**APDTRPAPGC**–NH_2	982.5	982.6	17.5	—
10	H–**APDTRPAPGC(***H–APDTRPAPGC–*NH_2**)**–NH_2	1964.2	1964.5	16.8	53.2

[a] Column: Phenomenex Jupiter C18 (250 × 4.6 mm, 5 μm, 300 Å); eluent A: 0.1% TFA/H_2O, B: 0.1% TFA/Acetonitrile–H_2O (80 : 20 v/v); gradient: 0 min 0% B; 5 min 0% B; 50 min 90% B; flow rate: 1 ml/min.

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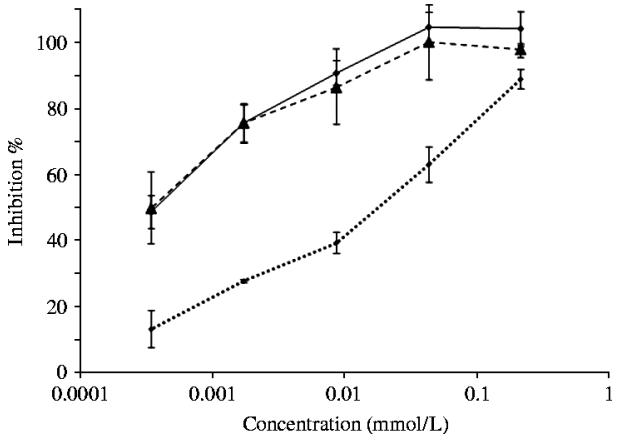

Figure 4 Inhibition of the binding of C595 mAb to Ac–[TKPK(H–APDTRPAPGC{CH_2–CO}–NH_2)G]$_4$–NH_2 target antigen in competition ELISA. H–(VTSAPDTRPAPGSTAPPAHG)$_2$–NH_2 (———), H–VTSAPDTRPAPGSTAPPAHC(CH_2-CO–VTSAPDTRPAPGSTAPPAHG–NH_2)–NH_2 (- - - -) and H–VTSAPDTRPAPGSTAPPAHG–NH_2 (·····).

MUC1 protein resulted in the improvement their immunogenicity [15]. It should be added that we found essentially no differences in the binding properties of 40-mer peptides with amide or thioether bond. From these results, it seems that the thioether bond between the two repeat units has no significant influence on mAb C595 recognition of the 40-mer MUC1 peptide.

Secondary Structure of Synthetic Peptides

The secondary structure of 20-mer and 40-mer (with thioether bond) peptides were studied in TFE and in water using electronic circular dichroism (ECD) spectroscopy. We found that shapes of the CD spectra for both peptides are similar in TFE and also in water. Data are summarized in Figure 5.

In TFE, the CD spectra of both peptides show a broad negative band centred at $\lambda = 197$ nm ($\pi - \pi^*$, $[\theta]_{MR} = -8500$), a shoulder at $\lambda = 202$ nm and a shoulder with lower intensity at $\lambda = 227$ nm ($n - \pi^*$). In water, only a weak $n - \pi^*$ band was found at $\lambda = 230$ nm. The $\pi - \pi^*$ band was red-shifted ($\lambda = 199$ nm) and showed higher intensity ($[\theta]_{MR} = -15\,000$).

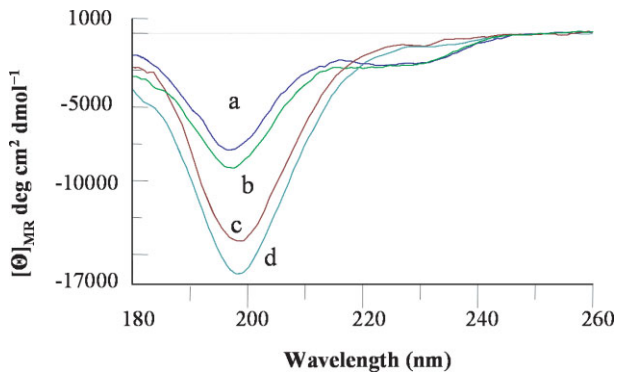

Figure 5 CD spectra of 20-mer peptide containing the repeat unit of MUC1 protein (a, d) and of its 40-mer dimer peptide with thioether bond (b, c) recorded in TFE (a, b) and in water (c, d).

Considering the chain length (20- or 40-amino acid residues) one could expect that both peptides adopt periodic (α-helix, β-sheet) conformation under appropriate conditions. In water, the pattern of CD spectra, detected for both peptides may be diagnostic of the presence of unordered (disordered) conformation or conformer mixture [28]. Our results presented here indicate that both peptides could form ordered conformations (β-turns or β-structure) in the presence of TFE considered as ordered structure inducing/stabilising solvent [29,30]. In TFE, the low band intensities of the CD spectra and the presence of two shoulders (at $\lambda = 202$ and 227 nm) reflected to the appearance of these structural elements [31].

Using the Yang's deconvolution method [32] to estimate the secondary structure, we found that a conformer mixture is present in TFE. For both 20-mer and 40-mer peptides, ~50% β-sheet, ~10% β-turn, and 40% random coil were calculated. In water, the random coil form was predominant (~55%), while the β-turn element (18%) slightly increased and the β-sheet form (25%) is decreased.

CD data suggest no essential differences in the conformer compositions between of 20-mer and 40-mer oligopeptides studied under condition used for binding analysis.

CONCLUSION

In this study, we reported the preparation and binding properties of monomeric and dimer MUC1 peptides containing the epitope region recognized by mAb C595. Two of these peptides contained only the epitope region (APDTRPAP) elongated by Gly or GlyCys residue at the *C*-terminal end. The other peptides contained a single or two copies of the whole 20-mer repeat unit (VTSAPDTRPAPGSTAPPAHG) of MUC1 protein. In the case of the 40-mer MUC1 peptides, amide or thioether bond was used to connect the two 20-mer units.

From the antibody binding assay, it turned out that the epitope (RPAP) is much better recognized by mAb 595 if it is a part of a longer sequence (20-mer). The presence of two copies of the short epitope peptide region (APDTRPAPGC) in the dimer construct did not result in improved binding capacity. However, connection of two 20-mer sequence increased the antibody binding dramatically. CD data obtained in water as well as in TFE indicated no significant differences in the steric arrangements between the 20-mer and 40-mer peptides. These findings might be supporting our proposal that increased antibody binding was probably due to multivalency. We also

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find that the type of linkage between the two 20-mer units (amide or thioether bonds) did not have significant influence on the monoclonal antibody recognition. In addition, the thioether bond formation from unprotected peptide precursors described in this communication could be considered as an appropriate and also practical technical approach to prepare MUC1 and possible other oligopeptides.

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