Synthesis of Cyclic Antigenic MUC1 Mimotopes

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Abstract

The immune system responds to antigens via specific sequences called epitopes. The antibody binding amino acid epitope PDTRP within the variable tandem repeat (VNTR) domain of the Mucin1 (MUC1) transmembrane epithelial glycoprotein has been found to be a tumor-associated antigen, capable of inducing an immune response. After the epithelial cell undergoes an epithelial mesenchymal transition (EMT), transitioning into a replicating tumor cell, the MUC1 glycoprotein becomes hypoglycosylated thus exposing the underlying VNTR domain to the extracellular environment and becoming immunologically active. We have synthesized a truncated cyclic mimotope (Aza-Pro-Asp-Thr-Pra-Lys) of the VNTR domain via solid-phase peptide synthesis and copper-catalyzed alkyne-azide cycloaddition (CuAAC) and isolated it via HPLC. The mimotope structure was characterized by 2D ^{1}H NMR via TOCSY and ROESY experiments. Although the cyclization appeared to be a success the ability of the synthesized mimotope to bind the mAb SM3 was inconclusive as no saturation transfer effects were detected via STD NMR but peak splitting was observed in the ^{1}H NMR spectrum of the peptide in the presence of antibody.

Introduction

Mucins (shown in **Fig. 1**) are a class of heavily *o*-glycosylated proteins, most commonly found on epithelial surfaces, which provide many protective cellular functions such as the formation of mucosal barriers [1]. The range of human mucins (MUC) spreads from MUC1 to MUC21, however the specific mucin this study is associated with is the MUC1 transmembrane protein. MUC1 has been detected as a carcinoma-linked antigen. This is believed to be caused by a loss in cellular polarity attributed in part to MUC1 protein. This loss of polarity triggers an epithelial-mesenchymal transition (EMT) producing aggressive cancer cells. Additionally, transmembrane mucins have been found to be overexpressed in cancer cells, leading to prolonged EMT activation and eventual malignancy [2,3]. Thus far it has been difficult to create an effective immunotherapy or vaccine for carcinomas as many tumors produce immunosuppressive effects [5,6].

It is believed that using truncated MUC1 peptide sequences could be an effective vaccination against carcinomas due to the nature of the *o*-glycosylation of cancer cell-associated mucins. These mucin proteins are overexpressed and hypoglycosylated, leaving short amino acid sequences exposed to the extracellular environment [4,5,7]. This potentially increases the efficacy of these proteins as antigens for the signaling of cytotoxic T cells if previously exposed to a vaccination by a short peptide epitope of MUC1 [4,5]. Furthermore, evidence shows that using a cyclized structural analog of an epitope may elevate the primary immune response when presented with a tumor cell exhibiting the natural epitope [11].

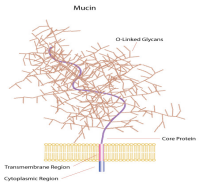

Fig. 1 Transmembrane mucin protein with *o*-glycosylation explicitly shown. This glycosylation is less dense in mucins associated with cancer cell membranes [8].

Objectives

Effective peptide antigens are being sought in the creation of vaccines and immunotherapies for the treatment of specific cancers. Peptides that are capable of binding to mucin monoclonal antibodies may be useful in the development of these therapeutic and prophylactic agents.

The objective of this study is to synthesize a truncated and cyclized epitope of the MUC1 protein (Aza-PDT-Pra-K) that is capable of interacting closely with the murine monoclonal antibody SM3 by measure of saturation transfer difference (STD) NMR.

Methods

Solid Phase Peptide Synthesis

The peptide used in this study (cyclo-Aza-Pro-Asp-Thr-Pra-Lys) was manually synthesized in the solid-phase using FMOC-chemistry [9]. Amino acids are bonded to "Wang" resin beads via a *p*-alkoxybenzyl group at their carboxyl end. Attached to the amine end of the amino acids are protective fluorenylmethyloxycarbonyl (FMOC) protective groups. The FMOC group of the resin-linked amino acid is removed to allow the amine group to react with the activated carboxyl group of the next amino acid to form the peptide bond. The process is repeated until the desired peptide sequence is obtained and then it can be cleaved from the resin.

Copper-Catalyzed Azide-Alkyne Cycloaddition

CuAAC, or "click chemistry", was performed to form a cyclic mimotope structure of 1,4-disubstituted 1,2,3-triazole ring. The reaction requires ascorbic acid and a copper(I) sulfate catalyst.

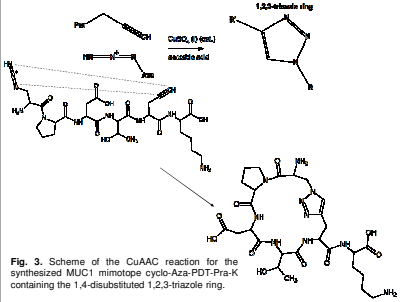

Fig. 3. Scheme of the CuAAC reaction for the synthesized MUC1 mimotope cyclo-Aza-PDT-Pra-K containing the 1,4-disubstituted 1,2,3-triazole ring.

Mass Spectrometry

Mass spectra were acquired from crude peptide sample to begin characterization. These spectra were compared to the theoretical masses of the peptide mimotope used in the study (MW 667u).

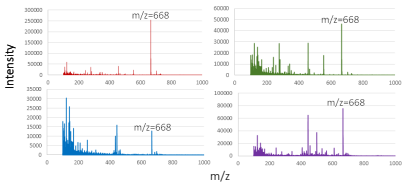

Fig. 4. Mass spectra of four separate HPLC peaks from the crude sample of cyAza-PDT-Pra-K. M/Z is given above the corresponding peak. Masses given in **Table 2**.

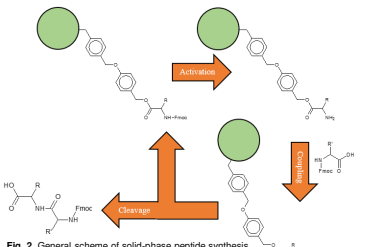

Fig. 2. General scheme of solid-phase peptide synthesis.

HPLC Purification

Purification of the synthesized peptide was carried out via reverse phase HPLC using a C_{18} column and water and acetonitrile mobile phases. Isolated peptide fractions were collected for further analyses using a UV/Visible light detection module. Results shown in **Fig. 5**.

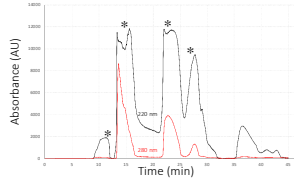

Fig. 5 HPLC chromatogram of peptide mimotope indicating collected fractions (*) and absorbance levels at 280nm (aromatic absorbance, red) and 220nm (protein absorbance, black).

NMR Spectroscopy

2D TOCSY and ROESY ^{1}H NMR were used to complete proton assignments for the peptide mimotope (**Fig. 6**). 1D ^{1}H NMR was used to verify the success of the CuAAC reaction by detection of the methylene proton of the newly formed 1,2,3-triazole ring (**Fig. 7**). STD NMR technique was used to determine peptide-antibody binding (**Fig. 8**). [9,10].

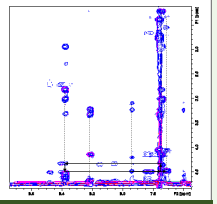

Fig. 6. Overlay of TOCSY (blu and ROESY (red) NMR spect and proton spin systems peptide mimotope Aza-PDT-Pr K.

Results

Peptide Synthesis, Cyclization, LCMS, and HPLC Purification

- Synthesis of the peptide Aza-PDT-Pra-K was successful with a functional yield, achieving the target theoretical mass as measured by LCMS (**Table 1**).
- Cyclization by CuAAC was successful as indicated by the appearance of a methylene proton resonance within the aromatic region of a 1D ^{1}H NMR spectrum (**Fig. 7**).
- The peptide appeared in four HPLC peaks over the course of the run-time, indicating that it did not interact favorably with the column. This likely led to the presence of contaminants in the samples.

Table 1. Theoretical and observed LCMS masses. $(M+H)^+$, $(M+Na)^+$, and $(M+K)^+$ are given and compared to the theoretical mass. Crude yield percentage is also given.

Peptide	Crude Yield (%)	Theoretical Mass (g/mol)	$(M+H)^+$ (m/z) (g/mol)	$(M+Na)^+$ (m/z) (g/mol)	$(M+K)^+$ (m/z) (g/mol)
Aza-PDT-Pra-K	~ 35	667	668	690	706

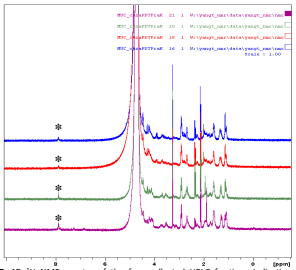

Fig. 7. 1D ^{1}H NMR spectra of the four collected HPLC fractions indicating the formation of 1,2,3-triazole groups via the resonance of the methylene proton (*).

Results

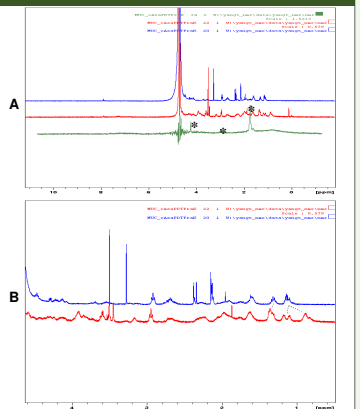

Fig 8. (A) (Green) STD ^{1}H NMR, (Red) ^{1}H NMR of Aza-PDT-Pra-K in the presence of mAb SM3, (Blue) reference ^{1}H NMR of peptide not in the presence of mAb SM3. Spectra were acquired in 20 mM phosphate, 5 mM NaCl, pH 7.0, 100% D_2O, 25 °C. **(B)** Dashed lines connect the protons that were shifted (peptide + mAb). Contaminants indicated by asterisks (*). No saturation transfer artifacts were observed on the STD spectrum, however, peak shifting was observed in the ^{1}H NMR spectrum of the peptide in the presence of antibody which can be caused by tight ligand binding, though inconclusive at this time.

Conclusions & Further Work

- Synthesis and cyclization of the peptide cyclo-Aza-PDT-Pra-K was successful.
- Antibody binding affinity of the mimotope is inconclusive at the time.
- Further exploration of potential binding affinity will be required.
- Bioconjugation of the synthesized mimotope to a carrier protein (BSA or KLH) to create a large immunogenic antigen is possible.

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