

Application Note

Characterization of Rituximab by Conformational Epitope Mapping and Substitution Scan

ABSTRACT

Rituximab (Rituxan™) is a monoclonal antibody targeting the CD20 receptor on B cells. To precisely define its binding site, we used high-density PEPPERCHIP® Peptide Microarrays for conformational epitope mapping and amino acid substitution analysis. Since Rituximab recognizes a conformational epitope within a 15-amino acid loop of CD20, we used constrained cyclic peptides cyclized via thioether bonds for structural mimicry.

While linear peptides showed no Rituximab binding, cyclic peptides produced strong signals, particularly for sequences containing the EPANPSEK motif. Signal intensity increased with loop size, with 10–13 amino acid peptides best replicating the native epitope structure.

A comprehensive substitution scan of the epitope peptide NIYNCEPANPSEK revealed that positions ⁹N, ¹⁰P, and ¹¹S were strictly conserved, while others allowed limited substitution. This high-resolution analysis not only confirmed the core epitope but also mapped tolerated variations.

These findings enhance our understanding of Rituximab's specificity and offer valuable insights for cross-reactivity assessments, biosimilar development, and patient stratification. The study demonstrates the power of cyclic peptide microarrays for detailed conformational epitope characterization in therapeutic antibody development.

INTRODUCTION

Peptide microarrays with a protein antigen displayed as set of overlapping peptides are the method of choice for the epitope mapping of antibodies and sera, and further enable the in-depth characterization of the underlying epitopes with respect to conserved and variable amino acids. However, many epitopes rely on protein folding and form conformational epitopes. Such conformational epitopes can hardly be detected with standard microarrays based on linear peptides. A common strategy to mimic conformational motifs is the introduction of structural constraints e.g. by peptide cyclization.

Typically, peptides are cyclized by disulfide formation between two cysteine side chains. However, this oxidative process is not well compatible with other oxidation-sensitive amino acids and can yield oxidized by-products. As an alternative and more flexible strategy for peptide cyclization, we applied an on-chip thioether formation between a

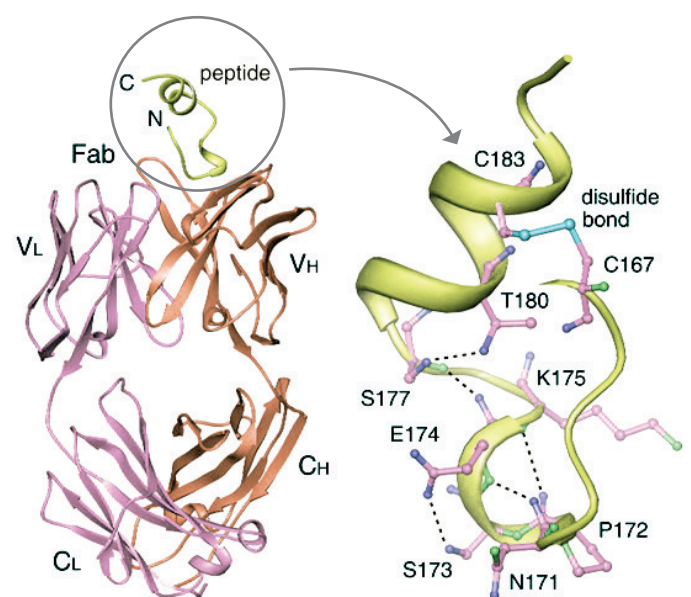


Figure 1. Structure of the Rituximab Fab-CD20 epitope-peptide complex. The Rituximab heavy chain is colored in orange and the light chain in pink. The CD20 epitope peptide is depicted in light green (figure modified from Du et al., 2007).

C-terminal cysteine side chain and the bromoacetylated N-terminus of the printed peptides, yielding constrained cyclic peptides with stable thioether bonds. This cyclization strategy is fully compatible with all amino acids and enables the generation of high density peptide microarrays with overlapping cyclic peptides without any restriction.

In this application note, we analyzed the binding behavior of Rituximab (Rituxan™), a therapeutic monoclonal antibody directed against the extracellular domain of the CD20 receptor. Based on crystal structures (Fig. 1), Rituximab is known to interact with the 15 amino acid loop NIYNCEPANPSEKNSPSTQYCYSIQ of CD20 (1). To gain an in-depth view of the actual conserved core motif of Rituximab, we started a high resolution epitope mapping study based on peptide microarrays with linear and constrained cyclic CD20 peptides. To cover various loop sizes, the amino acid sequence of the extracellular domain of CD20 was translated into 7, 10 and 13 amino acid peptides with maximum peptide-peptide overlap, and the resulting peptide microarray was assayed with Rituximab.

RESULTS AND DISCUSSION

The extracellular domain of CD20 was translated into 7, 10 and 13 amino acid peptides with the respective maximum peptide-peptide overlap of 6, 9 and 12 amino acids. Each peptide was further equipped with a C-terminal cysteine as chemical handle for the thioether cyclization. To account for possible side reactions of cysteine thiol side chains upon peptide cyclization, the same peptides were also printed after conservative exchange of cysteine by serine. The resulting peptide microarrays comprised 318 different peptides printed in duplicate and were framed by Flag (DYKDDDDKGG) and HA (YPYDVPDYAG) control peptides.

Two peptide microarray copies – one with linear peptides and one with constrained peptides after cyclization - were incubated with Rituximab at a concentration of 100 µg/ml. Both arrays were also stained with the secondary antibody in the presence of the anti-Flag antibody. Even at a high antibody concentration of 100 µg/ml, linear CD20 peptides did not show any response with Rituximab (Fig. 2). In contrast, we observed a very clear and strong response with the constrained cyclic CD20 peptides.

Data quantification was followed by generation of intensity plots for the Rituximab assays (Fig. 3). While we did not observe any response of Rituximab against linear CD20 peptides, the constrained cyclic peptides showed clear signals with excellent signal to noise ratios that were attributed to peptides with the consensus motif EPANPSEK. This is in accordance with the previously published co-crystal structure of Rituximab and the 15 amino acid loop NIYNCEPANPSEKNSPSTQYCYSIQ of

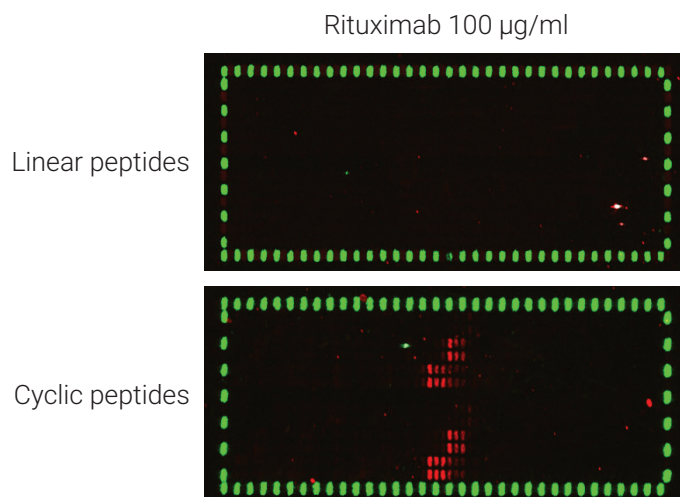


Figure 2. PEPperCHIP® Peptide Microarrays covering the extracellular domain of CD20 translated into overlapping linear (top) and constrained cyclic peptides (bottom). The top half of each array displays the native CD20 peptides with cysteine, the lower half the same CD20 peptides after conservative exchange of cysteine vs. serine. In the cyclic peptide microarray the first double-row in each half corresponds to the 7 amino acid peptides, the second and the third double-row to the 10 and 13 amino acid peptides. The arrays were assayed with 100 µg/ml Rituximab followed by staining with anti-human IgG (red) and anti-Flag (green) antibodies. While linear peptides did not show any Rituximab response, the constrained cyclic peptides exhibited clear epitope stretches. Flag control peptides framing the peptide array showed the expected well-defined spot pattern.

CD20 (1). Moreover we observed a clear dependency of the Rituximab response on peptide length and hence loop sizes: The poor reaction of the cyclic 7 amino acid peptides could be explained by the length of the actual epitope (8 aa). However, the increase in signal intensities from the 10 to the 13 amino acid loops likely resulted of a better mimicry of the native 15 amino acid loop in CD20.

Although the spot intensities of the constrained cyclic CD20 peptides were slightly higher after conservative exchange of oxidation-sensitive cysteine vs. the more stable serine, the difference was less remarkable and underlined the compatibility of the thioether-based peptide cyclization with all main amino acids.

To get a closer view on conserved and variable amino acids of the Rituximab epitope EPANPSEK as well as on tolerated and less tolerated amino acid exchanges, we also performed a PEPperMAP® Substitution Scan based on the constrained cyclic CD20 peptide NIYNSEPANPSEK. We generated a peptide microarray on which all amino acid positions of the given wild type peptide were exchanged by all 20 main amino acids. In contrast to a typical alanine scan, such a full substitution scan covers 20 times more data points with all possible amino acid exchanges, and also accounts for structural and/or functional similarities of amino acid side chains.

Staining with 100 µg/ml Rituximab and secondary antibody gave rise to a clear and well-defined substitution pattern (Fig. 4).

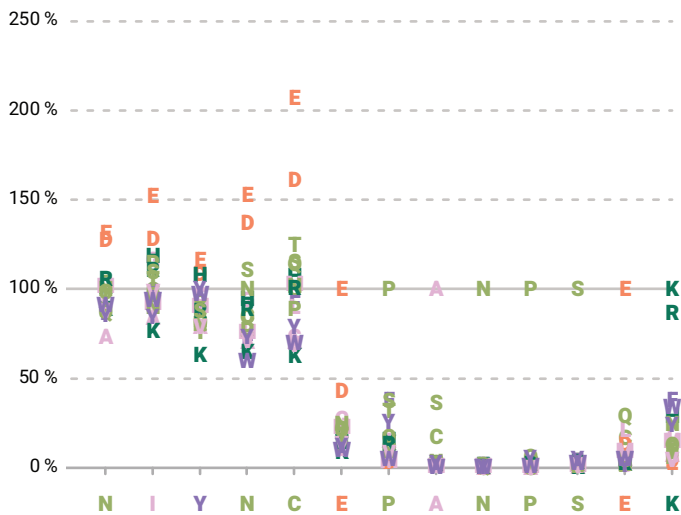


Figure 5. PEPperMAP® Epitope Substitution Scan of wild type peptide 'NIYNCEPANPSEK'¹³. The amino acid plot of the substitution scan of CD20 wild type peptide highlighted a conserved core motif ⁶EPANPSEK¹³ as well as a variable amino acid stretch ¹NIYNC⁵ at the N-terminus. The amino acid plot reflects the intensity ratio of an epitope variant compared to the wild type epitope at 100% and clearly highlights both the degree of sequence conservation as well as tolerated amino acid exchanges for each amino acid position.

enabled an in-depth view of the actual epitope of the chimeric mouse/human monoclonal anti-CD20 antibody Rituximab with detailed information on conserved and variable amino acids. Besides clear data about the absolute essential amino acids ⁹N, ¹⁰P and ¹¹S, the given data set also provides very precise knowledge on preferred and less preferred amino acid exchanges for each amino acid position of the Rituximab epitope EPANPSEK.

Such detailed knowledge of each individual amino acid in an epitope paves the way for a precise bioinformatic analysis of possible cross-reactions of a monoclonal antibody: instead of a standard blast search of the conserved core motif, the consideration of tolerated epitope sequence variants like DPANPSEK clearly levels the significance of in silico cross-reactivity analyses of monoclonal antibodies. The relative intensity data of the amino acid plot should even enable a semi-quantitative prediction of cross-reaction probabilities on a proteome-wide level.

Even more important may be the correlation of essential amino acid positions in the epitope of a therapeutic antibody with sequencing data of patients for patient group recruitment in clinical trials, or the differentiation between responders and non-responders before therapy. The identification of e.g. point mutations in a highly conserved epitope stretch can help minimizing the risk of failure in clinical trials and enables a personalized and more efficient treatment of patients with therapeutic antibodies. Last but not least, the detailed epitope data of the PEPperMAP® Substitution Scan helps to distinguish two similar monoclonal antibodies or a standard antibody from its biosimilar for regulatory purposes.

The present case study on the PEPperMAP® Conformational Epitope Mapping of the monoclonal anti-CD20 antibody including a detailed PEPperMAP® Substitution Scan of the conserved core motif EPANPSEK likely represents the most precise characterization of the binding behavior of Rituximab. The detailed analysis should further underline the potential of peptide microarray-based high resolution epitope mappings with constrained cyclic peptides for conformational epitope mapping.

BIBLIOGRAPHY

1. Du J et al. *J Biol Chem.* (2007) May 18; 282(20): 15073-80. PMID: 17395584