Characterization of Rituximab by Conformational Epitope Mapping and Epitope Substitution Scan

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 so-called 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 alternative and more flexible strategy for peptide cyclization, we applied an on-chip thioether formation between a 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 get an in-depth view on 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.

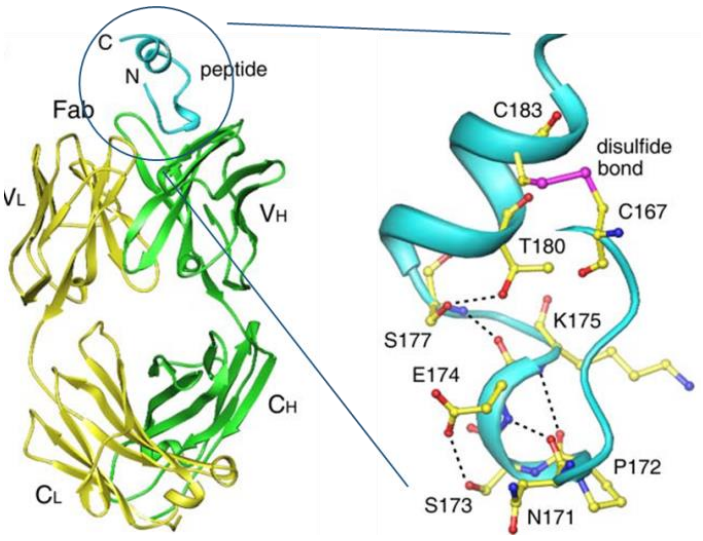

Fig. 1: Structure of the Rituximab Fab-CD20 epitope-peptide complex:[1] The Rituximab heavy chain is colored in green and the light chain in yellow. The CD20 epitope peptide is depicted in cyan.

[1] Du et al., J.Biol.Chem. 2007; 282, 15073-15080.

Results & 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.

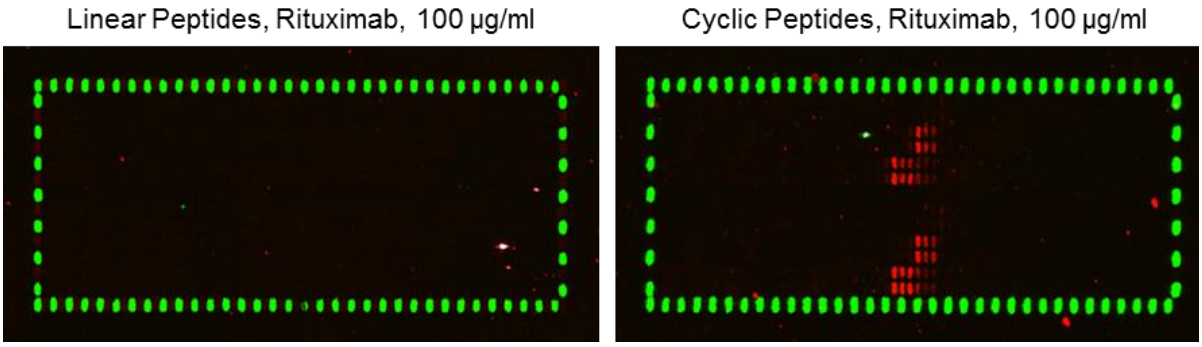

Fig. 2: PEPperCHIP® Peptide Microarrays covering the extracellular domain of CD20 translated into overlapping linear (left) and constrained cyclic peptides (right). The top half of each array displays the native CD20 peptides with cysteine, the lower half the same CD20 peptides after conservative exchange of cysteine vs. serine. 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.

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 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.

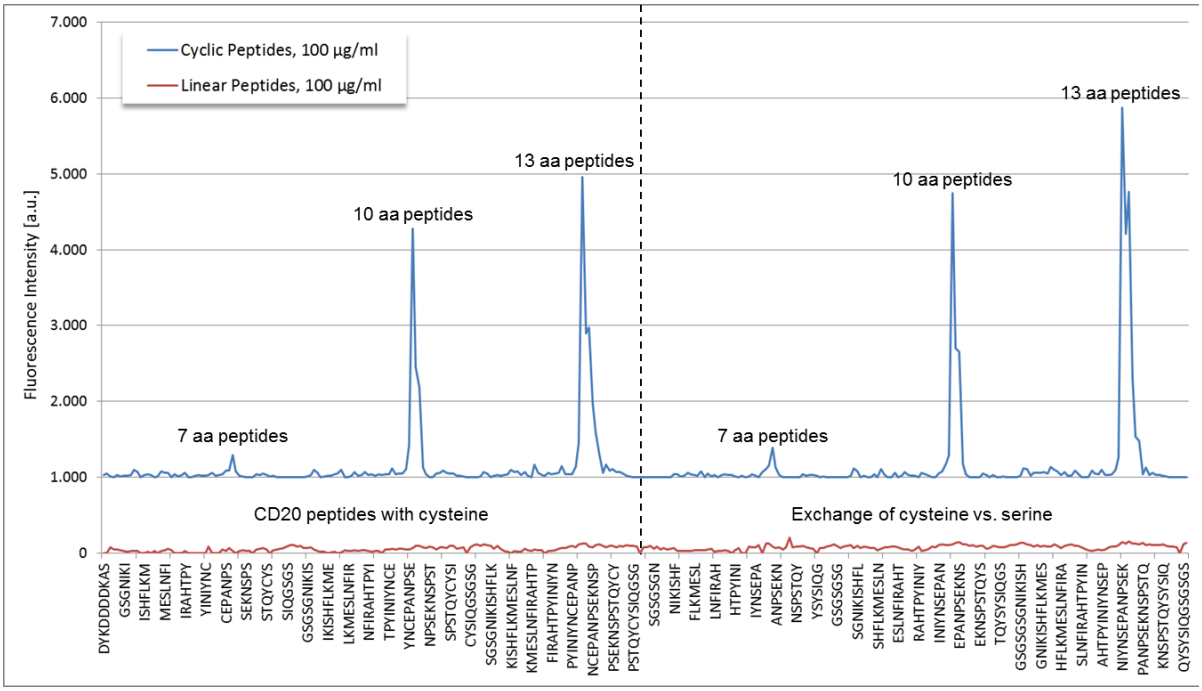

Fig. 3: The intensity plots of the Rituximab assays with linear and constrained cyclic CD20 peptides (the plot of the latter was leveled to provide a clearer data overview) showed clear epitope peaks for constrained cyclic peptides with excellent signal to noise ratios, whereas linear peptides did not reveal any epitope signal. The intensities of the Rituximab response increased with peptide loop sizes, and were slightly higher after conservative exchange of cysteine vs. serine.

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).

In accordance with the high resolution epitope mapping, we validated the conserved Rituximab core epitope ^{6}EPANPSEK^{13} and a variable stretch ^{1}NIYNC^{5} at the N-terminus of the wild type peptide. For an in-depth analysis of conserved and variable amino acids, we generated an amino acid plot with the wild type peptide at 100% and relative intensities for each amino acid exchange at all amino acid positions (Fig. 5). According to the amino acid plot, positions ^{9}N, ^{10}P and ^{11}S showed the highest

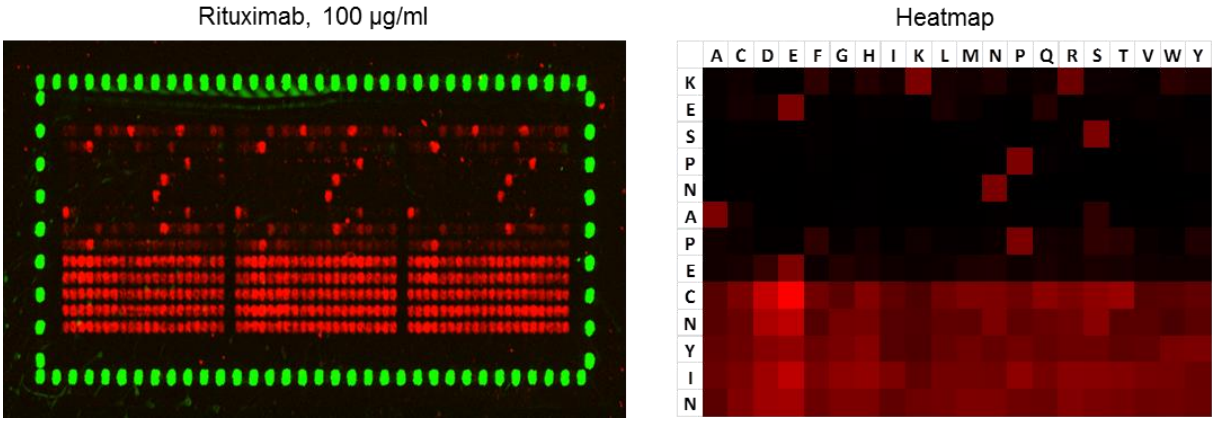

Fig. 4: Substitution Scan array of the wild type Rituximab peptide NIYNCEPANPSEK (left). Each amino acid position was gradually exchanged by the 20 L-amino acids from L-alanine (A) on left to L-tyrosine (Y) on right. Each row represented an amino acid position of the starting peptide ^{1}NIYNCEPANPSEK^{13}, each column exchanges by a certain amino acid. Three single spot subarrays were printed next to each other and framed by Flag control peptides. The array was assayed with 100 µg/ml Rituximab followed by staining with anti-human IgG (red) and anti-Flag (green) antibodies. The five widely invariant rows on bottom of each subarray hinted at variable amino acid positions close to the N-terminus of ^{1}NIYNCEPANPSEK^{13}, while the rows with few active peptide spots on top represented the conserved core motif close to the C-terminus. The same substitution and response pattern was also reflected by the heatmap with averaged spot intensities of all three subarrays (right).

degree of sequence conservation with a complete loss in antibody binding upon exchange by any other amino acid. Amino acid positions ^{6}E, ^{7}P and ^{8}A were well conserved; substitution by other amino acids like D (^{6}E), F, S or T (^{7}P) or S and C (^{8}A) resulted at least in a 50% loss of spot intensities and hence of Rituximab binding.

A similar degree of sequence conservation was found for C-terminal amino acid positions ^{12}E and ^{13}K; exchange of ^{12}E by Q caused an approx. 60-70% decrease of spot intensities, while other amino acids were even less tolerated. Amino acid position ^{13}K was susceptible for conservative exchange by R without any remarkable loss in antibody binding. Replacement by other amino acids, however, resulted in more than 50% reduced spot intensities and accordingly in a significantly reduced binding of Rituximab.

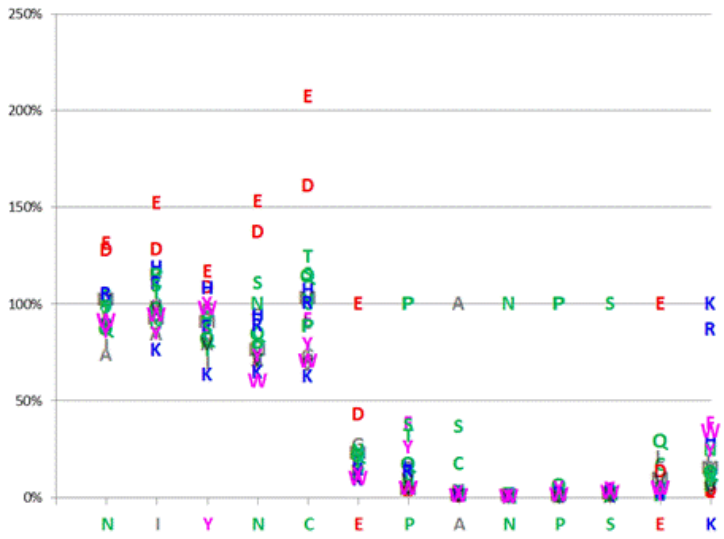

Fig. 5: The amino acid plot of the substitution scan of CD20 wild type peptide ^{1}NIYNCEPANPSEK^{13} highlighted a conserved core motif ^{6}EPANPSEK^{13} as well as a variable amino acid stretch ^{1}NIYNC^{5} at the N-terminus. The amino acid plot reflects the intensity ratio of an epitope variant compared 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.

Conclusion

Supplemental to the high resolution conformational epitope mapping, the PEPperMAP® Substitution Scan of the constrained cyclic CD20 peptide ^{1}NIYNCEPANPSEK^{13} enabled an in-depth view on 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 ^{9}N, ^{10}P and ^{11}S, 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 a detailed knowledge of each individual amino acid of 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 DPANPSER 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.