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Downsizing Nanobodies: Towards CDR Loop Mimetics Modulating Intracellular Protein-Protein Interactions

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Introduction

Monoclonal antibodies (ca. 150 kDa) display remarkable specificity and high affinity binding capabilities. However, their therapeutic application is associated with some limitations, including limited *in cellulo* access and high therapeutic costs. These drawbacks entailed the development of miniaturized functional antibody fragments (ca. 15-50 kDa) such as antigen-binding fragments (Fab), single-chain variable fragments (scFv) and recombinant variable single domains such as Nanobodies (Nb) [1]. In addition, smaller synthetic peptides based on (parts of) the antigen-binding site of antibodies or Nanobodies have been investigated as antibody mimetics, although so far with limited success [2].

In this context, we explored the feasibility of developing Nanobody-based peptide mimetics of limited molecular weight (1-2 kDa). Using a structure-based design strategy, several peptidomimetics were developed from the interacting complementarity-determining regions (CDR) of a Nanobody. This Nanobody modulates the nucleotide exchange activity on RAS proteins by interaction with the intracellular RAS:SOS1 complex. The significance of this protein-protein complex is related to the RAS protein. RAS proteins cycle between inactive GDP-bound states and active GTP-bound states, thereby controlling downstream cellular signaling pathways which impact cell proliferation, growth, differentiation, and survival [3]. The cycling between the active and inactive RAS protein states is regulated by guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs). This tight control is deregulated in case of activating mutations in RAS proteins, which may lead to the development of multiple human cancer types and developmental disorders [4]. Direct inhibition of RAS proteins has been perceived as a challenging task [5]. Therefore, modulation of RAS proteins through the regulatory GEF SOS1 represents an attractive alternative therapeutic target.

Results and Discussion

Upon discovery of a Nanobody which modulates the nucleotide exchange activity on RAS proteins by interaction with the intracellular RAS:SOS1 complex, we explored the feasibility to develop Nanobody mimetics based on its crucial complementarity-determining regions (CDR). This was guided through a biochemical nucleotide exchange assay, which follows the nucleotide exchange rate of RAS:GDP towards RAS:GTP catalyzed by SOS1. A co-crystal structure of the Nanobody bound to the RAS:SOS1 protein complex identified the CDR3 loop as an important contributor to the interaction by penetration within a hydrophobic pocket on SOS1 through a β-turn. A linear peptide representing the CDR3 amino acid sequence-maintained ca. 15% of the maximum activation rate of the Nanobody, although with a significantly weakened potency of 500 μM.

Subsequently, multiple peptide analogues were synthesized wherein amino acids residues were substituted. These substitutions indicated the importance of residues located at the β-turn portion of the CDR3 loop for the interaction with SOS1. Introduction of cationic residues at the Ile position resulted in efficacy improvements, especially in case of a D-Arg, which doubled the efficacy of the native peptide. A co-crystal structure of this peptide bound to the RAS:SOS1 complex showed a positioning of the peptide within the same binding pocket as the Nanobody CDR3 loop, although without stabilization of the β-turn structure.

Therefore, the peptide was cyclized by a lactam bridge at the terminal positions to potentially stabilize the formation of the β-turn conformation. Remarkably, this led to an improvement of the potency by a factor 7 compared to the native peptide. In this case, a co-crystal structure of this peptide

bound to the RAS:SOS1 complex shows a strong overlap of the top residues of the peptide with the Nanobody CDR3 loop, thereby validating the anticipated structural stabilization by introduction of the lactam tether. This co-crystal indicated a positioning of the D-Arg side chain towards a negative surface patch of SOS1.

To improve the contact of the D-Arg with SOS1, it was substituted by the extended analogues D- homoarginine and D-Phe(4'-guanidino). This led to either an improvement in the efficacy or potency of the peptides. For the peptide containing the D-Phe(4'-guanidino) residue, a co-crystal structure showed a reorientation of two Glu residues of SOS1 allowing efficient contacts with the amino acid side chain.

To further reduce the conformational flexibility of the peptide, the lactam bridge was substituted by a 1,4-disubstitued 1,2,3-triazole bridge. In addition, the Ala residue was deleted to provide an improved stabilization of the β-turn conformation. This resulted in a peptide showcasing 55% of the maximal activation by the parent Nanobody. Although similar binding positions and contacts were observed as the peptide with the lactam bridge in a co-crystal structure, this peptide allows the formation of more productive hydrogen bonds in the β-turn conformation, comparable to the parent Nanobody CDR3 loop.

By application of a structure-based design strategy, and guided by a biochemical GEF assay, we developed CDR3 loop mimetics able to mimic 55% of efficacy of the parent Nanobody, upon substitution with some (un)natural amino acids and introduction of cyclization tethers. In addition, selected peptidomimetics were found to structurally mimic the native Nanobody CDR3 loop, presenting similar binding poses upon interaction with the RAS:SOS1 complex (as demonstrated by X-ray analysis). With this study, we provided the first solid proof-of-principle that small peptidomimetics can be developed which mimic both structurally and functionally protein-protein interaction modulatory Nanobodies.

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