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Structure-guided engineering of Anticalins with improved binding behavior and biochemical characteristics for application in radio-immuno imaging and/or therapy

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ABSTRACT

Modern strategies in radio-immuno therapy and *in vivo* imaging require robust, small, and specific ligand-binding proteins. In this context we have previously developed artificial lipocalins, so-called Anticalins, with high binding activity toward rare-earth metal–chelate complexes using combinatorial protein design. Here we describe further improvement of the Anticalin C26 via *in vitro* affinity maturation to yield CL31, which has a fourfold slower dissociation half-life above 2 h. Also, we present the crystallographic analyses of both the initial and the improved Anticalin, providing insight into the molecular mechanism of chelated metal binding and the role of amino acid substitutions during the step-wise affinity maturation. Notably, one of the four structurally variable loops that form the ligand pocket in the lipocalin scaffold undergoes a significant conformational change from C26 to CL31, acting as a lid that closes over the accommodated metal–chelate ligand. A systematic mutational study indicated that further improvement of ligand affinity is difficult to achieve while providing clues on the contribution of relevant side chains in the engineered binding pocket. Unexpectedly, some of the amino acid replacements led to strong increases – more then 10-fold – in the yield of soluble protein from periplasmic secretion in *Escherichia coli*.

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1. Introduction

Today, recombinant antibodies (or immunoglobulins, Igs) represent an approved and potent class of biopharmaceuticals with broad application due to their high target specificity, therapeutic efficacy, safety, and accessibility via standardized manufacturing routes (Reichert, 2011). Available in a humanized or even fully human form, they can be applied in biomedical diagnostics as well as for the treatment of cancer, autoimmune and inflammatory diseases. Nevertheless, conventional antibodies suffer from several disadvantages, especially with regard to the high effort of cell culture production, their relatively complex biomolecular constitution, immunological side effects due to the effector functions of the constant region (Fc), and – in the context of *in vivo* imaging – their long circulation in plasma. The development of truncated forms of antibodies such as Fab fragments, single chain fragments (scFv) or diabodies is one approach to circumvent these problems (Holliger and Hudson, 2005). An alternative strategy is the use of non-Ig protein scaffolds with reprogrammed binding specificity (Gebauer and Skerra, 2009; Koide, 2010; Skerra, 2007b). Among those, the Anticalins constitute highly specific small binding proteins with potential as next generation biopharmaceuticals or as diagnostic reagents (Gebauer and Skerra, 2012; Skerra, 2007a; Skerra, 2008).

Anticalins are engineered proteins based on the scaffold of natural lipocalins, a class of small soluble proteins (160–180 residues) found in many organisms like vertebrates, bacteria, plants and, in particular, in humans (Åkerström et al., 2006). Usually, lipocalins are involved in the storage, transport or sequestration of a broad spectrum of biomolecules, e.g. vitamins, lipid-like substances, and hormones. Within the lipocalin family, the structural constitution is highly conserved, although the amino acid sequence identity is surprisingly low (Flower, 1996). A cup-shaped ligand pocket is formed by eight antiparallel β-strands that wind around a central axis and support four structurally flexible loops at the open end. The opposite end is closed by short loops, and an α-helix is attached to one side of the β-barrel.

Abbreviations: DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DTPA, diethylene triamine pentaacetic acid; PRIT, pretargeted RIT; RID, radio-immuno diagnostics; RIT, radio-immuno therapy; RMSD, root mean square deviation; SPR, surface plasmon resonance; SEC, size exclusion chromatography.

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Resting on the simple biomolecular architecture of lipocalins and their beneficial biochemical characteristics as small and robust plasma proteins, engineered Anticalins provide several advantages as a novel class of biopharmaceuticals in comparison with antibodies: (i) low risk of immunological side reactions due to the missing Fc region, (ii) facile and inexpensive production in bacteria or other microbial host organisms as a single polypeptide chain, (iii) shorter – yet, adjustable – circulation time in the blood due to their small size. Lipocalins can be reprogrammed with regard to ligand specificity – in a process resembling the selection of recombinant antibody fragments via phage display from synthetic libraries (Gebauer and Skerra, 2012) – using targeted random mutagenesis at defined amino acid positions that are likely responsible for binding of a prescribed ligand. In this manner, Anticalins have been selected against diverse targets, including small molecules, e.g. digoxigenin (Schlehuber et al., 2000) or Me·DTPA complexes (Kim et al., 2009), and large antigens, e.g. the cell surface receptor CTLA-4 (Schönfeld et al., 2009) or the extracellular matrix fibronectin ED-B (Gebauer et al., 2013), thus addressing a broad range of biomedically relevant molecular structures.

In radio-immuno therapy (RIT) and diagnostics (RID, *in vivo* imaging), tumors are targeted with radionuclides that have cell-killing function through their high energy radiation, inducing double-strand DNA breaks and triggering cellular apoptosis, or that provide long range radiation such as γ-photons which permit spatial detection. Conventionally, a metal–chelate complex is chemically coupled to a tumor-targeting protein, typically a highly specific antibody. Established chelators in clinical use are DOTA and DTPA as well as their derivatives, which can complex a broad range of rare-earth metals offering diverse characteristics concerning radioactive half-life, radiation type and range (Milenic et al., 2004). Today, several radionuclide-conjugated antibodies or antibody fragments are already approved by regulatory authorities, for example ibritumomab tiuxetan (Zevalin®) (Tomblyn, 2012), or are under preclinical development, like CHX-A''-DTPA-labeled MORAb-009 (Shin et al., 2011).

The main disadvantage of this direct labeling approach in conjunction with full size antibodies is the low signal-to-noise ratio due to the long circulation in blood and the poor tissue penetration of the large biomolecular conjugate, which leads to dose-limiting effects on sensitive organs such as bone marrow, liver, and kidney (Kaur et al., 2012; Steiner and Neri, 2011). Due to this handicap, antibody fragments in different formats, like Fab, scFv, diabodies or minibodies, are emerging as preferred reagents for *in vivo* imaging (Kenanova and Wu, 2006), including the approved Fab fragments ^{99m}Tc-Arcitumomab (CEA-Scan®), ^{99m}Tc-Sulesomab (Leukoscan®), and ^{99m}Tc-Igovomab (Indimacis®) (Boswell and Brechbiel, 2007; Kaur et al., 2012). However, the monovalent Ig fragments suffer from lower avidity and very rapid clearance from circulation. Furthermore, the scFv format is prone to renal uptake which limits clinical use. Even their divalent form, i.e. radiometal-conjugated diabodies, can cause renal damage in mice, despite improved tumor retention (Kenanova and Wu, 2006).

In this context, Affibodies – as one class of non-Ig protein scaffolds – have appeared as novel reagents for *in vivo* imaging and RIT (Löfblom et al., 2010; Miao et al., 2011). They offer a tiny size and good tissue penetration while lacking cross-reactivity with immune receptors. Similar to the approved Fab fragments, Affibodies are directly labeled with radionuclides – or their chelate complexes. Affibodies directed against the breast cancer target Her2 as well as the EGF receptor, which is overexpressed in various solid tumors, have shown promise in preclinical and early clinical studies (Ahlgren et al., 2009; Tolmachev et al., 2010). However, due to the bacterial origin of the scaffold (i.e. protein A from *Staphylococcus aureus*) an immune reactivity has to be expected. Generally, conventional methods for direct labeling – such as random iodination of Tyr side chains or Lys-/Cys-mediated chemical coupling of chelators – often leads to a loss in binding activity and, in contrast with peptides, the harsh conditions required for metal charging of DOTA, for example, pose a risk for protein denaturation.

Another solution to achieve high tumor to blood ratio is offered by upcoming pretargeting approaches, where the primary tumor targeting process is uncoupled from the tissue-specific deposition of the radionuclide (Sharkey et al., 2005). To this end, a bifunctional protein that provides both binding specificity toward a cell surface-exposed tumor marker and tight binding activity for a radiometal–chelate complex is initially applied. After accumulation at the site of the tumor and clearance of unbound protein from circulation, the metal–chelate complex is administered on its own, getting captured at the tumor while excess radioactivity is quickly excreted via the kidney. So far, this strategy was dominated by the use of bispecific antibodies (Sharkey et al., 2012), which are difficult to manufacture, or of complex conjugates between tumor-specific antibodies and other affinity reagents, in particular streptavidin (Goldenberg et al., 2006; Sato et al., 2005), thus providing challenges with regard to defined clinical product composition as well as immunogenicity. Consequently, there is a need for novel and smart bispecific protein reagents that can be easily prepared using convenient expression systems and that conform with the requirements of current biopharmaceutical drug development.

In this regard, Anticalins appear as particularly attractive building blocks. Due to their simple fold, comprising a single polypeptide chain with no or (usually) just one disulfide bond, they allow the facile manufacturing of bispecific fusion proteins, either by linking two different Anticalins to yield so-called Duocalins (Schlehuber and Skerra, 2001) or, for example, by fusion to an antibody fragment (scFv or Fab) or another alternative protein scaffold. For applications in the field of RIT and/or RID, we have previously generated an Anticalin with specificity for Me·DTPA complexes. This Anticalin (C26), which was derived from the natural human lipocalin 2 (Lcn2), specifically binds lanthanide (III) ions as chelate complexes with *p*-NH_2-Bn-CHX-A''-DTPA in a low nanomolar to picomolar affinity range (Kim et al., 2009).

Starting from a naive Lcn2 library with 12 randomized positions, several Me·DTPA specific Anticalin candidates had been selected using filamentous phage display. In a subsequent *in vitro* affinity maturation process, the binding characteristics of one promising candidate were further optimized using partial random mutagenesis, followed by bacterial colony and ELISA screening. The final Anticalin C26 recognizes Y·DTPA (conjugated to a carrier protein) with a dissociation constant of 400 pM. Crystal structure analyses of two Anticalin candidates derived earlier during this study, one of which will be presented here, have provided detailed insight into the complexation of the Y·DTPA ligand within the engineered binding pocket. This structural information has prompted us to further engineer the Me·DTPA specific Anticalin C26 in a semi-rational manner, which will be described in this report.

2. Materials and methods

2.1. Preparation of Me·DTPA chelate conjugates and complexes

365 nmol bovine pancreatic ribonuclease A (RNaseA; Fluka Chemie, Buchs, Switzerland) was dissolved in 1 mL $NaHCO_3$ pH 8.3 (>99.5%; Carl Roth, Karlsruhe, Germany) and reacted with a solution of 1.8 μmol (corresponding to fivefold molar amount) of [(*R*)-2-amino-3-(4-isothiocyanatophenyl)propyl]-*trans*-(*S*,*S*)-cyclohexane-1,2-diaminepentaacetic acid (*p*-SCN-Bn–CHX-A''-DTPA; Macrocyclics, Dallas, TX) in 10 μL dimethylformamide (DMF) over night at 4 °C under agitation. Excess reagents were removed by gel filtration on a PD-10 column (GE Healthcare, Freiburg, Germany)

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