

Euro Immunology 2017-Single domain antibodies for the knockdown of cytosolic and nuclear proteins- Thomas Boldicke- Helmholtz Centre for Infection Research

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Abstract

Single domain antibodies (sdAbs) from camels or sharks comprise only the variable heavy chain domain. Human single domain antibodies comprise the variable domain of the heavy chain or light chain. SdAbs are stable, non-aggregating. Ricin is an extremely potent biological toxin derived from the castor bean (*Ricinus communis*). It has a long history in the development of immunotoxins aimed at combating B cell lymphomas and other cancers. Yet, ricin is equally renowned as a biothreat agent, especially if dispersed by aerosol. Ricin's galactose/N-acetylgalactosamine-binding lectin subunit, RTB, mediates toxin endocytosis and retrograde transport to the endoplasmic reticulum (ER) of mammalian cells. In the ER lumen, ricin's enzymatic subunit, RTA, is liberated from RTB and retro-translocated into the cytoplasm where it inactivates ribosomes with remarkable efficiency. Activation of the ribotoxic stress response (RSR) and multiple stress-activated protein kinase (SAPK) pathways ensue, resulting in the triggering of programmed cell death pathways. In the context of the lung, ricin triggers acute lung injury characterized by a massive inflammatory response driven by IL-1, IL-6 and members of the tumor necrosis factor- α (TNF- α) superfamily, triggering destruction of the lung epithelium, vascular leak, and edema.

The catalytic mechanism by which RTA disables mammalian ribosomes was elucidated three decades ago when the X-ray crystal structure of ricin and its enzymatic activities were resolved more or less simultaneously. Endo and colleagues demonstrated that RTA is an RNA N-glycosidase (EC 3.2.2.22) that depurinates a single adenosine residue within the sarcin-ricin loop (SRL) of the 28S rRNA, an activity measurable in *in vitro* translation assays. The SRL, one of the longest conserved stretches of rRNA sequence, makes direct interactions with the GTP-binding domains of elongation factors like EF-Tu and is therefore indispensable for peptide elongation. The depurination reaction is confined to RTA's active site, a large solvent-exposed cleft on one face of the molecule that accommodates the protruding adenine (A) within the conserved GAGA motif of the mammalian SRL. The five critical residues associated with RTA's enzymatic activity have been defined by site-directed mutagenesis and include Tyr80, Tyr123, Glu177, Arg180, and Trp211. Tyr80 and Tyr123 serve to stabilize the adenine base substrate via a π -stacking network. Arg180 is involved in protonation of the adenine leaving group while Glu177 stabilizes the actual cleavage of the N-glycosidic bond.

The role of Trp211 in catalysis remains unknown. These catalytic residues, as well as the chemistry of the SRL depurination reaction is conserved among other members of the ribosome-inactivating protein (RIP) superfamily of toxins, including Shiga toxins 1 (Stx1) and 2 (Stx2) from foodborne *Escherichia coli*.

With the capacity to inactivate >1500 ribosomes per minute (10), RTA's active site is an obvious target to consider when designing therapeutics to arrest the effects of ricin toxin exposure. In fact, early efforts successfully identified substrate analogues (e.g., pteronic acid, guanine-like compounds) with modest RTA inhibitory activity *in vitro*, while other groups identified molecules capable of trapping RTA's active site in a closed conformation. However, issues related to solubility, limited potency and/or biodistribution have severely curtailed the use of those small molecule inhibitors in cell-based assays and animal models of ricin intoxication. High-throughput, cell-based screens run in parallel as a complementary means of identifying novel ricin inhibitors yielded compounds that targeted host proteins associated with toxin trafficking and SAPK pathways, but not ricin itself.

In the past decade, camelid-derived, single-domain antibodies, commonly referred to as VHHs or nanobodies, have received enormous attention for their potential as therapeutics against emerging infectious disease and biothreat agents, including botulinum neurotoxin (BoNT), anthrax toxin, and Shiga toxin. VHHs are small (13-16 kDa) immunoglobulin elements amenable to expression in *E. coli* and surface display on bacteriophage M13. VHHs are also highly soluble and thermostable. Of particular relevance to RTA is the reported propensity of VHHs to target active site clefts and enzymatic pockets, as shown for lysozyme, α -amylase and others. We recently described a collection of 21 VHHs that bind in immediate proximity to or overlapping with RTA's active site, as demonstrated by epitope mapping studies using hydrogen deuterium exchange (HDX). In this report we have characterized seven of those VHHs and demonstrate that three are potent inhibitors of RTA's enzymatic activity in *in vitro* assay and when expressed as intracellular antibodies ("intrabodies") within the cytoplasm of target cells. We then solved X-ray crystal structures of each of the VHHs in complex with RTA, which revealed direct interactions with the catalytic residues associated with depurination of the SRL.

Results

Identification of VHHs with potent RTA inhibitory activity

We recently identified, through a strategic series of masking and targeted elutions, a collection of 21 VHHs that recognized spatially-distinct epitopes along the rim of RTA's active site. A total of seven were chosen for further examination, ultimately because we were able to successfully solve the crystal structure of each in complex with RTA. Two of the VHHs, V2A11 and V6H8, derive from different alpaca libraries but share high

degree of CDR3 primary amino acid sequence identity (69%), possibly indicative of a similar mode of interaction with RTA. Three other VHHs, V8E6, V6A7 and V6A6, constitute a clonal family, as evidenced by >80% identity in CDR3. In addition, V6D4 was one of only two VHHs among the nearly two dozen previously characterized active site-targeting VHHs with detectable toxin-neutralizing activity in a Vero cell assay. The binding affinities of the seven VHHs for RTA were determined by surface plasmon resonance (SPR) and ranged from 0.3 nM to ~11 nM