Intracellular Neutralization of Ricin Toxin by Single Domain Antibodies Targeting the Active Site Pocket

Michael J. Rudolph^{1,*,#}, Timothy F. Czajka^{2,3,#}, Simon A. Davis¹, Chi My Thi Nguyen¹, Xiaoping Li⁴, Nilgun E. Tumer⁴, David J. Vance², and Nicholas J. Mantis^{*,2,3}

¹New York Structural Biology Center, New York, New York 10027; ²Division of Infectious Diseases, Wadsworth Center, New York State Department of Health, Albany, NY, 12208; ³Department of Biomedical Sciences, University at Albany, Albany, NY 12201 United States; ⁴, Department of Plant Biology, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901

[#], these authors contributed equally to this work

^{*}, to whom correspondence should be addressed; MJR, mrudolph@nysbc.org; NJM, nicholas.mantis@health.ny.gov

Running title: Occupancy of Ricin's Active Site

Abstract

The extreme potency of the plant toxin, ricin, is due to its enzymatic subunit, RTA, which inactivates mammalian ribosomes with near perfect efficiency. Here we characterized, at the functional and structural levels, seven alpaca single-domain antibodies (V_HHs) previously reported to recognize epitopes in proximity to RTA's active site. Three of the V_HHs, V2A11, V8E6 and V2G10, were potent inhibitors of RTA *in vitro* and protected Vero cells from ricin when expressed as intracellular antibodies ("intrabodies"). Crystal structure analysis revealed that the complementarity-determining region 3 (CDR3) elements of V2A11 and V8E6 penetrate RTA's active site and interact with key catalytic residues. V2G10, in contrast, sits atop the enzymatic pocket and occludes substrate accessibility. The other four V_HHs also penetrated/occluded RTA's active site, but lacked sufficient binding affinities to outcompete RTA-ribosome interactions. Intracellular delivery of high-affinity, single-domain antibodies may offer a new avenue in the development of countermeasures against ricin toxin.

Introduction

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 (1). Yet, ricin is equally renowned as a biothreat agent, especially if dispersed by aerosol (2). 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 (3, 4). 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 (5).

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 (6, 7). 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 (6). 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 (8). 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* (9).

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., pterioic acid,

guanine-like compounds) with modest RTA inhibitory activity *in vitro* (9), while other groups identified molecules capable of trapping RTA's active site in a closed conformation (11). 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 (12). 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 (13, 14).

In the past decade, camelid-derived, single-domain antibodies, commonly referred to as V_HHs 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 (15-18). V_HHs are small (13-16 kDa) immunoglobulin elements amenable to expression in *E. coli* and surface display on bacteriophage M13. V_HHs are also highly soluble and thermostable. Of particular relevance to RTA is the reported propensity of V_HHs to target active site clefts and enzymatic pockets, as shown for lysozyme, α -amylase and others (19, 20). We recently described a collection of 21 V_HHs that bind in immediate proximity to or overlapping with RTA's active site, as demonstrated by epitope mapping studies using hydrogen deuterium exchange (HDX) (21, 22). In this report we have characterized seven of those V_HHs 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 V_HHs in complex with RTA, which revealed direct interactions with the catalytic residues associated with depurination of the SRL.

Results

Identification of V_HHs with potent RTA inhibitory activity.

We recently identified, through a strategic series of masking and targeted elutions, a collection of 21 V_HHs that recognized spatially-distinct epitopes along the rim of RTA's active site (**Figure S1**) (21, 22). 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 (**Table 1; Figure S1**). Two of the V_HHs, 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 (**Figure S1**). Three other V_HHs, V8E6, V6A7 and V6A6, constitute a clonal family, as evidenced by >80% identity in CDR3 (**Figure S1**). In addition, V6D4 was one of only two V_HHs among the nearly two dozen previously characterized active

site-targeting V_HHs with detectable toxin-neutralizing activity in a Vero cell assay (**Figure S1**). The binding affinities of the seven V_HHs for RTA were determined by surface plasmon resonance (SPR) and ranged from 0.3 nM to ~11 nM (**Table 1: Figure S2**).

The effect of the seven V_HHs on RTA's capacity to inactivate ribosomes was evaluated in a cell-free, luciferase-based *in vitro* translation (IVT) assay, as described in detail in the Materials and Methods. RTA (EC₉₀; 0.75 nM) was mixed with V_HHs across a dose range and then added to the IVT cocktail. Luciferase production at 90 min served as the proxy for protein synthesis and ribosome activity. Three V_HHs (V2A11, V8E6 and V2G10) had high RTA inhibitory activity (>70%), one (V6A7) had intermediate activity (30-70%), and the remaining three V_HHs (V6A6, V6H8 and V6D4) had low or undetectable inhibitory activity (**Figure 1A**). The ability of the seven V_HHs to neutralize RTA was dose-dependent (**Figure 1B**) and correlated directly with binding affinity (K_D; **Tables 1, S1**). The relationship between binding affinity and RTA inhibition is exemplified by the three clonally related V_HHs, V8E6, V6A7 and V6A6, whose RTA neutralizing activities (high, medium and low, respectively) tracked inversely with their dissociation constants (0.42 nM, 4.2 nM and 10.9 nM, respectively; **Table 1**).

To confirm that the V_HHs actually interfere with RTA's RNA N-glycosidase activity, we tested the antibodies in an rRNA depurination reaction with yeast ribosomes as substrate (23). Overall, the trends were nearly identical to what was observed in the IVT assay: V2A11, V8E6 and V2G10 were potent inhibitors of RTA's depurination activity, while V6H8, V6A6 and V6D4 were weak inhibitors (**Figure 1 C, D**). V6A7 and V6H8 inhibited RTA in the depurination assay more effectively than in the IVT assay, possibly reflecting slight differences in the sensitivity and/or stringency of the assays.

In previous studies we and others have reported a relationship between antibody binding affinity and toxin-neutralizing activity, at least in cell-based assays (22). Therefore, we performed Spearman correlation analysis to examine the relationship between *in vitro* RTA inhibition and V_HH binding affinity at both high and low antibody:RTA molar ratios (**Figure 1E,F**). There was indeed a correlation between RTA inhibitory activity and V_HH dissociation constants (K_D), although a stronger relationship with RTA inhibitory activity and dissociation rates (K_{off}). These results indicate that the stability of the antibody-antigen complex is critical for potent inhibition of ricin in cell based killing assays and RTA in *in vitro* assays.

RTA-specific V_HH expressed as intrabodies render Vero cells resistant to ricin toxin. With the exception of V6D4, the V_HHs described above do not to have toxin-neutralizing activity in a cell-based cytotoxicity assay (Figure S1) (21, 22). Nonetheless, we hypothesized that the V_HHs, by virtue of the fact that they are postulated to bind epitopes in immediate proximity of RTA's active site, could protect Vero cells from the effects of ricin if they were successfully expressed in the cell cytoplasm as intracellular antibodies or "intrabodies." Intrabodies, which refers to the cytosolic or organelle-specific expression of antibody variable regions in the form of single chain Fv (scFv) or V_HHs, have been exploited as tools to knock-down intrinsic cytosolic targets like the EGF receptor, as well as extrinsic cytosolic targets like viral antigens and even botulinum neurotoxin (BoNT) (17, 24). Theoretically, RTA is an ideal intrabody target, since it is exposed to the cytosolic environment immediately following retro-translocation across the ER membrane.

To test this hypothesis, we constructed pCDNA3.1-based mammalian expression vectors encoding the cDNA for each of the seven RTA-specific V_HHs under control of the CMV promoter, essentially as done by Tremblay and colleagues for intracellular expression of BoNT-specific V_HHs (17). The antibodies were also designed to include a C-terminal E-tag (GAPVPYPDPLEPR) to enable detection in ELISA using commercially available anti-E tag antibodies. The resulting V_HH-encoding DNA expression vectors were emulsified in liposome nanoparticles (LNP) and used to transiently transfect Vero cells. To assess whether the V_HHs were functionally expressed in Vero cells, cell lysates were collected two days following transfection and evaluated by RTA and ricin ELISAs. Expression of six of the seven V_HHs was confirmed by ELISA (**Figures S3**). Only V6D4 was not detected in Vero cell lysates, possibly due to improper folding and/or instability in the intracellular compartment. As will be shown later in this manuscript, V6D4 is the only V_HH among the seven with a non-canonical disulfide bond between CDR2 and CDR3, which potentially renders the antibody susceptible to the reducing environment of the cytoplasm.

Nonetheless, to examine the capacity of the putative active-site targeting V_HHs to protect Vero cells against ricin-induced death, cells were transiently transfected as described above and then challenged with ricin 24 h later (**Figure 2A**). Vero cell viability was assessed after an additional 48 h incubation period. As controls, Vero cells were transfected with the BoNT-specific V_HH, ciA-H7 (17) or simply mock transfected with empty LNPs. Vero cells were treated

with an escalating scale of ricin toxin and a shift in EC₅₀ values relative to mock-transfected cells was interpreted as being the result of intracellular V_HH activity.

We found that Vero cells transfected with V_HHs V2A11, V8E6, or V2G10 were significantly more resistant to ricin, as compared to mock transfected cells. For example, cells transfected with V2A11 or V2G10 resulted in ~20-fold increase in EC₅₀ values, while transfection with V8E6 resulted in ~6-fold change (**Figure 2B-I**). These three V_HHs (V2A11, V8E6, or V2G10) were also the most effective at neutralizing RTA *in vitro*, as shown in **Figure 1**. Transfection with V6A7 rendered the Vero cells resistant to low dose (but not high dose) ricin challenge, whereas the three V_HHs with the lowest binding affinities for RTA, namely V6H8, V6A6, and V6D4, afforded no benefit. These results represent the first demonstration of intrabody-mediated targeting of RTA and highlight the potential application of V_HH-based therapies in combatting other structurally conserved RIPs like Shiga toxins 1 and 2, whose active sites are structurally conserved with RTA (25).

Structural analysis of RTA-V_HH complexes reveals antibody contacts with active site residues. To better define the molecular basis of intrabody-mediated ricin toxin neutralization, we solved the X-ray crystal structures of each of the seven V_HHs in complex with RTA, at resolutions ranging from 1.7 to 2.5 Å (**Table 1; Figure 3; Table S2**). All seven V_HHs possessed the canonical immunoglobulin fold, consisting of nine β-strands arranged in two β-sheets with CDRs 1, 2 and 3 on one face of the molecule along with either 2 or 3 ₃₁₀ helices (26). Furthermore, the structure of RTA in each complex consisted of seven α-helices (A-G), three ₃₁₀ helices (₃₁₀ a, ₃₁₀ b, ₃₁₀ c), and 10 β-strands (a-j) (**Figure S4**). RTA (PDB: 1RTC) superposition with the seven V_HH-RTA structures revealed RMSD values ranging from 0.6 to 0.7 Å, demonstrating that RTA does not undergo any significant conformational changes when complexed with the V_HHs.

Upon initial inspection, the most striking feature of the RTA-V_HH complexes was that five of seven the antibodies, namely V2A11, V6H8, V8E6, V6A7, and V6A6, physically occupied RTA's active site by virtue of their CDR3 elements (**Figure 3A-E**). The five RTA-V_HH complexes were structurally conserved, as evidenced by RMSD values between 0.5 to 2.0 Å when superpositioned (**Figure S5**). The five V_HHs, referred to as Mode 1, are further divisible into two sub-modes (A, B) based on RMSD. Mode 1A, represented by V2A11 and V6H8, have

an RMSD of 1.0 Å when superpositioned (**Figure S5**), while Mode 1B, represented by V6A6, V6A7, and V6E8 has an RMSD of 0.5 to 0.7 Å when superpositioned (**Figure S5**). The remaining two V_HHs, V2G10 (Mode 2) and V6D4 (Mode 3) form lids or caps over RTA's active site pocket, thereby distinguishing them from Mode 1. As will be detailed below, V2A11 (Mode 1A), V8E6 (Mode 1B) and V2G10 (Mode 2) are each potent inhibitors of RTA even though they attack the active site with slightly different binding modalities.

Occupancy of RTA's Active Site by V2A11 and V6H8 (Mode 1A)

Mode 1A consists of two clonally unrelated V_HHs with similar CDR3 sequences: V2A11, a potent inhibitor of RTA, and V6H8, a poor inhibitor of RTA. The V2A11-RTA complex resulted in a buried surface area (bsa) of 1953 Å² and an overall shape complementarity score as measured by the shape correlation (Sc) parameter of 0.617 (**Table 1**). The interaction is dominated by CDR3, which not only penetrates into RTA's active site but also interacts with residues integral to ricin's RNA N-glycosidase activity, Tyr80 and Tyr123, which are involved in stacking the adenine substrate within the SRL. Specifically, V2A11 residue Ser103 sits above RTA Tyr80, while V2A11 residue Trp100 comes within 3.5 Å of RTA Tyr123 (**Figures 4A**). As a result, V2A11 confines Tyr80 in a conformation that would prevent stacking of an adenine base between itself and Tyr123 (**Figure S6**). V2A11's CDR1 and CDR2 have only moderate interactions with RTA, as reflected by bsa of 254 Å² and 290 Å², respectively.

The V6H8-RTA interaction is similar. It results in a total bsa of 2080 Å² and has an Sc score of 0.684 (**Table 1**). The antibody-toxin interaction is also dominated by a CDR3 that penetrates RTA's active site. Moreover, V6H8's Tyr103 obstructs RTA's Tyr80, while V6H8's Trp100 comes within 3.9 Å of RTA's Tyr123 (**Figures 4B**). CDR1 and CDR2 interactions with RTA results in bsa of 401 Å² and 355 Å², respectively. The discrepancy in RTA binding affinity between V6H8 and V2A11 is conceivably due to a lower entropic penalty endured by V2A11 upon RTA binding relative to V6H8. V2A11's CDR1 and CDR2 interact to a lesser degree with RTA than V6H8's CDR1 and CDR2, essentially maintaining greater entropy after RTA interaction. The differences in CDR1 conformation can be explained by several non-covalent interactions unique to V2A11, including a salt bridge between Glu-26 and Lys-99, as well as two hydrogen bonds between the side chain hydroxyl groups of Ser-27 and Ser-29, along with Ser-30's side chain hydroxyl group with the carbonyl oxygen of Ser-27 (**Figure S7A**).

Collectively, these non-covalent interactions support the folding of V2A11's CDR1 residues 27 to 30 into a 3_{10} helix that projects away from RTA, relative to V6H8's CDR1, ostensibly generating higher configurational entropy within V2A11 when bound to RTA favoring tighter binding. Incidentally, if V2A11's CDR1 adopted a similar CDR1 configuration as seen in V6H8 when bound to RTA not only would V2A11 lose the aforementioned configurational entropy but V2A11 would also squander two hydrogen bonds formed by Ser-27 with Ser-29 and Ser-30 along with the Glu-26 to Lys-99 salt bridge while forming only one hydrogen bond between Ser-30 and RTA's Asn-122 as seen with V6H8's CDR1. This would reduce the enthalpic gain upon RTA binding and also diminish binding affinity for RTA. Moreover, there is a salt bridge between Glu-26 and Lys-99 in V2A11's CDR1 that cannot form in V6H8 because of a glycine residue at position 26 (**Figure S7**). The influence of this salt bridge on CDR1 structure is underscored the fact that V6H8's CDR1 shares structural similarity the CDR1 elements of the three V_H Hs in mode 1B (V8E6, V6A7, V6A6), which also have a Gly at residue 26 (**Figure S7**). Finally, V6H8 CDR2 residues 53 to 55 contact RTA to a greater extent than the corresponding residues in V2A11 due to the presence of two salt bridges (CDR2 Arg-55 and RTA Glu-127, and CDR2 Arg-56 and RTA's Glu-135), which ultimately constrains V2A11 CDR2 and further reduces V2A11's entropic penalty when binding RTA (**Figure S7**). Collectively, V2A11's relative higher binding affinity for RTA, compared to V6H8 can be attributed to a lower overall energetic penalty incurred upon engaging with RTA.

Occupancy of RTA's Active Site by V8E6, V6A7 and V6A6 (Mode 1B)

Within Mode 1B, there were notable parallels in how V8E6, V6A7 and V6A6 engaged RTA, which was not surprising considering the three V_H Hs share 85 to 91% amino acid sequence identity (**Figure S1**). V8E6 had the strongest binding affinity ($K_D = 0.42$ nM) for RTA and was the most effective at inhibiting RTA *in vitro* and when expressed as an intrabody (**Table 1; Figure 2**). The co-crystal structure revealed that V8E6 buried 2367 Å² of solvent exposed surface and had the highest shape complementarity in this subgroup with a score of 0.701, accounting for its high binding affinity and potent RTA inhibitory activity (**Figure 3C**).

V8E6's CDR3 formed 8 hydrogen bonds with RTA and contributed 1,188 Å² of buried surface area (**Table 1**). Like the two V_H Hs in described for Mode 1A, V8E6's CDR3 also occupies RTA's active site pocket. CDR3 residue Tyr103 obstructs RTA's Tyr80, while residue

Tyr104 obstructs RTA's Tyr123 (Figure 4C). CDR1 and CDR2 contributions are also significant with 8 H-bonds formed with RTA and a total of 972 Å² of buried surface area.

The co-crystal structure of V6A7-RTA revealed a buried surface area of 2,416 Å² (**Table 1; Figure 3D**) CDR3 forms 10 hydrogen bonds with RTA and accounts for more than half the total buried surface area (1325 Å²). V6A7's CDR3 also occludes key RTA active site residues: Tyr103 obstructs RTA Tyr80, while Tyr105 comes within 3.8 Å of RTA's Tyr123 (**Figure 4D**). As noted in **Table 1**, V6A7 has a slightly lower shape complementarity with RTA (0.672), as compared to V8E6 (0.701), likely contributing to their different binding affinities (4.2 vs. 0.42 269 nM).

Finally, V6A6 buried the largest amount of solvent exposed surface with RTA (bsa = 2,519 Å²), but had the lowest shape complementarity (Sc = 0.657) and the lowest overall binding affinity (10.9 nM) (**Table 1**). V6A6 established four fewer hydrogen bonds with RTA than V6A7, and two fewer salt bridges with RTA than V8E6, possibly explaining its weak binding affinity for RTA. Nonetheless, V6A6's CDR3 residues Tyr-103 and Tyr-104 engage active site residues: Tyr-103 occludes RTA residue Tyr80, while Tyr-104 stacks with RTA Tyr123 at a distance of 3.9 Å (**Figure 4E**). Therefore, binding affinity, rather than epitope specificity, accounts for the failure of V6A6 to inhibit RTA activity in vitro and intracellularly as an intrabody.

Conserved interactions of Mode 1 V_HHs with RTA active site

Taken as a whole, it is remarkable that all five V_HHs in Mode 1 employ CDR3 in a similar fashion to occupy RTA's active site, despite the very limited primary amino acid sequence identity (8-28%) among the CDR3 elements themselves. For example, in all five cases, CDR3 residues 102, 103, or 104 make close contact (2.9 to 3.0 Å) with Tyr80. A second CDR3 residue, Trp100 in V2A11 and V6H8, Tyr104 in V8E6 and V6A6, and Tyr105 in V6A7, are within 3.5 to 3.9 Å of Tyr123. V_HHs in Mode 1 also invariably form an H-bond between CDR3 and RTA's Arg180, the residue responsible for protonating the N-3 atom of the adenine substrate and thereby facilitating hydrolysis of the bond between adenine N-9 and C-1' of the ribose (27).

Ironically, the CDR3 elements also account for the relatively large RMSD (1.4 to 2.0 Å) between Modes 1A and 1B, compared to the RMSD (0.5 to 0.9 Å) within the individual Modes. Specifically, the difference in CDR3 configurations between V_HHs in Mode 1A and Mode 1B is

determined in large part by the residue located at position 100 (101 in V6A7). In Mode 1A that residue is a Trp, while in Mode 1B that residue is a Gly (**Figure S1**). A Gly at position 100 is consequential because it permits bulky residues to be positioned at residue 104 or 105 (e.g., Tyr-105 in V6A7; Tyr-104 in V8E6/V6A6), in addition to influencing the spatial location of abutting residues (Thr-102, Tyr-103, and Tyr-104 in V6A7; Ser-101, Tyr-102, and Tyr-103 in V6A6; Ser-101, His-102, and Tyr-103 in V8E6), relative to the corresponding residues in CDR3 of V2A11 and V6H8 (**Figure S8B**). The CDR3 configurations of Modes 1A and 1B V_HHs are also influenced by CDR1 in that in Mode 1A, residue 32 is a Tyr (V2A11) or a His (V6H8), which would sterically clash with a bulky residue located in CDR3 at position 104 or 105 (Figure S5). Altogether, local structural differences in CDR3 configurations result in appreciably different interactions with RTA between V_HHs in Modes 1A and 1B.

The CDR1 and CDR2 elements also contributed to the interface with RTA, but strictly outside of RTA's active site pocket. In general, CDR1 made contact with the loop region between β -strand h and α -helix C (referred to as loop h-C) while CDR2 interacted primarily with α -helix C, the loop between α -helices C-D, and helix 3_{10b}. Altogether, the level of interaction between each CDR1 and CDR2 differed to some degree with V2A11's CDR1 and CDR2 contributing the least (bsa = 544 Å², 2 H-bonds) and V8E6's CDR1 and CDR2 contributing the most (bsa = 972 Å², 8 H-bonds) to the interface with RTA. Additional contacts with RTA made by each CDR3 in Mode 1 involved comparable interactions with the α -helices B, C, E and G, β -strands f, i and j, helix 3_{10b}, as well as the loop regions between β -strand g and α -helix B (referred to as loop g-B), α -helices C and D (referred to as loop C-D), β -strands b-c (referred to as loop b-c) and β -strands e-f (referred to as loop e-f) (**Table 1**).

Interactions of V2G10 and V6D4 with RTA's active site.

We next analyzed the co-crystal structures of the remaining two complexes, V2G10-RTA and V6D4-RTA. V2G10, a potent inhibitor of RTA, and V6D4, a weak inhibitor of RTA, each formed a cap over RTA's active site (**Figure 3F-G**). Compared to the five V_HHs in Mode 1, V2G10 and V6D4 are rotated counterclockwise by ~99° and ~165°, respectively, around RTA's active site (**Figure S8**). In addition, their CDR3 elements are tethered to the surface of the V_HH molecule, physically restricting their ability to project into the active site cleft. In V2G10, tethering is the result of hydrophobic interactions between CDR3 residues Ala-118 and Tyr-124

and framework region (FR) residues Phe-44 and Phe-55, as well as a salt bridge between CDR3 Arg-119 and FR residue Asp-71. In the case of V6D4, the CDR3 element is restrained by a disulfide bond between CDR2 Cys50 and CDR3 Cys105 (**Figure S8**).

Overall, the V2G10-RTA interaction buried 1,965 Å² with an Sc score of 0.665 (**Table 1**). V2G10's CDR1 and CDR2 elements established 3 hydrogen bonds with RTA and bury a total of 892 Å² of surface area. CDR1 makes contacts with RTA α-helix C, helix 3₁₀ b, and β-strands i and j, while CDR2 interacts primarily with α-helix G and β-strands i and j. CDR3 contacts RTA's α-helices B, C, and G, helix 3₁₀ b, β-strand i, and the g-B loop, buries 1194 Å² of solvent exposed surface area, and forms a total of 7 hydrogen bonds with RTA. The CDR3 comes within 12 Å² of the catalytic residue, Tyr-80 (**Figure 5A**).

The V6D4-RTA interaction generated six hydrogen bonds and buried 1,746 Å² of surface area with an overall Sc of 0.689 (**Table 1**). V6D4's CDR1 and CDR2 made minimal contact with RTA, burying 12 Å² and no hydrogen bonds. In contrast, CDR3 buried 1,125 Å² and formed 6 hydrogen bonds. CDR3 interacted with RTA α-helices B, C, and G as well as loops c-d and e-f. In an interesting twist, the presence of a proline residue (Pro-106) in the proximal region of CDR3 creates a partially unrestricted bend within the V_HH main chain that positions Arg108 and Pro109 within ~3.5 Å of Tyr80 (**Figure 5B**).

The crystal structure of V6D4-RTA revealed that V6D4 FR residues (Tyr-45, Gly-46, Tyr-59) and one CDR3 residue (Ile-107) made contact with RTA's α-helix B (Asn-97, Gln-98, Ala-101) burying 324 Å² while forming 1 hydrogen bond between FR residue Tyr-59 and Asn-97 in RTA's α-helix B (**Figure S9**). Although V6D4 is a weak inhibitor of RTA *in vitro*, it was the only V_HH among the seven that had demonstrable toxin-neutralizing activity in a cell-based assay (**Figure S1**), which we speculate may be due to contact with RTA's α-helix B, a known neutralizing hotspot. In this regard, it is interesting to point out that previous epitope mapping studies by HX-MS did not differentiate V2G10 from the Mode 1 family of V_HHs, but did successfully classify V6D4 as being unique, largely because of its interaction with α-helix B (21).

Evidence for distinct germline-dictated CDR3 configurations within alpaca derived V_HHs

A closer examination of CDR3 configurations between Mode 1 (V2A11, V6H8, V6A6, V6A7, V8E6) and Modes 2 (V2G10) and 3 (V6D4) is warranted, because these groups of V_HHs appear

to be representative of two CDR3 archetypes that exist in alpacas (*Vicugna pacos*). Mode 1 antibodies like V2A11 are representative of a class of V_HHs in which the CDR3 element projects away from the antibody surface in an “extended” conformation, while V2G10 is representative of V_HHs in which the CDR3 is “pinned” to the surface of the antibody (**Table S3**). While our sample size remains small (seven deposited to PDB, plus six pending for “extended”; 11 deposited to PDB, plus 10 pending for “pinned”), the overall alignment is remarkable (**Table S3**; **Figure S10**). Within our collection, the pinned-back CDR3 elements are, on average, 5 residues longer than the extended CDR3 elements. Moreover, the two different CDR3 configurations appear to be influenced by germline V_H gene usage: the extended elements predominantly derive from IGHV3S53 and the pinned-back from IGHV3-3 (28). The fact that V2A11, V8E6 and V2G10 are equally effective at occluding RTA’s active site using divergent molecular approaches to RTA’s active site is a demonstration of the versatility of antibody selection.

DISCUSSION

RTA is a near perfect enzyme that once unleashed in the cytoplasm of mammalian cells inactivates ribosomes with an efficiency theoretically limited only by diffusion (3, 6, 12). RTA’s active site is a solvent exposed cleft on one side of the molecule that accommodates the protruding adenine (A) within the conserved GAGA motif of the mammalian SRL. The adenine base is stabilized within RTA’s active site by a π -stacking network involving Tyr80 and Tyr123. RTA residues Glu177 and Arg180 promote the actual cleavage of the N-glycosidic bond that liberates the adenine moiety, thereby inactivating the ribosome. While RTA’s active site is an attractive drug target, in silico-based efforts to identify small molecule inhibitors of ricin have been stymied on several fronts (12). Even the best substrate analog inhibitors to date have IC₅₀s only in the micromolar range in cell-free assays. Moreover, while pterin-based molecules that fit snugly into RTA’s active site have been identified, the compounds themselves are poorly soluble and incompatible with cell and animal models of ricin toxicity (12, 29). In an effort to circumvent the issue of solubility, high-throughput, cell-based screens were performed with commercially available small molecule libraries. While compounds were identified that were capable of protecting cells from ricin-induced killing, the molecules invariably targeted host cell processes, rather than the toxin itself (13, 14, 30).

It is therefore highly significant that we have discovered three V_HHs with potent RTA inhibitory activity: V2A11, V8E6 and V2G10. Two of the three antibodies, V2A11 and V8E6, representing binding Modes 1A and 1B, respectively, physically occupy RTA's active site by virtue of their CDR3 elements, as revealed by the X-ray crystal structure of the V_HH-RTA complex. In this respect, V2A11 and V8E6 are textbook examples of the propensity of V_HHs to target concave epitopes such as the enzymatic pockets of hen egg white lysozyme and α -amylase (19, 20, 31-33), the CD4-binding pocket of HIV-1 gp120 (34), and the receptor-binding pocket of poliovirus (35). The proclivity of camelid heavy chain-only antibodies for enzymatic clefts has been attributed to their "prolate" shape and rigidification of CDR3 through FR interactions, as well as non-canonical disulfide bonds that alleviate the entropic penalty that would normally be associated with extended CDR3 elements (15, 33). The interactions of V_HHs with active site pockets can be so exquisite that they compete with small molecule substrate analogs and mimic native substrates at the atomic level (19, 20, 36).

Indeed, we found that V_HHs in Mode 1A and 1B had CDR3 residues that directly interacted with RTA's catalytic residues Tyr80 and Tyr123, steering Tyr80 into a configuration that would effectively perturb the π -stacking network required for depurination of SRL (**Figure S6**). Mode 1 V_HH CDR3 elements further perturbed RTA's RNA N-glycosidase machinery by forming H-bonds with Arg180, effectively pulling this residue away from the target N-3 atom within the adenine ring. By way of comparison, when RTA is bound to a substrate analog, AMP, Arg-180 is 3 Å from the N-3 atom (PDB: 3HIO), whereas Arg-180 is 3.9 Å away from the N-3 atom when RTA is bound to V2A11 (**Figure S6**). The greater distance between Arg-180 and substrate when RTA is bound to Mode 1 V_HHs would compromise proton transfer to the N-3 atom within the adenine ring, effectively impeding hydrolysis of the N-9 to C-1' bond within the adenosine residue within the SRL substrate (27).

The other three V_HHs within Mode 1, V6H8, V6A7 and V6A6, engaged with RTA active site residues in an identical fashion as V2A11 (in the case of V6H8) and V8E6 (in the case of V6A7 and V6A6), but were ineffective as RTA inhibitors. The primary difference between the potent and impotent antibodies is binding kinetics: V2A11 and V8E6 have equilibrium dissociation constants (K_D) of <1 nM and off-rates of <6 x 10⁻⁴ (1/s), whereas V6H8, V6A7 and V6A6 have K_D values of 4-10 nM and off-rates ranging from 4-20 x 10⁻³ (1/s). These results reveal a

minimum binding/dissociation threshold necessary to effectively neutralize RTA, a topic that will be discussed further below.

The third potent inhibitor of ricin, namely V2G10, sits atop and caps (rather than penetrates) RTA's active site. This alternative mode of attack (vis a vis V2A11 and V8E6) is due to V2G10's CDR3 element being tethered to the surface of the antibody and, therefore, restricted from projecting away from the core immunoglobulin fold and interacting with concave epitopes (33). We speculate that the pinned back CDR3 configuration is predetermined by the IGHV3-3 VH gene usage and represents one of at least two alpaca VH archetypes. Despite these different binding modalities, V2G10's K_D (0.28 nM) and off-rate (6.2×10^{-4} 1/s) were identical to those of V2A11 and V8E6 and therefore over the minimum threshold required to inhibit RTA activity.

All three potent inhibitors of RTA, V2G10, V2A11 and V8E6, also rendered Vero cells resistant to ricin intoxication when expressed as cytoplasmic "intrabodies". While intrabodies in the form of single chain Fv (scFv) or VHs have been under investigation for more than a decade as tools to deplete ("knockdown") viral and oncogenic proteins in mammalian cells, there is only a handful of examples targeting toxins, and only one instance aimed at a toxin's enzymatic activity (37). Specifically, Tremblay and colleagues identified two VHs against botulinum neurotoxin (BoNT) serotype A that blocked the toxin's protease activity *in vitro* (17). When expressed as intrabodies in neuronal cells, the VHs co-localized with the toxin and reduced protease-mediated degradation of BoNT/A's substrate, SNAP25. At this point in time, we speculate that V2G10, V2A11 and V8E6 exert their effects on ricin toxin by interacting with RTA immediately following its translocation across the ER membrane and preventing subsequent interactions with the ribosomes. The window of opportunity for the antibodies to intervene may be extremely short, considering that there is evidence that ribosomes promote RTA refolding and stabilization (38). Moreover, the results presented here certainly argue that high affinity binding (slow off-rates) are pre-requisite for intracellular anti-toxin activity. With the advent of mRNA-based, VH-expression platforms for passive vaccination (at least in mice) (39), the prospect of using VHs as therapeutics for fast acting biothreat agents is no longer farfetched.

Experimental Design

Cloning, expression and purification of VHs. The PCR amplicons for all 7 VHs

were subcloned into the pSUMO expression vector which contained an N-terminal deca-histidine and SUMO tag, as described (40). RTA (residues 1-268) was subcloned into the N-terminally deca-histidine tagged MCSG7 expression vector. All cloning was performed using a standard ligase independent cloning protocol. The V_HHs and RTA were expressed in *E. coli* strain BL21(DE3)-pRARE. The transformed bacteria were grown at 37°C in TB medium and induced with IPTG (0.1 mM) at 20°C and grown for ~16 h to mid-log phase (optical density of 0.6 at 600 nm). Cells were harvested by centrifugation and resuspended in 20 mM HEPES [pH 7.5] and 150 mM NaCl. The cell suspension was sonicated and centrifuged at 30,000 g for 30 min. The supernatants were subjected to 1 mL nickel affinity column followed by a Superdex 200 16/60 gel filtration column on an AKTApurify (GE Healthcare). The elution buffer consisted of 0.5M imidazole in binding buffer. The gel filtration buffer consisted of 20 mM HEPES [pH 7.5], 150 mM NaCl, and 20 mM imidazole. V_HH- or RTA-containing fractions were pooled and subject to TEV protease cleavage (1:10 weight ratio) for 3 h at room temperature in order to remove fusion protein tags. The cleaved protein was passed over a 1 mL Ni-NTA agarose (Qiagen) gravity column to remove TEV protease, cleaved residues, and uncleaved fusion protein. To generate each RTA-V_HH protein complexes, RTA was mixed with V_HHs in a 1:1 stoichiometry then concentrated to of 10 mg/ml.

***In vitro* measures of RTA enzymatic activity.** IVT assays were performed as described, with minor alterations (41). V_HHs diluted into 1% bovine serum albumin in PBS (w/v) were mixed at defined stoichiometric ratios with 0.75 nM RTA (Vector Laboratories, Burlingame, CA) in triplicate then added to an ice cold mixture containing luciferase mRNA (3.7 µg/mL; Ambion, Inc., Austin, TX) and Retic Lysate IVT™ Kit (ThermoFisher Scientific). The cocktail was incubated for 90 min at 30°C and then chilled on ice for 5 min before being transferred to wells of an opaque, 96-well microtiter plate with an equal volume (20 µL) of Bright-Glo™ luciferase substrate (Promega, Madison, WI) at room temperature. Luminescence was detected immediately using SpectraMax L (Molecular Devices, San Jose, CA). Percentage of RTA inhibition was scaled with 0 at the average luminescence of RTA control reactions and 100 at the average luminescence of positive control reactions.

The depurination inhibition activities of V_HHs were measured by qRT-PCR (42). Briefly RTA (1.0 nM) was mixed with 4, 20 or 100 nM V_HHs in 100 μ L volume at room temperature. Yeast ribosomes (50 nM) were added and the mixture was incubated at 25 °C for 5 min. The reaction was stopped by the addition of 100 μ L 2X RNA extraction buffer. RNA was purified from the reaction mixture and the depurination was measured using a previously published qRT-PCR method (43). For each set of reactions, one sample without the addition of RTA was set as the 100% inhibition control and one sample without the addition of V_HH was set as the no inhibition control. The percentage of depurination inhibition was calculated based on the controls. The data is shown as mean $\pm$ SD from three different biological replicates.

Crystallization and data collection. All RTA-V_HH crystals were grown by sitting drop vapor diffusion at 20°C using a protein to reservoir volume ratio of 1:1 with total drop volumes of 0.4 μ L. Crystals of the RTA-V2A11 complex were produced using a crystallization solution containing 100 mM Tris (pH 7.0), 200 mM calcium acetate, and 20% PEG 3,000. Crystals of the RTA-V2G10 complex were produced using a crystallization solution containing 100 mM MES (pH 5.0) and 20% PEG 6,000. Crystals of the RTA-V1D3 complex were produced using a crystallization solution containing 100 mM sodium acetate (pH 4.6) and 1.1 M di-ammonium tartrate. Crystals of the RTA-V8E6 complex were produced using a crystallization solution containing 100 mM MES (pH 6.0), 170 mM zinc acetate, 6% PEG 8,000, and 0.5% ethyl acetate. Crystals of the RTA-V6A7 complex were produced using a crystallization solution containing 200 mM potassium sulfate and 20% PEG 3,350. Crystals of the RTA-V6H8 complex were produced using a crystallization solution containing 100 mM citric acid and 15% PEG 6,000. Crystals of the RTA-V6D4 complex were produced using a crystallization solution containing 100 mM MES (pH 6.0), 400 mM zinc acetate, and 11% PEG 8,000. Crystals of the RTA-V6A6 complex were produced using crystallization buffer containing 100 mM imidazole (pH 8.0), 200 mM calcium acetate, and 15% PEG 8,000. All crystals were flash frozen in liquid nitrogen after a short soak in the appropriate crystallization buffers supplemented with 25% ethylene glycol. Data were collected at the 24-ID-C and 24-ID-E beamlines at the Advanced Photon Source, Argonne National Labs as well as beamline 8.2.2 at the Advanced Light Source, Berkeley Lab. All data was indexed, merged, and scaled using HKL2000 (44) then converted to structure factors using CCP4 (45).

Structure determination and refinement. The RTA-VHH complex structures were solved by molecular replacement using the program Phaser (46). Molecular replacement calculations were performed using the coordinates of the ricin a chain as a search model for RTA (PDB code 1RTC) and the VHH coordinates D10 as the search model for each VHH (PDB code 3EZJ). The resulting phase information from molecular replacement was used to autobuild the polypeptide chain for each RTA-VHH complex using the program ARP (47). Further manual model building was performed with COOT (48) combined with structural refinement employing the PHENIX package (49). Twinned refinement was performed for the RTA-V2G10 complex using the twin operator $-h-2^*l,-k,l$ with a twinning fraction of 0.052. During refinement a cross-validation test set was created from a random 5% of the reflections. Data collection and refinement statistics are listed in Table S1. Molecular graphics were prepared using PyMOL (Schrodinger) (DeLano Scientific LLC, Palo Alto, CA).

Accession Numbers. The structures generated in this study were deposited in the Protein Data Bank (PDB; <http://www.rcsb.org/pdb/>). The accession numbers are presented in **Table 1** and **Table S1**.

Surface Plasmon Resonance (SPR). Rabbit anti-E-tag polyclonal antibody (Bethyl Laboratories #A190-133A) was immobilized at 9000 RU on a Series S CM5 Chip. V_HHs with a C-terminal E-tag (diluted in running buffer) were captured on the chip surface at a maximum of 100 RU at a flow rate of 10 μ L/min using Biacore T200 (GE Healthcare, Chicago, IL). Serial dilutions of RTA in running buffer were injected at a rate of 50 μ L/min. After each injection, the chip surface was regenerated with 10 mM glycine-HCl pH 2.0 at 30 μ L/min for 40-45 s. Sensorgrams were normalized by subtracting baseline RU values from a reference flow cell (absent captured V_HH) and analyzed by fitting the data to the 1:1 Langmuir binding model using the Biacore T200 Evaluation Software (GE Healthcare). Plots were created by importing the sensorgram data into GraphPad Prism 8.2 software.

Vero cell culture, transfection, and lysis. Vero cells (ATCC, Manassas, VA) were cultured in Dulbecco's minimal essential medium (DMEM) with fetal bovine serum (10% v/v) and

penicillin/streptomycin at 37°C (5% CO₂). Cells were transfected in 6-well (ELISAs) or 96-well plates (cytotoxicity assays) as recommended for Lipofectamine™ 3000 transfection protocol (Life Technologies, Carlsbad, CA). For ELISAs, cells were washed 48 h post-transfection twice in ice-cold PBS. RIPA Lysis Buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 1% Na-deoxycholate; 150 µL) was added and cells were incubated for 5 minutes on ice with occasional gentle rocking to ensure well coverage. Cells were transferred to chilled 2 mL screw cap tubes with 1 mm glass beads by scraping and pipetting followed by homogenization at 5 m/s for 5 s. Cell lysates were replaced on ice for 30 min with one more homogenization step, followed by centrifugation at 15,700 x g for 10 min at 4°C.

For cytotoxicity assays, cells were transfected for 24 h before ricin treatment. Medium was aspirated from cells, replaced with 100 µL ricin toxin serially diluted in DMEM, and incubated at 37°C for 2 h. Ricin was then aspirated from cells and replaced with 100 µL DMEM. Cells were incubated for 48 h at 37°C. Viability was determined using Cell Titer-Glo® (Promega) and a Spectramax L Microplate Reader (Molecular Devices) and measured as a percentage of live control cells (transfected, but not treated with ricin).

ELISAs. To detect intrabody levels, 96-well ELISA plates (Immulon™ 4HBX; ThermoFisher) were coated overnight at with 1 µg/mL SyH7 (anti-RTA mAb) (50) or 4 µg/mL asialofetuin (ASF; Sigma-Aldrich) in PBS. Plates were washed and blocked for 2 h at room temperature. Following block, ricin or RTA (1 µg/mL in PBS) was applied to ELISA plates for 1 h. Transfected cell lysate was added in triplicate and serially diluted across plate for 1 h. Plates were washed and HRP-conjugated anti-E-tag antibody (prepared following manufacturer's guidelines; Bethyl Laboratories #A190-132P, Montgomery, TX) was applied for 1 h. Plates were washed and 100 µL TMB (SureBlue 3,3',5,5'-tetramethylbenzidine; Kirkegaard & Perry Labs, Gaithersburg, MD) was added for 8-10 min followed by stop solution (1M phosphoric acid). The ELISA plates were analyzed using a SpectroMax250 spectrophotometer equipped with Softmax Pro 5.4.5 software (Molecular Devices, Sunnyvale, CA). Purified V_{HH} protein with a C-terminal E-tag was used as a positive binding control for each transfection. Plates were washed following each step in PBS-Tween (0.1%). Cell lysates and secondary antibody were diluted in block buffer (wash buffer with 2% goat serum).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 8.2 software for Windows (San Diego, California). For *in vitro* translation assays, a Student's t-test was used to compare to RTA control values. For intrabody cytotoxicity results, a two-way ANOVA was used with the Sidak post hoc test to compare transfected and LNP-vehicle control cell viabilities at each ricin concentration.

Data Availability. All results associated with this study are included in the manuscript and supplementary information. As noted above, the crystal structures generated in this study were deposited in the PDB.

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Competing interests: The authors have no financial or other competing interests to declare.

Data and materials availability: The DNA sequences encoding the V_HHs described within this manuscript will be deposited into GenBank.

FIGURE LEGENDS

Figure 1. Abrogation of RTA's RIP activity in vitro by single domain antibodies. (A, B) *In vitro* translation assays were performed by mixing RTA (0.75 nM) with (A) 100 nM or (B) 20 nM of indicated V_HH and then adding the mixture to a cocktail containing rabbit reticulocyte lysate and luciferase mRNA, as detailed in the Materials and Methods. (C, D) Depurination assays were done with purified yeast ribosomes mixed with RTA (1.0 nM) and (C) 100 nM or (D) 20 nM of indicated V_HH, as described in the Materials and Methods. For both sets of experiments, results were normalized to RTA treatment without antibody (0%) and reactions without the addition of RTA positive controls (100%). Reactions were performed in triplicate. (E, F) Spearman correlation analysis was performed to determine correlation between IVT inhibition and the respective binding affinities (E) and off-rates (F) for each V_HH. Significance for the IVT experiments was calculated using a Student's t-test comparison to the RTA control. **** p<0.0001, *** p<0.001, ** p<0.005, * p<0.05

Figure 2. Protection of Vero cells from ricin toxin by V_HH intrabodies. (A) Cartoon of the experimental set-up. Vero cells were seeded in 96-well plates on day 1 and transiently transfected with V_HH-encoding pcDNA3.1 plasmids on day 2. Ricin challenge occurred on day 3 and cell viability was assessed on day 5. (B-I) Cell viabilities of Vero cells transfected with vehicle control (LNP; black circles) or V_HHs (open circles) at indicated toxin concentrations. Shown are two biological replicates with three technical replicates each. Horizontal red lines indicate 50% viability. (J) Significance was calculated using a two-way ANOVA with the Sidak correction test at each ricin concentration to compare viability of cells following intrabody transfection and LNP mock transfection. In two instances (indicated by parentheses in panel J), Vero cell viability of V_HH transfected cells was less than mock transfected cells, suggesting that V_HH expression actually imparts a burden on cells viability. Asterisks: **** p<0.0001; *** p<0.001; ** p<0.005; * p<0.05.

Figure 3. Structures of RTA-V_HH complexes. Structures of RTA (gray surface) in complex with (A) V2A11, (B) V6H8, (C) V8E6, (D) V6A7, (E) V6A6 (F) V2G10, and (G) V6D4 depicted as ribbon diagrams. Each V_HH is colored cyan, with CDRs 1, 2, and 3 colored blue, yellow, and red, respectively. RTA active site residue Tyr-80 is colored green.

Figure 4. Mode 1 V_HH interactions within the RTA's active site residues. The C α -traces of RTA (green) in complex with (A) V2A11, (B) V6H8, (C) V8E6 (D) V6A7, and (E) V6A6. The V_HH CDR3 elements are colored red. V_HH residues forming interactions with RTA active site residues Tyr-80 and

Tyr-123 are drawn as sticks. All stick representations are color coordinated to their respective main chain color.

Figure 5. Structural analysis of V6D4 and V2G10 with RTA's active site residues. Close-up images of the interface between RTA (green) and (A) V2G10 and (B) V6D4. The V_HHs CDR3 elements are colored red. V_HH residues forming interactions with RTA active site residues Tyr-80 are drawn as sticks. All stick representations are color coordinated to their respective main chain color.

Tables

Table 1. Summary of V_HH-RTA binding and interface information

V _H H	K _d ^b	PDB ^c	H-bonds ^a				Buried Surface Area (Å ²)			
			Total	CDR1/2/3	SB ^d	SC ^e	Total	CDR1	CDR2	CDR3
V2A11	0.31	6OBC	10	2/0/7	9	0.617	1953	254	290	1210
V6H8	4.01	6OBE	6	1/0/3	8	0.684	2080	401	355	1257
V8E6	0.42	6OBG	19	5/3/8	5	0.701	2367	452	520	1188
V6A7	4.2	6OBM	24	8/4/10	3	0.672	2416	367	493	1325
V6A6	10.9	6OBO	20	5/3/10	3	0.657	2519	475	494	1323
V2G10	0.28	6OCA	10	0/3/7	10	0.665	1965	405	487	1194
V6D4	9.51	6OCD	9	0/0/6	3	0.689	1746	12	0	1125

^a, hydrogen bonds; ^b, nM; ^c,PBD ID; ^d, salt bridges; ^e, shape complementarity score

Figure 1

Figure 2

J. Vero cell resistance to ricin toxin following V_HH transfection

Ricin	V _H H							
ng/mL	V2A11	V6H8	V8E6	V6A7	V6A6	V2G10	V6D4	ciA-H7
5000	ns	ns	ns	ns	ns	ns	ns	ns
1250	***	ns	ns	ns	ns	ns	ns	ns
313	****	ns	ns	ns	ns	***	ns	ns
78	****	ns	****	*	ns	****	ns	ns
20	****	ns	****	****	ns	****	ns	ns
5	****	(****)	****	***	ns	****	ns	(****)

Differences in percent (%) cell viability between mock transfected and V_HH-transfected cells at doses of ricin indicated in left column. Statistical analysis was performed using 2-way ANOVA with Sidak post-hoc test. Asterisks indicate Vero cells were significantly more resistant to ricin, as compared to mock transfected cells. Parentheses indicate that cell viability was significantly reduced, as compared to mock transfected cells. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$

Figure 3

Figure 4

Figure 5

A

B