Optimal Neutralization of *Centruroides noxius* Venom Is Understood through a Structural Complex between Two Antibody Fragments and the Cn2 Toxin*

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The current trend of using recombinant antibody fragments in research to develop novel antidotes against scorpion stings has achieved excellent results. The polyclonal character of commercial antivenoms, obtained through the immunization of animals and which contain several neutralizing antibodies that recognize different epitopes on the toxins, guarantees the neutralization of the venoms. To avoid the use of animals, we aimed to develop an equivalent recombinant antivenom composed of a few neutralizing single chain antibody fragments (scFvs) that bind to two different epitopes on the scorpion toxins. In this study, we obtained scFv RU1 derived from scFv C1. RU1 showed a good capacity to neutralize the Cn2 toxin and whole venom of the scorpion *Centruroides noxius*. Previously, we had produced scFv LR, obtained from a different parental fragment (scFv 3F). LR also showed a similar neutralizing capacity. The simultaneous administration of both scFvs resulted in improved protection, which was translated as a rapid recovery of previously poisoned animals. The crystallographic structure of the ternary complex scFv LR-Cn2-scFv RU1 allowed us to identify the areas of interaction of both scFvs with the toxin, which correspond to non-overlapping sites. The epitope recognized by scFv RU1 seems to be related to a greater efficiency in the neutralization of the whole venom. In addition, the structural analysis of the complex helped us to explain the cross-reactivity of these scFvs and how they neutralize the venom.

Scorpion sting accidents are very common worldwide and are considered a public health problem (1, 2). Scorpion venoms contain various components, and the toxins that affect mammalian sodium channels are the most medically important components (3, 4). These toxins cause severe intoxication and, in extreme cases, death. Commercial antivenoms (mainly of equine origin) inhibit poisoning because they are polyclonal and polyvalent (5). These properties enable them to recognize different epitopes on the toxins, and due to the high sequence identity among the toxins, the antivenoms are also cross-reactive. In Mexico, the venom of four species of scorpion is used to immunize horses to produce the neutralizing antivenom against eight dangerous Mexican scorpion species (5).

At this time, new strategies other than the use of animals for the generation of antivenoms against scorpion stings are being sought to produce recombinant proteins as novel antivenoms (5). The most promising way to obtain these new proteins is the phage display of libraries of antibody fragments from various sources (e.g. camelids and humans, among others) (6–8). These fragments, which were obtained by immunizations or in vitro maturation, have the ability to neutralize the effect of venoms. One of the most widely used formats is the single chain variable fragment (scFv), consisting of the variable domains of the heavy and light chains joined by a peptide linker, with (Gly4-Ser)3 used most often (9). The scFv format offers great advantages, including the ease with which it can be manipulated and optimized, and its use in cases of acute poisoning in mice has demonstrated its significant neutralizing capacity, due to its rapid distribution and elimination (10). In addition, the human origin of these scFvs makes them promising agents for a new generation of antivenoms.

From a library of scFvs displayed on phages, two scFvs (3F and C1) were isolated against the most abundant and lethal component (Cn2 toxin) of venom from the Mexican scorpion *Centruroides noxius* (6). Cn2 is a small toxin (66 amino acids) (Fig. 1A) that exclusively binds and modifies the gate functions of the human sodium channel Nav1.6 (11). We were able to obtain the scFv LR after three cycles of directed evolution of the parental scFv 3F and incorporating a V101F mutation into one

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*The abbreviations used are: scFv, single chain antibody fragment; CDR, complementarity-determining region; Vδ, light chain variable domain; Vδ, heavy chain variable domain; Cn, toxin from *Centruroides noxius* Hoffmann; Css, toxin from *C. suffusus*; CLI, toxin from *Centruroides limpidus limpidus*; PDB, Protein Data Bank; C. noxius, *Centruroides noxius* Hoffmann.*
of the derived variant scFvs (9004G) (12) (Fig. 1B). This scFv recognizes and neutralizes the main toxins from the venoms of the *C. noxius* and *Centruroides suffusus suffusus* scorpions (Cn2 and Csl2, respectively), with affinities in the picomolar range, and removes any poisoning symptoms. In neutralization tests using 3 LD50 of fresh whole venom in mice, scFv LR was capable of inhibiting nearly all symptoms caused by the *C. suffusus suffusus* venom. However, for the neutralization of *C. noxius* venom, some symptoms of intoxication were still observed, although survival rates between 90 and 100% were reached (rescue and preincubation assays) (12). These symptoms may be due to the presence of other toxins from the venom, which are less abundant but are not neutralized by the scFv LR. To resolve this limitation, we sought to generate a second neutralizing antibody directed against a non-overlapping site on the toxin. This strategy aims to achieve two goals: imitating the polyclonal character of the commercial antivenom but not its complexity and exploiting the cross-reactivity of the obtained scFvs. This last phenomenon is frequently due to the high sequence identity among the toxins from *Centruroides* scorpions. We expected that the neutralization of a second epitope could eliminate the remnant symptoms. A new scFv, which was obtained from a mutagenic library of scFv C1 (13) and screened against toxin Cn2, was isolated and named 3H (Fig. 1B). Therefore, the same library of scFv C1 was screened against the Cll1 and Cll2 toxins (Fig. 1A) from the venom of *Centruroides limbipidus limbipidus*. scFv 202F was obtained after two cycles of directed evolution against toxin Cll1 (Fig. 1B). This scFv was able to neutralize the Cn2 and Cll1 toxins (13).

In this paper, we describe the generation of a new scFv (RU1), obtained by improving scFv 202F (13) through the incorporation of the Y110H substitution that was previously identified in the 3H1 variant (Fig. 1, B and C). scFv RU1 exhibited the ability to fully neutralize 2 LD50 of the Cn2 toxin and 3 LD50 of fresh venom, without leaving any detectable intoxication symptoms. scFv RU1 was used in conjunction with scFv LR in regular neutralization (preincubation) and poisoning rescue assays in mice injected with whole fresh venom from *C. noxius*. The results showed that these two scFvs complement each other. When administered together, they showed a better neutralization capacity than when used independently, even at low doses (toxin/scFv at a molar ratio of 1:5).

The three-dimensional structure of the ternary complex scFv LR-Cn2-scFv RU1 obtained by crystallization and x-ray diffraction revealed that both antibodies bind to the Cn2 toxin in diametrically opposite regions. scFv LR (derived from 9004G) maintained the same binding site on the Cn2 toxin in the ternary complex compared with that in the binary complex scFv 9004G-Cn2, as reported previously (14). A new epitope, which was recognized by the scFv RU1, was identified. The existence of this second epitope in Cn2, which is associated with its toxic effects, suggests the existence of other potential toxic epitopes present in the related β-toxins, which can be targeted to inhibit their toxic effects on sodium channels.

### Experimental Procedures

#### Venoms from *C. noxius* and *C. limbipidus limbipidus*

Fresh venom was obtained from individuals of either species by electrical stimulation. The samples were diluted in tetra-distilled water and centrifuged at 10,000 rpm for 10 min at 4 °C. The insoluble material was discarded, and the toxin-containing supernatant was recovered and spectrophotometrically quantified (∆ = 280 nm).

#### Toxins

The toxins were purified from the scorpion venoms using methodologies described previously. Cll1 was obtained from the venom of *C. limbipidus limbipidus*, and the Cn2 toxin was from *C. noxius* Hoffmann (15–17).

#### Construction of the scFv RU1 by Site-directed Mutagenesis

The 202F DNA sequence was modified at position 110 (Tyr → His). The primer 5’-GGGCCGACTGGCACTTTCG-3’ and the primer DIR (6) were used to generate a megaprimer in a standard PCR, as described previously (6), using the scFv 202F DNA as a template. The amplified DNA fragment was purified from an agarose gel. This megaprimer, the REV oligonucleotide (6), and the same template (202F), were used to amplify the full DNA segment encoding scFv RU1 using PCR. The PCR product was purified, digested with the SfiI and NotI enzymes, and then ligated into the expression vector pSyn1 using PCR. The PCR product was purified, digested with the SfiI and NotI enzymes, and then ligated into the expression vector pSyn1 which had been treated with the same enzymes. TG1 *Escherichia coli* cells were electroporated with the ligation product. The plasmids from several clones were purified and sequenced. A *bona fide* construct (without any unexpected change) was selected for the subsequent procedures.

#### Antibody Expression and Purification

Protein expression and purification was performed as described previously (6). The final purification procedure was performed by gel filtration chromatography on a Superdex™ 75 column (GE Healthcare). The buffer was 1× PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7.4), and the flow rate was 0.5 ml min⁻¹. The protein concentration was determined spectrophotometrically at λ = 280 nm.

#### Surface Plasmon Resonance Measurements

The kinetic constants of scFv 202F binding to the immobilized Cn2 or Cll1 toxins were determined using a Biacore biosensor system (Biacore X, GE Healthcare). The buffer was 1× PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7.4), and the flow rate was 0.5 ml min⁻¹. The protein concentration was determined spectrophotometrically at λ = 280 nm.
surfaces were regenerated with 10 mM HCl. The kinetic constants were determined using the corresponding sensograms, which were corrected by subtracting the values from both the reference flow cell and the blank buffer injection. The Langmuir (1:1) model from BIA Evaluation software version 3.1 was used to determine the kinetic constants.

**Competition Assays Using Surface Plasmon Resonance**

Surface plasmon resonance binding assays were performed to test whether the scFvs interacted with different epitopes on Cn2 toxin. The sensor chip was prepared as described above. Three saturating samples (30 µl of 0.5 mM) of scFv LR were consecutively injected onto a Cn2-coated chip at a rate of 20 µl min⁻¹ in HBS-EP buffer to ensure that the available sites were saturated. Then 30 µl of 1 mM RU1 scFv were injected, and the sensogram was analyzed. The same experiment was performed, but the epitope recognized by scFv RU1 was preincubated with scFv LR. As controls, separate samples of the scFvs recognizing Cn2 were injected and compared with a mixture of the two scFvs in competition. The results were validated by comparing the values of $k_{max}$.

**Neutralization Tests**

**Toxins**—To evaluate the neutralization ability of scFv RU1, 1 LD₅₀ of the Cll1 toxin (1.7 µg of Cll1 toxin per 20 g of mouse) or the Cn2 toxin (0.25 µg of Cn2 toxin per 20 g of mouse) were used. Groups of 8–20 female CD1 mice weighing ~20 g were intraperitoneally injected with 1 LD₅₀ of Cll1 or 2 LD₅₀ of Cn2 (control groups). In the experimental groups, the neutralization activity of scFv RU1 was tested by the administration of a preincubation mix of scFv and each toxin at a molar ratio of toxin/antibody 1:10. Additional experiments were performed to test the protection limit against the Cn2 toxin were performed at molar ratios of 1:5 and 1:2. The mixtures were preincubated for 30 min at room temperature (~25 °C) prior to injection. The intoxication symptoms were followed until death or remissions occurred. The survivor animals were observed for 2 days.

**Whole and Fresh Venom of C. noxius**—Two different trials were performed to test the neutralization activity of the scFvs against freshly prepared whole venom. In trial 1, an amount of venom equivalent to 3 LD₅₀ was preincubated with scFv RU1 or both LR and RU1 at several toxin/antibody molar ratios. These ratios were calculated relative to the main toxin in the venom. The LD₅₀ of the venom of C. noxius is ~2.5 µg/20-g mouse, where toxin Cn2 represents ~6.8% of the total toxic content (18). The mixtures (venom and scFv(s)) were preincubated for 30 min at room temperature before their injection into mice. Trial 2 was designed to determine the ability of scFv RU1 alone and in combination with scFv LR to rescue mice that had been previously poisoned with 3 LD₅₀ of venom. A time span of 5–10 min was allowed to elapse before the mice were injected with different amounts of the antibodies representing different toxin/antibody molar ratios in terms of the concentration of the main toxin in the venom.

**Crystallization of the scFv LR-Cn2-scFv RU1 Complex**

The purified proteins, the Cn2 toxin, scFv LR, and scFv RU1, were mixed in an equimolar ratio of 1:1:1, which corresponded to 263 µg of Cn2 and 1 mg of each scFv, all solubilized in 1× PBS. The mixture was incubated overnight at 4 °C. The next day, the ternary complex was purified on a Superdex 75 column, with a flow rate of 0.5 ml min⁻¹. A main peak at an elution time of 17 min was detected, which corresponded to the ternary complex. Other complexes (binary) and individual proteins were not observed. The recovered sample was concentrated to 8 mg ml⁻¹ total protein. The crystallization conditions were initially screened at 18 °C by the microbatch method using different screening kits (Crystal Screens I and II and Index Screen) from Hampton Research (Aliso Viejo, CA) and Wizard III and IV from Emerald Biosystems (Bainbridge Island, WA). Protein droplets consisting of 1 µl of the ternary complex and 1 µl of the reservoir solution were covered with paraffin oil. Small crystals of the ternary complex were observed after 2 months in condition 18 of Wizard IV, consisting of 0.1 m SPG (succinic acid, sodium dihydrogen phosphate, and glycine in a molar ratio of 2:7:7), pH 8.5, and 25% (w/v) PEG 1500. After optimization, bipyrimal crystals of the ternary complex appeared and grew to dimensions of ~0.10 × 0.10 × 0.05 mm by the hanging drop vapor diffusion method, with a reservoir solution consisting of 0.1 m SPG, pH 8.5, and 20% (w/v) PEG 1500 and a growth period of 1 month.

**Data Collection and Processing**

The data were collected from the crystals of the ternary complex on beamline X6A of the National Synchrotron Light Source, Brookhaven National Laboratory (Upton, NY), using an Area Detector Systems Corp. Quantum 270 detector. The x-ray diffraction data for the ternary complex were collected from a single crystal at λ = 0.9795 Å. The crystal-to-detector distance was maintained at 365 mm, with an oscillation range per image of 1° and an exposure time of 30 s. For data collection under cryogenic conditions, the crystal was briefly soaked in a cryo-protectant solution consisting of 0.1 m SPG, pH 8.5, 20% (w/v) PEG 1500, and 20% (v/v) PEG 400. Subsequently, the crystal was loop-mounted and flash-cooled in liquid nitrogen. The diffraction images were integrated using XDS (19), and scaling was performed with SCALA from the CCP4 suite (20).

**Structure Determination and Refinement**

The structure of the ternary complex was determined by molecular replacement, assuming a P2₂,₁,₂ space group, as suggested by POINTLESS (21). Molecular replacement trials were performed in Phaser-MR (22) using the structure of the 9004G-Cn2 complex (PDB entry 2YC1) as a search model. The initial phases were obtained by dividing the 9004G-Cn2 complex into two parts and submitting these two models as separate search ensembles. The structure was refined using Phenix (23) and was alternated with manual building/refinement in Coot (24). Five percent of the data were randomly chosen and reserved to determine $R_{free}$. To improve the low-resolution refinement in Phenix and decrease the gap between $R_{work}$ and $R_{free}$, the structure of the 9004G-Cn2 complex (determined at a 1.9 Å resolution; PDB entry 2YC1) was used as a reference model. Therefore, reference model restraints, secondary structure-dependent restraints, and Ramachandran-based restraints were added to the refinement process for the ternary complex.
Cn2 Toxin Is Neutralized over Two Different Epitopes

The values in parentheses correspond to the highest resolution shell.

**Table 1**

| Parameters                      | Ternary complex |
|--------------------------------|----------------|
| Data collection statistics     |                |
| Space group                     | P22,2          |
| Unit-cell dimensions            |                |
| a, b, c (Å)                     | 45.6, 74.6, 140.2 |
| a, b, c, t (Å)                  | 90.0, 90.0, 90.0 |
| Resolution range (Å)            | 39.6–3.10 (3.27–3.10) |
| No. of reflections              | 88,588         |
| No. of unique reflections       | 9184 (1331)    |
| Completeness (%)                | 100.0 (100.0)  |
| Rmerge (%)                      | 13.6 (39.9)    |
| I/σ(I)                          | 17.5 (6.4)     |
| Multiplicity                    | 9.6 (9.8)      |
| No. of complexes in asymmetric unit | 3          |

Refinement statistics

| Resolution range (Å) | 38.21–3.10 (3.27–3.10) |
| Rwork/Rfree (%)      | 18.5/22.9              |
| No. of atoms         | Protein: 4040, Ion/ligand: NA, Water: 6 |
| Mean B-values (Å²)   | Protein: 45.6, Ion/ligand: NA, Water: 32.0 |
| Ramachandran plot (%)| 96.3                    |
| Additional allowed regions | 3.5         |
| Disallowed regions   | 0.2                    |

* Rmerge = Σhk(ihkik) − (Σikhkik)/Σikhkik, where Σhk(ihkik) and (Σikhkik) represent the diffraction intensity values of the individual measurements and the corresponding mean values. The summation is over all unique measurements.

**Results**

Two very important and dangerous scorpion species live in Mexico, *C. noxius* and *C. limpidis limpidus*. The main toxins of these venoms are Cn2 for *C. noxius* and Cll1 and Cll2 for *C. limpidus limpidus* (Fig. 1A). Other toxins that are present in lower amounts in the venoms may also modify the activity of the sodium channels and may cause intoxication symptoms if they are not completely neutralized. In a previous paper (13), scFv 202F (Fig. 2B) was obtained after two cycles of directed evolution of the parental scFv C1 against toxin Cll1. scFv 202F significantly improved the recognition of Cll1 and Cn2 and was able to neutralize both toxins. This result was surprising because these two toxins differ by 10 amino acids. Moreover, Cll2, which has a higher identity with Cll1, was not neutralized (Fig. 1A). In a subsequent stage, we attempted to increase the affinity of the scFv C1 against the Cn2 toxin to identify key mutations that could guide the recognition of a particular toxin. scFv 3H was isolated by screening the mutagenic library of scFv C1 against the Cn2 toxin. This variant had been previously isolated against Cll1 (13). The scFv 3H with only two changes with respect to the parental C1 (Table 2) showed a significant increase in its recognition of Cn2 and in the ELISA. This clone was subjected to new cycles of directed evolution against the Cn2 toxin. However, the obtained variants did not significantly improve the recognition of Cn2. Therefore, the Y110H mutation, which is present in 3H, was introduced into scFv 202F by

in phenoredictine. In addition, non-crystallographic symmetry restraints were applied during the early stages of refinement and later removed. The refinement cycles were complete when R_work and R_free values lower than 0.19 and 0.23, respectively, were obtained. A few water molecules were first automatically located using ARP/wARP (25) and then validated in Coot. No electron density was observed for residue Ser66 of Cn2 and residues Gly125–Gly138 in the interdomain linker between the VH-VL domains of RU1 and the VH-VL domains of LR. Model validation was performed with MolProbity (26). The data collection and refinement statistics are summarized in Table 1.

**Structural Analysis**

The interfaces were analyzed with the PISA and PIC servers (27, 28) using the default values. The electrostatic surface potentials were calculated with the APBS (29) plugin (M. G. Lerner and H. A. Carlson, University of Michigan, Ann Arbor, MI) for the PyMOL software (PyMOL Molecular Graphics System, version 1.2r3pre, Schrödinger, LLC) using the PDB2PQR (30) server to generate the PQR files with the CHARMM force field (31, 32) and PROPKA (33) settings. The graphic representations were prepared with the PyMOL and Chimera (University of California, San Francisco) packages (34).

**Model of the scFv RU1-Cll1 Complex**

To understand the cross-reactivity of scFv RU1 to Cn2 and Cll1, a model of the scFv RU1-Cll1 complex was prepared, based on the structure of the scFv RU1-Cn2-scFv LR ternary complex. The LR scFv structure was deleted from the pdb file of the ternary complex to obtain a scFv RU1-Cn2 complex. There are 10 amino acid differences between the sequences of the toxins Cn2 and Cll1. Nevertheless, the last amino acid in the sequence of the toxin Cn2, which corresponds to one of the changes between these two toxins, is not observable in the Cn2 structure. Using the Chimera program, the remaining nine changes were introduced into the Cn2 structure to construct the Cll1 toxin, with the goal of generating a model of the scFv RU1-Cll1 complex. The introduced changes were L51, D7N, K81, N95, L17Y, A45G, I56V, N62K, and R64T.

The model of the scFv RU1-Cll1 complex was submitted to simulation annealing and energy minimization procedures. The model of the RU1 scFv-Cll1 complex was prepared using the protein preparation wizard of the Maestro suite (35–37); the model of the complex was soaked with a buffering box size of 10 Å per side and an NaCl concentration of 0.15 M. Using the Viparr utility from the Desmond program, the charmm22star and tip3p_charmm force field parameters were adjusted to the soaked RU1 scFv-Cll1 model system. The Desmond SA procedure was applied to this system in six stages (30 ps at 10 K, 100 ps at 100 K, 200 ps at 300 K, 300 ps at 400 K, 500 ps at 400 K, and 1000 ps at 300 K) using the NVT ensemble and relaxing the model system before simulation. Then the energy of the model system was minimized, with 4000 maximum iterations and a convergence threshold of 0.5 kcal/mol/Å, using the steepest descent algorithm to a threshold of 25 kcal/mol/Å, followed by the limited-memory Broyden-Fletcher-Goldfarb-Shannon algorithm.
site-directed mutagenesis, resulting in scFv RU1 (Table 2 and Fig. 1C).

**Affinity Determination**—The binding kinetics of the scFvs 3H, 202F, and RU1 that recognize the Cn2 and Cll1 toxins were determined using different affinity assays performed by surface plasmon resonance (Biacore) (Table 3). In general, the three tested scFvs exhibited greater affinity constants (\(K_D\)) for the Cn2 toxin than for the Cll1 toxin. Similarly, scFv RU1 showed higher affinities for the two toxins. These results show that the Y110H mutation in scFv RU1 improved its affinity constant (\(K_D\)) for both toxins as a result of a decrease in the kinetic rate of dissociation (\(k_{off}\)). This slower dissociation rate results in an increase in the interaction time of this scFv with both toxins. This last parameter is the average time that an scFv stays bound to its target and named residence time; \(TR = 1/k_{off}\) (Table 3).

**Neutralization of the Cll1 and Cn2 Toxins**—These assays allowed us to confirm that scFv RU1 was also able to neutralize the Cn2 and Cll1 toxins, similar to its predecessor (scFv 202F) (Table 4). One LD_{50} of the Cll1 toxin was fully neutralized by scFv RU1 at a molar ratio of 1:10 (toxin/scFv). Two LD_{50} were used in the assays of neutralization of the Cn2 toxin, because RU1 showed a higher affinity for this toxin. The results revealed that all of the mice were protected when they were challenged at a molar ratio of 1:10 (toxin/scFv), whereas 75 and 40% were protected at molar ratios of 1:5 and 1:2, respectively.

**Biacore Competition Assays**—The competition assays were performed on a Biacore using two methods. In the first method, the Cn2 toxin binding sites were saturated with scFv RU1, and then scFv LR was injected (Fig. 2A). The resonance signal (in resonance units (RU)) obtained in this method was comparable with that of scFv LR without competition (Fig. 2C). Likewise, the binding sites were first saturated with scFv LR, followed by scFv RU1 injection (Fig. 2B). The second method consisted of a simultaneous injection of the two antibodies. The resulting
bound resonance units were close to the sum of the two independent responses (Fig. 2C). These results clearly indicated that the scFvs recognized different sites on the toxin. This is significant because the toxins were neutralized through the binding of the scFvs to non-overlapping sites, which improved their efficiency as an antivenom.

Neutralization of the Venom of C. noxius—After determining the individual affinities and the neutralization capacities of the antibodies to the Cn2 toxin, whole venom neutralization tests were performed using two methods: 1) preincubation mix and 2) a poisoning rescue, as described under “Experimental Procedures.” Table 5 shows the results of the neutralization assays, where three LD50 of fresh C. noxius venom were used. In the control group, symptoms appeared between 10 and 15 min postinjection, and the mice died within 30–90 min. All of the mice were protected when the scFvs were injected at a molar ratio of 1:10 (Table 5, top). Although it exhibited a reduced affinity for Cn2, scFv RU1 did not leave any noticeable symptoms of intoxication compared with scFv LR. The neutralizing capacity of a mixture of the two scFvs (RU1 and LR) was also evaluated at molar ratios of 1:5 and 1:10. The results showed that both mixtures were significantly effective because no symptoms of intoxication were detected. In rescue tests, the neutralization capacity of RU1 was also remarkable because it induced the rapid recovery of the intoxicated animals and a complete elimination of the poisoning symptoms (Table 5, bottom). The combination of both scFvs rescued the intoxicated mice, even at very low antibody concentrations. The rapid disappearance of the symptoms was evident, indicating that both neutralizing antibodies contributed to alleviate poisoning effects in a complementary manner. Finally, the injection of high amounts of the scFvs (1:40 each) showed that they are
equally efficient compared with the lower ratios (no side effects related to the surplus antibody were observed).

Crystalization of the Ternary Complex—The ternary complex scFv RU1-Cn2-scFv LR was crystallized to determine how the two scFvs simultaneously bind to the Cn2 toxin. Initially, several tests were performed to generate crystals of scFv RU1 with the Cn2 toxin. However, these crystals were not of sufficient quality to obtain x-ray diffraction patterns that would allow us to obtain a good structural model of the binary complex. In contrast, the samples of the ternary complex rapidly formed high-quality crystals.

The 3.1 Å crystal structure of the scFv RU1-Cn2-scFv LR ternary complex corresponded to the orthorhombic P2_1_2_1 space group (Table 1). The heavy chain (V\_H) of scFv RU1 is composed of residues Gln\_1–Ser\_124, whereas its light chain (V\_L) includes the residues Asn\_140–Ala\_250. On the other hand, the V\_H of scFv LR is composed of Gln\_1–Ser\_124, and its V\_L includes the residues from Glu\_1 to Arg\_240 (Fig. 1C). Electron density maps were not observed for the residues corresponding to the sequence of the peptide linker that connects the V\_H and V\_L domains of both scFv RU1 and scFv LR or for the residues corresponding to the C-Myc and the His\_6 tags in both molecules (Fig. 1C). Electron density maps are also missing for residues Arg\_240 of scFv LR, G250 of scFv RU1, and Ser\_260 of the Cn2 toxin. The structure of the ternary complex showed good geometric parameters, with only the Arg\_240 residue of scFv RU1 in the disallowed region of the Ramachandran plot (Table 1), probably due to its location next to residue Pro\_195 and some packing restraints. scFvs RU1 and LR bind at diametrically opposed sites on the Cn2 toxin (Fig. 3A). An important observation is that the contribution of the V\_H domains is far superior to that of the V\_L domains for both scFv interactions with the Cn2 toxin. The embedded areas at the binding interface are as follows: V\_H LR (629 Å²), V\_L LR (277.5 Å²), V\_H RU1 (703.4 Å²), and V\_L RU1 (195.2 Å²).

The scFv LR Binding Site on the Cn2 Toxin—scFv LR is located at the same place on the Cn2 toxin as its predecessor (9004G) (14). Specifically, the binding site of scFv LR is located near the N terminus of the α-helical region of the Cn2 toxin, and the scFv RU1 binding site is located near the C terminus of this helix (Figs. 1A and 3A). A V101F mutation was incorporated into scFv 9004G, resulting in scFv LR. This change did not affect the way in which LR binds to the Cn2 toxin (Fig. 3A). The structure of the Cn2 toxin in the ternary complex is virtually the same as that in the 9004G-Cn2 crystallographic structure (14), with a root mean square deviation between both Cn2 toxin structures of only 0.507 Å. This is also similar to the NMR solution structure already reported for this toxin (38). The epitope on the Cn2 toxin that is recognized by LR corresponds to a segment that spans from the β1 strand to the α-helix, contacting residues Tyr\_4, Asp\_7, Tyr\_14–Leu\_19, and Asn\_22, and includes part of the α-helix and the β2-turn, binding to residues Tyr\_24 and Arg\_27 and residues Tyr\_32–Ala\_46, respectively. The water content in the ternary complex was significantly reduced compared with the Cn2-scFv 9004G complex (PDB 4V1D). Fifteen contacts mediated by water molecules were identified in this binary complex, whereas the same residues, located at the interface between the Cn2 toxin and scFv LR in the ternary complex, establish direct contacts through hydrogen bonds.

Structural Features of the Interaction between the Cn2 Toxin and scFv RU1—As previously stated, scFv RU1 binds to the opposite site on the Cn2 toxin in the ternary complex, with respect to scFv LR (Fig. 3A). The structures of the V\_H and V\_L domains of scFv RU1 show the common structural characteristics of the variable domains of an immunoglobulin. Although the resolution of the ternary complex reached 3.1 Å, the electron density is well defined at the interface regions between toxin Cn2 and scFv RU1 (see “Results”). Using a simulated annealing omit map, we verified the correctness of the model. The electrostatic surface potential at the scFv RU1-Cn2 toxin interface is remarkably complementary (Fig. 3B), which correlates with the affinity observed between these molecules.

The epitope recognized by scFv RU1 is composed of three regions of the Cn2 toxin: the N-terminal region, including residues Leu\_5, Val\_16, and Lys\_8; the α-helix C-terminal end/β-turn region, including residues Gln\_31, Gln\_32, Tyr\_33, and Lys\_35, and the C-terminal portion of the toxin, including residues Tyr\_53, Gln\_54, Ile\_56, Leu\_60, and Asn\_62. Several residues in the three CDRs of V\_H from scFv RU1 and some residues located at framework region 3 participate in the interaction with the Cn2 toxin (Fig. 3C). Only CDR3 of the V\_L chain from scFv RU1 contributes to the recognition of the Cn2 toxin. Detailed information about all of the interactions between the residues from scFv RU1 and the Cn2 toxin is shown in Table 6 (see also Fig. 3, C and D).

The scFvs derived from the parental scFv C1 underwent a maturation process that has allowed us to identify specific mutations that enabled them to improve their neutralization capacity and maintain their cross-reactivity toward toxins from related scorpions. The combination of the changes in scFvs 3H and 202F that were present in scFv RU1 is shown in Table 2. The D54G, S56G, M105L, and Y110H mutations, located at CDR2 and -3 of the V\_H of scFv RU1, are involved in the majority of the contacts with the Cn2 and Cll1 toxins. The analysis of the structure of the ternary complex revealed that the glycine resi-

### TABLE 5

**Neutralization tests**

Top, neutralization challenge with 3 LD\_50 of whole fresh soluble venom of C. noxius. An amount of freshly prepared venom equivalent to 3 LD\_50 was injected into the mice. Alternatively, a mixture that had been preincubated with the indicated toxin/antibody molecular ratio of the antibody was used. Bottom, rescue from a challenge with 3 LD\_50 of whole soluble venom of C. noxius. At 5–10 min postintoxication, the indicated molecular ratios of antibody relative to Cn2 were injected.

| Preincubation | Molar ratio, toxin/scFv \(^a\) | Survivors/total |
|---------------|-------------------------------|----------------|
| Control       | 0/10                          |                |
| RU1           | 1:10                          | 18/18          |
| LR            | 1:10                          | 10/10\(^b\)    |
| RU1 + LR      | 1:5                           | 10/10          |
| RU1 + LR      | 1:10                          | 10/10          |

| Rescue        | Molar ratio, toxin/scFv \(^a\) | Survivors/total |
|---------------|-------------------------------|----------------|
| Control       | 0/10                          |                |
| RU1           | 1:10                          | 10/10          |
| RU1           | 1:20                          | 10/10          |
| LR            | 1:10                          | 6/10           |
| LR            | 1:18                          | 9/10           |
| RU1 + LR      | 1:40                          | 10/10          |
| RU1 + LR      | 1:20                          | 8/8            |
| RU1 + LR      | 1:5                           | 10/10          |

\(^a\) Molar ratios of each scFv, assuming that Cn2 corresponds to 6.8% of C. noxius venom.

\(^b\) Some poisoning symptoms.
dues at positions 54 and 56 of CDR2 improved the binding of this part of the surface of scFv RU1 with the Cn2 toxin. Two hydrogen bonds between the backbones of Gly54 and Gly56 residues and residue Gln31 of the Cn2 toxin were generated (Fig. 3C and Fig. 4A). Furthermore, the M105L and Y110H mutations are located at CDR3 of VH, which is a key region for the interaction with the toxins. The M105L mutation favored a local hydropathic increase (leucine hydropathic index = 3.8, whereas that of methionine is 1.9) (39). Residue Tyr33, which is present in the Cn2 and Cll1 toxins and located in the vicinity of Leu105, established a new hydrophobic interaction and enhanced the binding strength of scFv RU1 with both toxins. The second mutation at the CDR3 (Y110H) induced the stabilization of this loop through the formation of a new hydrogen bond between the side chain of His110 and the adjacent residue Asp108 (Table 6 and Fig. 4A).

Model of scFv RU1 Binding to the Cll1 Toxin—An in silico model of the Cll1 toxin bound to scFv RU1 was generated to determine the differences in the affinity of scFv RU1 to the Cn2 and Cll1 toxins. The residues that differ between the Cn2 and Cll1 toxins were modified accordingly, followed by an energy minimization. The model shows that most of the contacts with scFv RU1 are maintained because of the high homology between the two toxins, which explains the cross-reactivity with RU1. The contacts at the interfaces of scFv RU1 with the Cn2 toxin (structural data) and the Cll1 toxin (theoretical

FIGURE 3. Analysis of the structure of the ternary complex scFvLR-Cn2-scFvRU1. A, the structure of the ternary complex is represented by ribbons. This representation shows the simultaneous binding of both scFvs to the toxin. B, analysis of the complementary electrostatic surfaces between scFv RU1 and the Cn2 toxin. The negatively and positively charged surfaces are colored red and blue, respectively. C, hydrogen bonds located at the interface between scFv RU1 and the Cn2 toxin. The hydrogen bonds are represented by blue lines. The colors used to identify the different interactions and protein regions are as follows: orange for the Cn2 toxin, blue for the RU1 VH domain, green for the RU1 VL domain, yellow for CDR1 of VH, light brown for CDR2 of VH, and dark brown for CDR3 of VH and VL. D, hydrophobic and cation-Pi interactions at the interface between scFv RU1 and the Cn2 toxin. The color codes are the same as in C. Magenta lines, hydrophobic interactions; green lines, cation-Pi interactions.
model) are shown in Table 6. There are fewer contacts present in the scFv RU1-CII1 complex compared with the scFv RU1-Cn2 complex, which may explain the lower affinity of scFv RU1 for CII1 (Table 3). A loss of four hydrogen bonds and two ion-Pi interactions as well as the gain of one hydrophobic contact in the scFv RU1-CII1 complex were detected compared with the scFv RU1-Cn2 complex (Table 6). The effect of the differences in the sequences of toxins (Fig. 1A) for the interaction with scFv RU1 is detailed in Fig. 4B.

**Discussion**

The progress on the characterization of scorpion venoms in terms of identifying the main harmful toxins has provided us the opportunity to implement a rational strategy to generate new antibodies and/or antibody fragments that are exclusively directed against the toxic components. Therefore, the aim of this study was to improve the neutralization capacity of scFvs derived from the parental scFv C1 toward the Cn2 and CII1 toxins. At the same time, we sought to understand the molecular basis of these improvements on the fragments’ neutralization capacities.

The scFv C1 variants were generated using strategies described previously (12, 13) and those described in this study. We learned that only a few changes were required to obtain scFvs that neutralize the Cn2 and CII1 toxins. To understand the influence of these changes, the scFvs were expressed in bacteria and characterized in terms of their affinity (Biacore) toward these toxins (Table 3). scFv 3H, which contains only two changes with respect to the parental scFv C1 (Table 2), exhibited reduced affinities to the Cn2 and CII1 toxins compared with scFv 202F or RU1 (Table 3). A combination of the changes accumulated in scFvs 3H and 202F, which were incorporated into scFv RU1, improved the interaction with both toxins as a consequence of a slower dissociation rate ($k_{off}$) during the molecular interactions with both toxins. The slower dissociation rate is reflected as an increase in the interaction times between scFv RU1 and the toxins (Table 3). We had already reported that scFv 202F neutralized the CII1 and Cn2 toxins (13). The improvements in the binding strength of scFv RU1 explain its ability to optimally neutralize not only the CII1 and Cn2 toxins but also up to 3 LD$_{50}$ of fresh whole *C. noxius* venom. The rescue assays of previously poisoned animals revealed that a single intraperitoneal administration of 5 µg/mouse of each of the scFvs progressively reduced intoxication symptoms, and the mice recovered to their normal conditions in ~30 min. These results emphasize that the use of two complementary neutralizing scFvs ensures total protection against the effects of the venom and rescues the animals that were previously poisoned.

The simultaneous binding of the scFvs LR and RU1 to Cn2 was demonstrated by surface plasmon resonance assays (Fig. 2), which explained the complementary neutralization activity of the two scFvs (RU1 and LR), prompting us to identify the second interaction site on the Cn2 toxin. The first binding site on the Cn2 toxin, which was recognized by scFv LR and was confirmed in this work, allowed us to reinforce the available information related to the toxin areas that interact with the sodium channel and the proposed neutralization mechanisms (14, 40). The new binding site on the Cn2 toxin, which was structurally opposite the one recognized by scFv LR, is composed of the N-terminal segment, including residues Leu$^5$, Val$^6$, and Lys$^8$; the α-helix C-terminal end/β-turn portion, including residues Gln$^{31}$, Gln$^{32}$, Tyr$^{33}$, and Lys$^{35}$; and the C-terminal region, including residues Tyr$^{42}$, Gln$^{43}$, Ile$^{46}$, Leu$^{48}$, and Asn$^{49}$. As a consequence of these results, new questions arise about the mechanisms of toxin-channel interactions, which can be complex and diverse. Several studies have focused on the interaction of the Css4 toxin (from *C. suffusus suffusus* scorpion venom) with the Na$_{1.2}$ sodium channel (41, 42). The results presented in these reports suggest that the effects of this toxin on the Na$_{1.2}$ channel could be due to a specific interaction, but there are nine types of sodium channels in mammals (43), implying that there might be another type of interaction mechanisms with sodium channels. Moreover, the local variability of toxin sequences could determine different specificities and affinities for sodium channels.

It has been shown that the Cn2 toxin is specific for the Na$_{1.6}$ channel (11). Considering that scFv RU1 neutralizes the Cn2 toxin but binds to a region on the Cn2 toxin that has not been implicated in the interaction with the Na$_{1.6}$ channel, we can hypothesize that the inhibition of the channel activity could be associated with an as yet undescribed mechanism. A plausible alternative of the interaction could involve a process with two
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FIGURE 4. Representation of the maturation processes that led to the recognition and neutralization of the Cn2 and Cll1 toxins. A, the mutations that led to scFv RU1 (D54G, S56G, M105L, and Y110H) are represented. Orange, Cn2; blue, VL of scFv RU1; green, VH of scFv RU1; yellow, CDR1 of VH; light brown, CDR2 of VH; dark brown, CDR3s of VH and VL. The glycines located in CDR2 of the VH of scFv RU1 are indicated in magenta. The hydrogen bonding interactions are represented as continuous blue lines. The cation-Pi, and hydrophobic interactions are indicated as green and magenta dotted lines, respectively. B, overlapping of the indicated segments of the scFv RU1-Cn2 and scFvRU1-Cll1 complexes. The differences in the amino acid sequences of Cn2 and Cll1 are shown. The hydrophobic interactions involved in Cll1 recognition are also shown. The scFv RU1-Cn2 complex is represented as ribbons in light colors; VH is shown in light blue; VL is in light green, and Cn2 is in orange. The scFv RU1-Cll1 complex is also represented as dark colored ribbons; VH; in dark blue; VL in dark green, and Cll1 in dark red. The Vh and Vl CDR3 loops are shown in dark brown. The CDR1 and CDR2 loops of the VH regions are shown in light yellow and dark brown, respectively. The residues that differ in the Cn2 and Cll1 toxins are indicated in the same colors as the toxin. The residues of the toxins are separated by a slash, where the first one corresponds to Cn2 and the second one to Cll1. The hydrogen bonds, hydrophobic interactions, and cation-Pi interactions are represented as in A. Due to the change in the amino acid at position 9 (Asn replaced by Ser) in the Cll1 toxin relative to the Cn2 toxin, the hydrogen bond formed between Lys8 at framework region 3 of Vh and Asn9 of Cn2 toxin was lost. Additionally, due to the changes of Lys8 for Leu8 and Lys62 for Asn62 in Cll1 with respect to Cn2, three hydrogen bonds established within the CDR3 of the VH (Ile236 with Lys8 and Ser234 (OG) with Asn62 (OD1) and with Asn62 (OD2)) were also lost. In addition, due to the change of Lys8 for Leu8, Cll1 lost two cation-Pi interactions (Lys8 of Cn2 with Tyr59 and Trp231 of VH and VL chains of scFv RU1, respectively). Finally, due to the change of Lys8 for Leu8 in Cll1, a new hydrophobic contact with Ile236 was formed.

FIGURE 5. A, electron density map 2Fo – Fc (a-weighted, simulated annealing) at the interface between scFv RU1 VH and the Cn2 toxin. The Cn2 toxin and its corresponding residues are colored orange. The Vh domain of scFv RU1 is colored blue, with its CDR1 in yellow, CDR2 in light brown, and CDR3 in dark brown. Vl domain of scFv RU1 is colored green, with CDR3 in brown. The disulfide bridge between Cys103 and Cys106 that is present in CDR3 of the Vh chain is indicated. B, differences between the CII2 and Cn2 toxins at the interface with scFv RU1. The Cn2 toxin is colored orange; the Vh and Vl domains of scFv RU1 are colored blue and green, respectively; and CDR1 and CDR2 of the Vh domain are colored yellow and light brown, respectively. The CDR3 loops of the Vh and Vl domains are colored dark brown. The residues comprising the hydrophobic patch at the interface are labeled. The differences in the residues at the interface between the Cn2 and CII2 toxins are labeled and separated by a slash, where the first residue corresponds to Cn2 and the second to CII2.

steps. In the first step, the toxins would bind through the epitope recognized by RU1 to an as yet undefined site in the channel. In the second step, the toxins would bind through the epitope recognized by LR (on a site of the channel that has been previously identified). Similar alternatives have been proposed for other mechanisms of interaction between channels and toxins (41, 44). We used the complementary approaches of x-ray determination of the ternary complex scFv LR-toxin Cn2-scFv RU1 and modeling of the theoretical complex of scFv RU1 with the Cll1 toxin to accomplish the aims of this study. The analysis of the structure of the ternary complex allowed us to explain the effect of the selected mutations during the maturation process of scFv RU1. The mutations at CDR2 (D54G and S56G) and CDR3 (M105L and Y110H) of the Vh region improved the recognition of the Cn2 and Cll1 toxins (Figs. 3 and 4). The CDR3 of the Vh from scFv RU1 is a very long loop composed of 17 residues. This CDR3 is stabilized by a disulfide bond formed by Cys105 and Cys106, which is clearly visible in the electron density map of the ternary complex (Fig. 5A). As discussed previously, the interaction, through a hydro-
The histidine residue at this position hinders the formation of a hydrogen bond with Ile\textsuperscript{236} of the V\textsubscript{i} chain from scFv RU1. Additionally, the incorporation of a hydrophilic residue in the hydrophobic core of the patch at the interface between the toxin and the scFv RU1 disfavored its binding to Cll2 (Fig. 5B).

scFv RU1 also cross-reacted with other toxins and some toxic fractions from different venoms of the genus Centruroides (data not shown). These observations emphasize the potential importance of this new interaction site, considering that the Cll1 and Cn2 toxins exhibit different specificities for sodium channels (Cll1: Na\textsubscript{1.1}, Na\textsubscript{1.2}, Na\textsubscript{1.3}, Na\textsubscript{1.4}, Na\textsubscript{1.5}, and Na\textsubscript{1.6}; Cn2: Na\textsubscript{1.6}) (11, 45). The neutralization properties of this scFv RU1 can be still improved by directed evolution and screening by phage display.

The determination of the crystal structure of the ternary complex permits us to reflect on several important points. 1) The fact that a small toxin, such as Cn2, can be neutralized by two antibodies that recognize different epitopes suggests that, despite their small size, toxins can be neutralized by antibodies that could bind to different epitopes. The total area of the Cn2 toxin is 4601 Å\textsuperscript{2}, and the total percentage of surface covered by the two scFvs is 39.25%. This result suggests that there may be other neutralizing epitopes around the two that have already been described. However, considering the relatively small surface of the Cn2 toxin, they might overlap. 2) The analysis of the structure of the ternary complex helped us to understand how the maturation processes allowed the scFv RU1 to "fit" onto the surfaces of the Cll1 and Cn2 toxins through the formation of a set of interactions that could not be established by the parental scFv C1. 3) The rational use of the approaches here described can be exploited to improve the scFv interactions with the toxins from other scorpions. The incorporation of a few key mutations seems to determine the level of recognition because the regions contacted by both scFvs are highly conserved. The set of interactions that could not be established by the parental scFv RU1 can be improved by directed evolution and screening by phage display.

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Optimal Neutralization of *Centruroides noxius* Venom Is Understood through a Structural Complex between Two Antibody Fragments and the Cn2 Toxin

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