Published in final edited form as:
Biochem J. ; 427(3): 369–376. doi:10.1042/BJ20100074.

Isolation and characterization of selective and potent human Fab inhibitors directed to the active-site region of the two-component NS2B–NS3 proteinase of West Nile virus

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Abstract

There is a need to develop inhibitors of mosquito-borne flaviviruses, including WNV (West Nile virus). In the present paper, we describe a novel and efficient recombinant-antibody technology that led us to the isolation of inhibitory high-affinity human antibodies to the active-site region of a viral proteinase. As a proof-of-principal, we have successfully used this technology and the synthetic naive human combinatorial antibody library HuCAL GOLD® to isolate selective and potent function-blocking active-site-targeting antibodies to the two-component WNV NS (non-structural protein) 2B–NS3 serine proteinase, the only proteinase encoded by the flaviviral genome. First, we used the wild-type enzyme in antibody screens. Next, the positive antibody clones were counter-screened using an NS2B–NS3 mutant with a single mutation of the catalytically essential active-site histidine residue. The specificity of the antibodies to the active site was confirmed by substrate-cleavage reactions and also by using proteinase mutants with additional single amino-acid substitutions in the active-site region. The selected WNV antibodies did not recognize the structurally similar viral proteinases from Dengue virus type 2 and hepatitis C virus, and human serine proteinases. Because of their high selectivity and affinity, the identified human antibodies are attractive reagents for both further mutagenesis and structure-based optimization and, in addition, for studies of NS2B–NS3 activity. Conceptually, it is likely that the generic technology reported in the present paper will be useful for the generation of active-site-specific antibody probes for multiple enzymes.

Keywords

antibody; Fab; flavivirus; IgG; serine proteinase; West Nile virus (WNV)

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AUTHOR CONTRIBUTION

Sergey Shiryaev characterized the purified scFv and Fab antibodies. Ilian Radichev performed mutagenesis and isolated the mutant NS2B–NS3 proteinase constructs. Boris Ratnikov performed the experiments that were designed to determine the inhibitory constants of the antibodies. Alexander Aleshin and Boguslaw Stec contributed to the modelling of the NS2B–NS3 proteinase structure. Katarzyna Gawlik contributed to the initial stages of the project. Christian Frisch and Achim Knappik performed the bio-panning procedures and isolated the scFv and Fab antibodies from the HuCAL GOLD® library. Alex Strongin developed and co-ordinated the project and wrote the paper. All authors discussed the results and their implications, and commented on the manuscript at all stages.

INTRODUCTION

There is a need to develop inhibitors of mosquito-borne and tick-borne flaviviruses, including WNV (West Nile virus). There have already been multiple cases of mosquito-borne WNV in the United States. Unfortunately, there are no effective countermeasures or vaccines as yet. WNV is an enveloped positive-stranded 11-kb RNA virus. The genomic RNA of WNV encodes a polyprotein precursor that consists of three structural proteins, C (capsid protein), prM (precursor membrane protein) and E (envelope protein), and seven NS proteins (non-structural proteins), NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, arranged in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5.

Polyprotein processing by the viral two-component NS2B–NS3pro (proteinase domain of NS3) and also by the host-cell secretase and furin is required to generate individual viral proteins [1–3]. The full-length NS3 is a multifunctional protein in which the N-terminal 184 amino-acid residues represent the NS3pro and the C-terminal sequence codes for the helicase, nucleoside triphosphatase and RNA triphosphatase activities, all of which are co-ordinately regulated through localization within membrane compartments in the infected cell. NS2B functions as an essential cofactor of NS3pro. The cofactor activity of the 48-amino-acid central portion of NS2B is roughly equivalent to that of the entire NS2B sequence [4–7]. Structural studies have determined that NS2B wraps around NS3pro, completing, in a precise and well-defined fashion, the structure of the active site [8,9]. In agreement, deletion of the NS2B sequence inactivates the functional activity of NS3pro [10,11]. These unique cofactor–protease-domain interactions are common for multiple flavivirus types [7,12–15].

NS3pro is responsible for the cleavage of the internal sites within C, NS3 and NS4A, and also at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 boundaries. Because inactivating mutations of the NS3pro cleavage sites in the polyprotein precursor abolished viral infectivity [12,14–28], it is clear that the NS3pro function is vitally important for the virus and that NS3pro antagonists have merit as viral drugs. Multiple competitive and non-competitive peptide and small-molecule inhibitory scaffolds of NS3pro have recently been identified [29–36]. There are, however, obstacles, including inefficient cell penetration and poor solubility and stability, to their development as an antiviral therapy. Additional, unconventional, approaches are required to overcome these obstacles.

Because of their unique ability to bind specifically to nearly any antigen, antibodies provide a much sought after and excellent scaffold for designing inhibitors targeted to the individual members of a family of homologous enzymes. Over the past two decades, recombinant technology has enabled the production of engineered antibody fragments such as Fab or scFv (single-chain variable fragment) antibodies [37–41]. An scFv antibody is a small engineered antibody, in which the VH (variable heavy chain) and VL (variable light chain) of the antibody molecule are connected by a short flexible polypeptide linker.

Phage-display technology has been used successfully for the isolation of specific scFvs or Fabs from human repertoire libraries [42]. For phage-display selection, the Fab format is preferred because scFv fragments usually oligomerize and, as a result, generate multimers [43,44]. Fabs usually do not oligomerize and are more stable compared with scFvs. The identified Fabs can be genetically manipulated further and linked to different tags or enzymes for detection or labelling purposes or equipped with new diversity in the CDRs (complementarity-determining regions) in order to improve affinity and specificity [45,46].

In the present paper, we report an innovative and effective antibody-selection technology. As a proof-of-principal, we used this technology and the phage Fab antibody library [47] to select the inhibitory active-site-targeting antibodies to a viral proteinase (WNV NS2B–NS3pro). This

generic technology, by analogy, may be useful for the efficient generation of active-site-specific antibody probes for enzymes.

MATERIALS AND METHODS

Reagents

Reagents were purchased from Sigma–Aldrich (Milwaukee, WI, U.S.A.), unless indicated otherwise. The Pyr-RTKR–AMC (pyroglutamic acid-Arg-Thr-Lys-Arg–7-amino-4-methylcou-marin) fluorescence-quenched cleavage peptide was purchased from Bachem (King of Prussia, PA, U.S.A.). Goat anti-human F(ab′)2 fragment conjugated with HRP (horseradish peroxidase) was from AbD Serotec (Oxford, UK). Aprotinin was purchased from Serological Corporation (Norcross, GA, U.S.A.) and then additionally purified by using gel-exclusion chromatography on a Superdex-75 column (GE Healthcare, Piscataway, NJ, U.S.A.).

The WNV and DV2 (Dengue virus serotype 2) NS2B–NS3pro expression constructs

The WNV (strain NY99) and the DV2 (strain 16681) cDNAs were generously given by Dr Richard Kinney (Centers for Disease Control and Prevention, Fort Collins, CO, U.S.A.) and Dr Michael Diamond (Department of Internal Medicine, Washington University, St Louis, MO, U.S.A.) respectively. The WT (wild-type) DV2 cDNA was used in PCR reactions followed by routine gene-engineering manipulations to generate the DV2 NS2B–NS3pro constructs, which included the 48-residue NS2B cofactor (amino acids 1393–1440) linked via a nona-peptide linker GGGGSGGQQ to the NS3pro portion (amino acids 1476–1687). Similarly, the WNV cDNA was used to generate the WNV NS2B–NS3pro constructs, which included the 48-residue NS2B cofactor (amino acids 1423–1470) linked via a GGGGSGGGG linker to the NS3pro sequence (amino acids 1506–1689) [6]. The design and purification of the autolytic-site-deficient WNV NS2B–NS3pro K48A construct, the H51A construct, the G22S construct and D32A/D33A/D34A mutants (DDD/AAA) with the mutations in the NS2B cofactor sequence have been described previously [4–6,48]. The T52V and R76L mutations in the NS3pro portion were inserted into the WT WNV NS2B–NS3pro construct. As a result, the NS2B/NS3pro boundary was autocleaved in these constructs, albeit NS2B then remained non-covalently associated with the NS3pro domain [7]. The sequence of the constructs is shown in Figure 1. The constructs were re-cloned into the pET101/D-TOPO expression vector (Invitrogen, San Diego, CA, U.S.A.).

Proteinase expression and purification

Competent *Escherichia coli* BL21 CodonPlus® (DE3)-RIPL cells (Stratagene, San Diego, CA, U.S.A.) were transformed with the individual recombinant pET101/D-TOPO vectors. Transformed cells were grown in Luria–Bertani broth at 37 °C to reach D_{600} = 0.6. The protein expression was then induced at 37 °C using 1 mM isopropyl β-D-thiogalactoside for an additional 6 h. The cells were collected by centrifugation, resuspended in 20 mM Tris/HCl, pH 8.0, containing 1 M NaCl and 1 mg/ml lysozyme and disrupted by sonication (30s pulse, 30s interval, six pulses) on ice using a Misonix 3000 sonicator (Farmingdale, NY) at a power setting according to the manufacturer's instructions for 'liquid sample volume'. Cell debris was removed by centrifugation. The WNV and DV2 constructs were purified from the supernatant fraction using HiTrap Co^{2+}-chelating chromatography on a 5 ml column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The His_6-tagged NS2B–NS3pro constructs were eluted using a 0–500 mM gradient of imidazole concentrations. The fractions were analysed using SDS/PAGE followed by Coomassie staining, and also by Western blotting with a His_6-tagged antibody (Clontech, Mountain View, CA, U.S.A.).

Proteinase assays with fluorogenic peptide

The assay for NS2B–NS3pro peptide-cleavage activity was performed in 0.2 ml of 20 mM Tris/HCl buffer, pH 8.0, containing 20 % (v/v) glycerol and 0.005 % Brij 35. The cleavage-peptide (Pyr-RTKR–AMC) and enzyme concentrations were 25 μM and 10 nM respectively. The reaction velocity was monitored continuously at λ_{ex} = 360 nm and λ_{em} = 465 nm on a Spectramax Gemini EM fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.). All assays were performed in triplicate in wells of a 96-well plate. The assays were repeated multiple times and the results were reproducible from day to day.

The concentrations of catalytically active NS2B–NS3pro in the purified samples were always quantified by active-site titration prior to kinetic studies [7]. Active-site titration of NS2B–NS3pro was performed with aprotinin (K_i = 20 nM). Briefly, 10 nM NS2B–NS3pro was incubated with increasing concentrations of aprotinin. Residual activity of NS2B–NS3pro was then measured by determining the rate of cleavage of Pyr-RTKR–AMC. The results were plotted against the amounts of aprotinin and a line was fitted through the data points. The intercept on the *x*-axis is equal to the concentration of active enzyme. The concentration of active NS2B–NS3pro was close to 100 % when compared with the protein concentration.

Determination of the K_i values of the inhibitory antibodies

The NS2B–NS3pro constructs (50 nM) were pre-incubated with increasing concentrations of the antibodies for 30 min at room temperature (22 °C) in 0.1 ml of 20 mM Tris/HCl buffer, pH 8.0, containing 20 % glycerol and 0.005 % Brij 35. The Pyr-RTKR–AMC substrate (25 μM) was then added in 0.1 ml of the same buffer. All assays were performed in triplicate in wells of a 96-well plate. IC_{50} values were calculated for each antibody by determining the antibody concentration needed to inhibit half of the maximum cleavage activity of NS2B–NS3pro against Pyr-RTKR–AMC. The measured IC_{50} values were used to calculate the K_i values. K_i values of the competitive antibody inhibitors were derived using the Cheng–Prusoff equation, $K_i = IC_{50}/(1 + [S]/K_m)$, where K_i is the binding affinity of the inhibitor, IC_{50} is the functional strength of the inhibitor, [S] is the substrate concentration and K_m is the affinity of the substrate for the enzyme. GraphPad Prism was used as a fitting software. Because the [S] and K_m values of the Pyr-RTKR–AMC substrate that we used were 25 μM and 43–71 μM respectively [5,7], the actual difference between the IC_{50} and K_i values of the inhibitory antibodies was less than 25 %.

ELISA

Wells of a Nunc-Immuno MaxiSorp 96-well flat-bottomed plate (Thermo Fisher Scientific, Rochester, NY, U.S.A.) were coated in triplicate for 18 h at 4 °C using the WNV or DV construct (0.1 ml/well; 1 μg/ml). Control wells were coated with 1 μg/ml BSA. After washing with PBS, non-specific binding sites were saturated by incubation with 0.2 ml of 1 % BSA for 2 h at room temperature. The wells were then washed using PBS/0.05 % Tween 20. The 2 μg/ml antibody solution (0.1 ml) in PBS/1 %BSA/0.05 %Tween 20 was added to the wells. After a 2 h incubation at room temperature, the wells were washed using PBS/0.05 % Tween 20. Goat anti-human F(ab′)2 fragment (1:5000 dilution in 1 % BSA/0.05 % Tween 20 in PBS; 0.1 ml) was then added to the wells and the incubation was continued for 2 h at room temperature. After washing with PBS/0.05 % Tween 20 and incubation with 0.1 ml of TMB/M (TMB Super Sensitive One-Component HRP Microwell Substrate solution; BioFX, Owings Mills, MD, U.S.A.), the reaction was stopped by the addition of 0.05 ml of 1 M HCl, and the intensity of the colour reaction was measured at 450 nm using a Spectra Fluor Plus fluorescence plate reader (Tecan, Mannedorf, Switzerland).

Generation of human NS2B–NS3-specific antibodies from the HuCAL (human combinatorial antibody library) GOLD® library

The synthetic phage-display HuCAL GOLD® library was designed, developed and tested by MorphoSys (Martinsried/Planegg, Germany). Recombinant antibodies were isolated from a one-billion-gene sub-library of the HuCAL GOLD® collection of 15 billion antibody genes [47] by three rounds of selection (panning) using the immobilized recombinant WT NS2B–NS3pro protein as a bait [46,47,49–52]. Prior to selection, the phage library was blocked with the NS2B–NS3pro H51A construct in order to deplete the antibodies to the common epitopes present in both the immobilized NS2B–NS3pro and the H51A mutant. BSA and the ubiquitin–His_6 and CD33–His_6 constructs were used as controls to eliminate false-positive antibodies and antibodies to the His_6 tag. After three rounds of selection, the enriched Fab gene pool was sub-cloned into an expression vector that leads to the expression of bivalent Fab mini-antibodies [53]. Single *E. coli* colonies were screened through ELISAs using both the NS2B–NS3pro K48A and the H51A mutant, and the antibodies specific for the WT protein were expressed in *E. coli* and then purified using metal-chelating chromatography.

Western blotting

Following the transfer to the Immobilon P membrane (Millipore, Bedford, MA, U.S.A.), the membrane was incubated for 16 h at 4 °C with the primary antibodies AbD05320, AbD05321, AbD05322, AbD05444, AbD05445 and AbD05446 (0.25 μg/ml) or the AbD05323 antibody (0.5 μg/ml). The secondary antibody (1:1000 dilution) was goat anti-human IgG F(ab′)2 fragment conjugated with HRP. TMB/M was used as a substrate.

RESULTS

The structure of the two-component flaviviral proteinase

Multiple previous studies have showed that the 48-residue central NS2B domain linked to the N-terminus of NS3 via an artificial flexible linker (GGGGSGGGG) is active in the *in vitro* protease assays [54,54a]. However, the NS3pro activity usually cleaves the initial $K^{48}G\downarrow$GGGSGGGG linker sequence, leading to the presence of the non-covalently associated NS2B cofactor and the NS3pro domain in the samples. The K48A mutation of the C-terminal amino-acid residue of the NS2B sequence inactivated the autolytic cleavage site. As a result, the NS2B–NS3pro K48A mutant is resistant to autoproteolysis and is represented by the intact single-chain NS2B–NS3pro construct in the samples. In the additional WNV mutant, called H51A, an alanine residue was substituted for the catalytically essential His^{51} of the NS3pro active site. As a result of this mutation, the H51A construct became catalytically inert and was not autocleaved.

NS3pro from DV and WNV share 50 % sequence identity. Despite the limited number of amino-acid substitutions proximal to the catalytic triad, the two proteinases display significant differences in their substrate-cleavage preferences and, accordingly, in the structure of the active-site region. Active-site differences between WNV and DV exist at Thr^{52} (Val^{52} in DV) and Arg^{76} (Leu^{76} in DV). To explore the potential role of the Thr^{52} and Arg^{76} residues, we constructed chimaeric proteins with replacements of DV residues into the WNV protein, leading to the construction of the T52V and R76L mutants.

Additional mutants used, G22S and DDD/AAA, involved the modifications of the NS2B–NS3pro K48A sequence that might affect either the folding or the interactions of NS2B with NS3pro in the proximity of the active-site region or both parameters (Figure 1).

WT DV and WNV NS2B–NS3pro, together with the WNV/DV chimaeras, were expressed in *E. coli* with C-terminal His_6 tags and isolated from the soluble fraction by metal-chelating

chromatography. The cleavage kinetics of the Pyr-RTKR–AMC fluorescent peptide substrate was measured to confirm the catalytic potency of the constructs (Table 1). The purified constructs were used as baits in the antibody selection and characterization procedures.

Identification of the active-site-targeting antibodies

To identify the Fabs capable of binding to the active-site region of WNV NS2B-NS3pro, we used a bio-panning procedure that involved both the catalytically active NS2B–NS3pro K48A construct with the intact active-site sequence and the inert NS2B–NS3pro H51A mutant with the inactivated active site (Figure 2).

We tested several different panning methodologies, each of which included three consecutive rounds of phage selection. In these procedures, the NS2B–NS3pro K48A construct was immobilized on microtitre plates as bait. In the first approach, we blocked the phage library using the H51A mutant construct (20 μg/ml). The obtained antibodies (AbD05320 and AbD05321) bound both the K48A protein and the mutant, indicating that the blocking was not stringent enough. In the course of performing the second methodology, we reduced the amount of the immobilized NS2B–NS3pro K48A construct from 50 μg/ml in the first round to 5 μg/ml in the second round and, finally, to 1 μg/ml in the third round. Again, blocking of the library was performed with the H51A mutant (20 μg/ml). Using this strategy, we isolated two additional antibodies (AbD05322 and AbD05323); the latter was specific for the K48A construct. In the third approach, we blocked the library using a high concentration (100 μg/ml) of the H51A mutant. As a result, we selected three additional antibodies (AbD05444, AbD05445 and AbD05446). Overall, as a result of the bio-panning of the HuCAL GOLD® library, seven individual antibodies were identified (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/427/bj4270369add.htm). These seven antibodies were isolated from the respective recombinant *E. coli* cells and the purified antibody samples were characterized further.

Characterization of the antibodies

To determine whether the antibodies were resistant to NS2B–NS3pro proteolysis, the purified antibody samples (4 μg; 8 μM each) were co-incubated for 2 h at 37 °C with the purified NS2B–NS3pro K48A construct (1 μg; 1.4 μM; the enzyme/substrate molar ratio was 1:6) in 10 mM Tris/HCl buffer, pH 8.0, containing 20 %(v/v) glycerol and 0.005 %Brij 35. The digested samples were separated by gradient SDS/4–20 % PAGE. All of the antibodies were completely resistant to NS2B–NS3pro proteolysis (results not shown).

To determine the inhibitory potency of the antibodies, increasing concentrations of the purified AbD05320, AbD05321, AbD05322, AbD05323, AbD05444, AbD05445 and AbD05446 samples were each co-incubated for 60 min at room temperature with NS2B–NS3-K48A (10 nM). The residual proteolytic activity of the protease was then measured using the Pyr-RTKR–AMC fluorescent peptide substrate. A high-level inhibition of the proteolytic activity of NS2B–NS3-K48A was observed at a 300 nM concentration of AbD05320, AbD05322, AbD05323, AbD05444 and AbD05445, whereas AbD05321 and AbD05446 were less inhibitory (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/427/bj4270369add.htm).

To analyse the inhibitory characteristics of the antibodies in more detail, we determined the K_i values of the antibodies (Figure 3). For these purposes, NS2B–NS3-K48A (10 nM) was pre-incubated with the increasing concentrations of the antibodies for 60 min at room temperature in 10 mM Tris/HCl buffer, pH 8.0, containing 20 % (v/v) glycerol and 0.005 % Brij 35. The Pyr-RTKR–AMC substrate (25 μM) was then added to the 0.1 ml reactions. Then, the K_i values of AbD05320, AbD05321, AbD05322, AbD05323, AbD05444, AbD05445 and AbD05446 were determined to be 264, 400, 170, 31, 58, 35 and 288 nM respectively. Because the

antibodies bind to the active-site region of NS2B–NS3pro, Lineweaver–Burk plots, as a result, demonstrated strictly competition-type inhibition by the antibody inhibitors. The representative results are shown in Figure 3. Because excessively high concentrations of the antibodies were required for the V_0 values to reach clear plateaus, the incomplete plots approaching the plateaus were used.

To determine whether the antibodies selectively bind to the WNV and DV constructs, we used a Western blotting procedure (Figure 4). The purified WNV K48A, H51A, T52V, R76L, G22S and DDD/AAA constructs (100 ng of each) were added to the *E. coli* soluble protein fraction (20 μg of total protein) and the samples were analysed by Western blotting with the AbD05320, AbD05321, AbD05322, AbD05323, AbD05444, AbD05445 and AbD05446 antibodies followed by a goat anti-human IgG F(ab′)2 fragment conjugated to HRP and a TMB/M substrate. Because the NS2B/NS3pro boundary is autocleaved in the T52V and R76L mutants, the resulting NS3pro domain has a low molecular mass compared with other mutants that were constructed using the autolytic-site-deficient K48A background.

Although the G22S and DDD/AAA mutations in the NS2B cofactor did not notably affect the binding efficiency of all of the antibodies, the presence of H51A, T52V and R76L mutations in the NS3pro active-site region significantly decreased the binding efficiency of AbD05322 (K_i = 170 nM), AbD05323 (K_i = 31 nM) and AbD05444 (K_i = 58 nM). In turn, AbD05320, AbD05321, AbD05445 and AbD05446 partially cross-reacted with the H51A and additional tested mutants (Table 2).

To corroborate these results using the native proteinase constructs (rather than partially denatured ones as in the Western-blotting experiments), we performed an assessment of the antibody binding with the WNV and DV constructs using ELISA. After coating wells of a 96-well plate with the constructs, the antibodies were allowed to bind to the immobilized material. The level of the resulting immune complexes was determined using the goat anti-human Fab fragment conjugated with HRP (Figure 5). None of the selected anti-WNV antibodies recognized the closely related DV and HCV proteinases, as well as human serine proteinases, including furin (results not shown). Because AbD05323 and AbD05444 did not interact with the H51A, R76L and T52V WNV constructs, these two antibodies were clearly different when compared with the other anti-WNV antibodies we analysed. Overall, we concluded that the AbD05323 and AbD05444 antibodies target the active-site region of the two-component WNV NS2B–NS3pro in a selective and focused manner.

DISCUSSION

Because of their unique ability to bind specifically to nearly any antigen, antibodies provide an excellent scaffold for designing inhibitors targeted to the individual members of a family of homologous enzymes. Over the past decade, recombinant technology has enabled the production of engineered antibody fragments such as Fabs or single-chain scFvs. Phage-display technology allows the presentation of the scFv or Fab on the phage surface and this technology has been used successfully for the isolation of specific antibodies from human repertoire (immune, non-immune or synthetic) libraries via several enrichment cycles. The identified scFvs/Fabs can be genetically manipulated further for improved specificity and affinity. The scFv–His_6 antibody format can be readily modified and transformed into a two-chain Fab format tagged with FLAG or streptavidin tags or vice versa.

There is a need to develop inhibitors of flaviviruses, including Dengue haemorrhagic fever, yellow fever, Japanese encephalitis viruses and WNV. Today, there are millions of cases of Flaviviridae infections worldwide and multiple cases of WNV in the United States. The two-component NS2B–NS3pro is the only proteinase encoded by the flaviviral genome. This

proteinase activity is essential for the proteolytic processing of the viral polyprotein precursor and virus infectivity. NS2B–NS3pro consists of two components of which the NS3pro portion represents the proteinase domain and the NS2B part is a cofactor. NS3pro alone is inactive. Because of its irreplaceable role in the virus life cycle, NS2B–NS3pro is a highly promising target for drug design.

We successfully used the phage Fab human synthetic antibody HuCAL library to isolate selective and potent function-blocking, active-site-targeting antibodies to WNV NS2B–NS3pro. For this purpose, we developed a novel technology, which, by analogy, allows the rapid and efficient identification of active-site-targeting antibodies to any enzyme. In our methodology, we tried to identify only those antibodies that were able to bind to the WT proteinase, but which were then incapable of binding with the proteinase mutant bearing a point mutation of the catalytically essential active-site residue (in the case of NS2B–NS3pro, the histidine residue of the catalytic triad). As a result, the active-site-targeting antibodies were readily selected from the antibody library. Conceptually, the use of the focused panning technology we described in our report may facilitate the identification of the active-site-targeting recombinant antibodies to any enzyme.

From the seven initially isolated Fabs, AbD05323 (K_i = 31 nM) and AbD05444 (K_i = 58 nM) appeared to be the most promising. These two Fabs were able to bind to the WT WNV proteinase, but not the to proteinase with the H51A, T52V and R76L mutations in the active-site region. The Fabs AbD05323 and AbD05444 were then transformed into an scFv format. These low-nanomolar-range inhibitory human antibodies are both novel molecular tools for the determination of the NS2B–NS3pro active-site parameters and a valuable guiding scaffold for the design of the anti-flaviviral inhibitors. Because the NS3pro viral enzyme activity is in close association with the endoplasmic-reticulum membrane inside the cell, a limited level of cell penetration, unfortunately, remains a major obstacle in using the anti-NS3pro antibodies for clinical purposes. However, the selected Fab antibodies may be used *in vitro* for the determination, in better detail, of the NS2B–NS3pro active-site parameters. In addition, the structural parameters of the antibody–protease active-site interface may guide a scaffold for the design of the selective, small-molecule, anti-flaviviral inhibitors. On the basis of the available structure of the inhibitory complex of the trypsin-like matriptase serine proteinase with the E2 Fab antibody, which is a 15 pM inhibitor of matriptase [55,56], we believe that further refinement of the 30–50-nM-range inhibitory NS2B–NS3pro antibodies is possible and that this refinement can be accomplished by using either mutagenesis of CDR-H (heavy-chain variable regions) or DNA shuffling or both.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING

This work was supported by the National Institutes of Health [grant numbers AI061139, AI055789 (to A.S.)].

Abbreviations used

C	capsid protein
CDR	complementarity-determining region
DV2	Dengue virus serotype 2

E	envelope protein
HRP	horseradish peroxidase
HuCAL	human combinatorial antibody library
NS	non-structural protein
NS3pro	proteinase domain of NS3
prM	precursor membrane protein
Pyr-RTKR–AMC	pyroglutamic acid-Arg-Thr-Lys-Arg–7-amino-4-methylcoumarin
scFv	single-chain variable fragment
TMB/M	TMB Super Sensitive One-Component HRP Microwell Substrate Solution
WNV	West Nile virus
WT	wild-type

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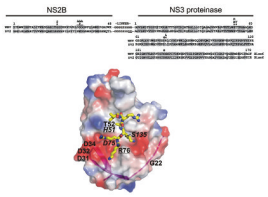

Figure 1. Structure and sequence alignment of the NS2B cofactor and the NS3 proteinase domain of WNV and DV2

Upper panel: sequence alignment of the NS2B–NS3pro constructs; homologous amino-acid-residue positions are shaded. The asterisks above the sequences indicate His^{51}, Asp^{75} and Ser^{135} of the catalytic triad. The arrows indicate mutations (G22S, DDD/AAA, H51A, T52V and R76L). The G22S and DDD/AAA constructs also contained the K48A mutation that inactivated the Lys^{48}↓Gly autolytic cleavage site where glycine is the N-terminal residue of the linker. Lower panel: the structure of WNV NS2B–NS3pro (PDB accession code 2IJO) [8]. The original position of the His^{51}, Asp^{75} and Ser^{135} of the catalytic triad (italicized) and the mutant positions are shown. The mutations that affect the binding of the proteinase with the antibodies are underlined. The surface is coloured by electrostatic potential (negative, red; positive, blue). The magenta ribbon shows NS2B contributing to the NS3pro active site. The positioning of a substrate-mimetic peptide (KRKARI) is shown in yellow. The model was presented using PyMol software (DeLano Scientific; http://www.pymol.org).

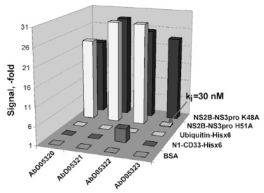

Figure 2. Selection process of the antibodies directed to the region proximal to the essential His^{51} of the catalytic triad

The purified NS2B–NS3pro K48A construct was used for selection of the phage antibody library, whereas the inert H51A mutant with the mutation of the active-site His^{51} was used for library blocking and counter-screening. The constructs were C-terminally tagged with a His_6 tag. The antibodies, which recognize the K48A enzyme and which do not recognize the mutant, were selected from the antibody library. BSA, ubiquitin–His_6 and CD33–His_6 were used as controls to eliminate false-positive antibodies. The AbD05323 antibody (indicated by an arrow) was selected in this particular experiment. Hisx6, His_6; ki, K_i.

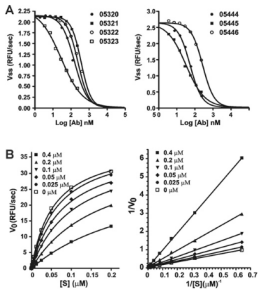

Figure 3. Catalytic parameters of the inhibitory antibodies

(**A**) Before the addition of the Pyr-RTKR–AMC substrate (25 μM), the purified WNV proteinase (10 nM) was co-incubated for 30 min with increasing concentrations of the antibodies. The cleavage of the Pyr-RTKR–AMC peptide by the proteinase was monitored to determine the K_i values. (**B**) Left-hand panel: competitive inhibition of NS2B–NS3pro proteolysis of Pyr-RTKR–AMC NS2B–NS3pro by the AbD05444 antibody (0–4 μM). Right-hand panel: Lineweaver–Burk plot of the results. The inhibitory antibody was added to the reactions. The initial velocity of the Pyr-RTKR–AMC cleavage was then measured in triplicate. Note that the K_m values, but not the V_{max} values, are affected by the inhibitor. RFU, relative fluorescence unit; V_0 and Vss, steady-state rate.

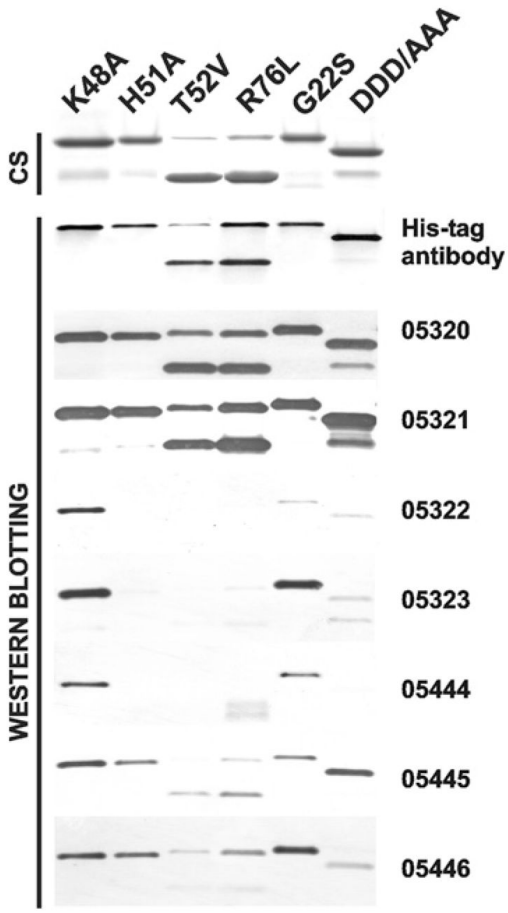

Figure 4. The H51A, T52V and R76L WNV NS2B–NS3 mutants do not interact with the AbD05323 and AbD05444 antibodies

The purified mutant constructs were analysed by SDS/PAGE (4 μg/lane) followed by Coomassie staining (CS) and Western blotting (100 ng/lane) with a His_6-tagged antibody and the selected AbD05320, AbD05321, AbD05322, AbD05323, AbD05444, AbD05445 and AbD05446 antibodies. Note that AbD05323 and AbD05444 did not bind to the H51A, T52V and R76L constructs, which exhibit mutations in the proximity of the active site.

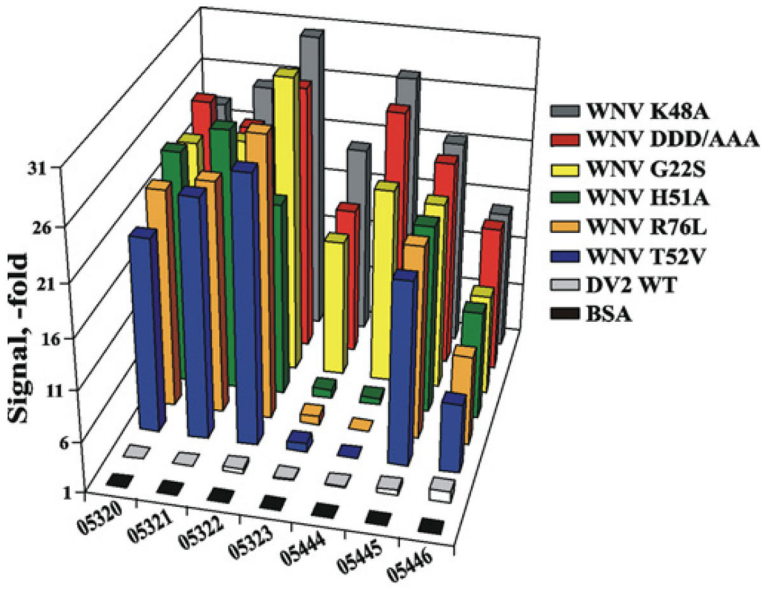

Figure 5. ELISA of the NS2B–NS3pro constructs

The antibodies were allowed to interact with the native K48A and mutant WNV co nstructs and with the WT DV2 constructs. The binding efficiency is expressed as a fold increase relative to the control (BSA).

Table 1

Kinetic parameters of the Pyr-RTKR–AMC cleavage by the DV2 and WNV constructs

The G22S, DDD/AAA and H51A WNV mutants have no activity because the G22S and DDD/AAA mutations affect the interactions of the NS2B cofactor with the NS3pro domain [48] and because the H51A mutation inactivates the proteinase. Results are means ± S.D.

NS2B–NS3pro	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
WNV WT	71 ± 15	6.3 ± 0.35	88 ± 12
WNV K48A	58.8 ± 10	5.25 ± 0.25	89.3 ± 11
WNV T52V	0.13 ± 0.006	0.013 ± 0.0006	100 ± 10
WNV R76L	89 ± 16	7.3 ± 0.4	82 ± 11
DV2 WT	3.6 ± 0.2	0.02 ± 0.001	5.5 ± 0.5

Table 2

The K_i values (nM) of the antibodies against the original WNV NS2B–NS3pro K48A construct and the WNV NS2B–NS3pro mutants

	K_i value (nM)						
Construct	**AbD05320**	**AbD05321**	**AbD05322**	**AbD05323**	**AbD05444**	**AbD05445**	**AbD05446**
K48A	264	400	170	31	58	35	289
T52V	263	416	763	624	863	754	811
R76L	282	432	720	684	847	733	828