Title: Selection and characterization of single domain antibodies against human CD20

Jinny L. Liu^{a*}, Dan Zabetakis^a, Ellen R. Goldman^a and George P. Anderson^{a*}.

- a. Center for Bio/Molecular Science and Engineering, Naval Research Laboratory, 4555 Overlook Ave. SW. Washington DC 20375.

*corresponding authors: Jinny.liu@nrl.navy.mil; 202-404-6122;
george.anderson@nrl.navy.mil; 202-404-6033

Abstract

CD20 is a membrane protein with four integral membrane regions and a large extracellular loop between residues 142 and 187, which serves as a target binding region for rituximab (RTX) and most other anti-CD20 monoclonal antibodies. It is highly expressed in B-lymphoma cells and B lymphocytes and often serves as a target for immunotherapy. In this study, we developed single domain antibodies (sdAbs) for the sensitive detection of CD20. To achieve this, an immune sdAb library was prepared from llamas immunized with a fusion between the large loop from CD20 and Hoc, a highly antigenic protein from the T4 bacteriophage, (CD20-Hoc). By subtracting binders to recombinant Hoc during the biopanning, potential anti-CD20 sdAbs were selected, sequenced, and characterized for their binding affinity to CD20-Hoc fusion versus Hoc. Twenty five clones grouped into three different families based on CDR3 sequence were identified as potential CD20 binders. The binding kinetics of representative sdAbs from each class and RTX were evaluated by surface plasmon resonance (SPR). Most of the sdAbs that were evaluated show binding affinities to CD20-Hoc in the nM range, and class A sdAbs, exhibited ≥ 40 -fold increase in affinity for CD20-Hoc versus Hoc. When the binding of the sdAb to CD20 on SU-DHL-4 cells was evaluated by flow cytometry, only class A sdAbs displayed strong binding to CD20 and recognized DHL cells in a concentration dependent manner.

Key words: CD20; Single domain antibody; immune library; biopanning; Hoc

Abbreviations: RTX, Rituximab; SdAb, single domain antibody; CDR, complementarity determining region; FR, framework region; Bt, Biotinylated; NA-PE, NeutrAvidin – phycoerythrin conjugate; Ab, antibody; complement-dependent cytotoxicity (CDC).

1. Introduction

The Human CD20 molecule consists of 297 amino acids and is a non-glycosylated 33-35kD integral membrane protein expressed at high levels in B lymphocytes and most B-cell lymphomas (Tedder and Engel, 1994). It is part of the MS4A family of proteins, which are predicted to have four intermembrane regions and contains their C and N-termini within the cell. It is an ideal target for antibodies (Abs) as it does not shed from B-cells and is not internalized after antibody treatment (Cragg et al., 2005). More evidence shows that Ab treatment triggers complement-dependent cytotoxicity (CDC) and binding to CD20 triggers cell signaling that affects cell viability (Teeling et al., 2006; Dean et al., 2002). In fact, Rituximab (RTX), a human/mouse chimeric anti-CD20 antibody, was the first therapeutic monoclonal Ab approved by Food and Drug administration (FDA) for the treatment of B-cell Non-Hodgkin lymphomas (NHLs). Although RTX has been widely used in the treatment of NHL, only 48% of patients respond and complete responses are less than 10% (Cheson and Leonard, 2008). Thus, there is still a need to develop alternative CD20 specific Abs that may provide better therapeutic results or be useful for diagnostic and imaging applications.

A large extracellular CD20-loop formed by residues 142 to 187 between the third and fourth intermembrane region is the target binding domain for most of the anti-CD20 monoclonal Abs, including RTX, which specifically binds the epitope constituted by residues 163-187 (Teeling et al., 2006; Du et al., 2007). Therefore, the CD20-loop is often chosen as an alternative antigen to raise anti-CD20 Abs, especially as the full length of recombinant CD20 tends to aggregate in the expression host bacteria or cells (Anbouhi et al., 2012). To ensure the loop displays with proper secondary conformation and to increase its antigenicity, the CD20-loop is often expressed as a fusion with a carrier protein for use in immunizing the animals.

Single domain antibodies (sdAbs, also called nanobodies) are small antigen binding domains (~15 kD) that consist of the heavy chain variable region (VHH) derived from heavy chain antibodies (HcAb) exclusive to camelids. Although sdAbs have been evaluated for therapeutic and imaging applications (Unciti-Broceta et al., 2013; Muyldermans 2013), sdAbs specific to human CD20 have not previously been described. Most current CD20 immuno-reagents are mAbs and their scFv derivatives (Press, 2000; Foon, 2000; Schultz et al., 2000). Due to their smaller size, sdAbs have improved tumor penetration compared to full length conventional

antibodies. An additional consequence of this property is their rapid normal tissue clearance, making them compatible for use with short half-life radionuclides such as ^{68}Ga and ^{18}F for PET imaging and ^{211}At for targeted radiotherapy (Vaneycken et al., 2011; Pruszyński et al., 2012). SdAbs are able to direct treatment to the tumors with minimal off target impact, which should greatly enhance the prospects for patients. The availability of anti-CD20 sdAbs for both imaging and therapeutic applications would be a significant advancement, as they can be prepared with high homogeneity and their small size will allow for the facile development of fusion constructs in the future (Liu et al., 2016). Fusion constructs, such as with the biotin binding protein rhizavidin/**streptavidin** have the potential to yield a new generation of imaging and treatment options that could augment or supplant current immunoreagents. In addition, sdAbs have superior properties such as solubility and thermostability, thus they have the potential to provide reagents with improved shelf life and less degradation or aggregation prior to patient use (**Revets et al., 2005; Siontorou 2013**)

In this study, we immunized llamas with a fusion of the CD20 large loop region with a highly antigenic carrier protein, **T4 Hoc (highly antigenic outer capsid protein)** and developed an immunized llama phage display library capturing the VHH repertoire of the animals for further selection of anti-CD20 sdAbs. The sdAbs selected from this library were characterized by binding to a genetic fusion of the CD20 peptide with Hoc, a highly antigenic protein from the T4 bacteriophage, by surface plasmon resonance (SPR) and CD20 displayed on B-lymphoma cells by flow cytometry.

2. Material and Method

2.1 Preparation of recombinant Soc, Hoc, and CD20-Hoc fusion

Synthesized oligo-nucleotides encoding the CD20 peptide (Fig. 1A) with flanking NcoI and XhoI sites were inserted into a T7 expression plasmid, pet22b, containing a pelB leader sequence to direct expressed proteins to the periplasmic space to produce the plasmid CD20 p22b. The T4 Hoc gene (NCBI gene ID: 1258642) (Fig. 1B), 1128 bp in length and amplified by PCR, was cloned to CD20 p22b plasmid through the C-terminal XhoI site, creating the CD20-Hoc p22b plasmid. The sequence for the recombinant CD20-Hoc p22b plasmid was confirmed by Sanger sequencing (Fig. 1C). **Similarly, fusions of CD20 with Soc (NCBI gene ID 1258783), T4 small outer capsid protein, were also constructed (CD20-Soc p22b).** For expression of unfused Soc and Hoc, the Soc or Hoc gene were cloned to pet22b through NcoI and XhoI sites. T4 Hoc or Soc gene was amplified using T4 genomic DNA as a PCR template. T4 genomic DNA is a gift from Dr. Lindsay Black at University of Maryland at Baltimore. The recombinant Soc, Hoc, and CD20-Hoc plasmids were each then co-transformed into Tuner (DE3) with the pHelp plasmid (Hayhurst and Harris, 1999). Transformed bacteria were then grown at 30 °C for 3 hrs before the temperature was decreased to 25 °C. Chaperone production was induced by the addition of 80 µg/mL of Arabinose, followed 30 min. later by 0.25 mM IPTG induction of the Soc, Hoc or CD20-Hoc production. After 2.5 hrs of IPTG induction, bacterial cells were centrifuged down and the pellets were subjected to osmotic shock and IMAC extraction according to the previously published protocol (Hayhurst et al., 2003; Tuner et al., 2015). Following IMAC extraction, recombinant proteins were further purified from other protein contaminants or aggregates through Superdex 200 10/300 GL column (GE Healthcare) on a BioLogic DuoFlow chromatography system (Bio-Rad).

2.2 Immunization of llamas and construction of CD20 immunized phage display library

Purified CD20-Hoc was used to immunize two llamas (Triple J Farms, Bellingham, WA). Following immunization, peripheral blood lymphocytes were obtained from the buffy coat and the total RNA was isolated using QIAamp RNA Blood Mini kit (Qiagen Inc, Valencia, CA)

according to manufacturer's protocol. Purified RNA was converted to cDNA using superscript RTIII (Life Technologies, Grand Island, NY) and oligo-dT according to manufacturer's protocol. The heavy chain variable domain fragments were amplified from PCR reaction using degenerated primers (Ghahroudi et al., 1997) and our previously published modified protocol (Goldman et al., 2006). The immune library derived from llamas immunized with CD20-Hoc was obtained by cloning the variable domain fragments into the phage display vector, pECAN21 (Goldman et al., 2006, Liu et al., 2007). Gel purified variable domain PCR fragments and pECAN21 DNA cut with SfiI (New England Biolab Inc, Ipswich, MA) were ligated overnight at 15°C with a 3:1 ratio of insert to vector. The ligation mixture was then transformed to XL1 Blue cells (Agilent Technologies Inc, Clara, CA) using electroporation. Phage displaying sdAbs were prepared from the library according to the previous protocol (Liu et al., 2013). Representative clones were sequenced to assess the library quality and diversity.

Llama polyclonal antibody was purified from each animal using caprylic acid followed by MEP HyperCel hydrophobic charge induction chromatography (Pall, East Hills, NY) as previously described (Anderson et al., 2008). Prior to evaluation the polyclonal antibody was biotinylated using a 10-fold excess of Sulfo-NHS-LC-LC-Biotin (ThermoFisher), with excess biotin being removed using a 2 mL Zeba spin desalting column 7K MWCO (ThermoFisher).

2.3. Biopanning, selection of CD20 binders and analysis of binder sequences

To select CD20-specific sdAbs, purified CD20-Hoc (10 µg/mL) was used to coat 4 wells on a 96 well plate for biopanning. Two methods were used for biopanning to ensure the selection of sdAbs against CD20. Two rounds of biopanning were performed using each of the methods. Method 1 (M1) involved biopanning with the subtraction of phage that bound empty wells before the addition of phage to CD20-Hoc coated wells. Method 2 (M2) consisted of biopanning with the subtraction of phage which bound to Hoc coated wells before the addition of phage to CD20-Hoc coated wells and the presence of Hoc (10 µg/mL) in the suspension during the incubation with coated CD20-Hoc. The resulting phage was defined as R1 phage if it resulted from one round panning and R2 if it was from the second round panning. The original phage library was defined as R0. Ninety six colonies were selected from each R2 plate and grown in LB containing ampicillin and glucose in sterile 96 well plates for screening sdAbs binding to each antigen (CD20-Hoc and Hoc; 1 µg/mL) using a modified monoclonal phage ELISA (Griffiths et al.,

1993). In addition a MagPlex assay was utilized to re-evaluate selected phage displayed sdAb clones as described previously (Anderson et al., 2007). Positive sdAb display plasmids were isolated and sequenced by Eurofins MWG Operon (Huntsville, AL). Amino acid sequences were obtained and aligned using multalin (<http://multalin.toulouse.inra.fr/multalin/>) to compare the variations in the complementarity determine regions (CDRs) (Corpet, 1988). Based on the homology of CDR 3, single domain antibodies were grouped into five classes as indicated in the supplemental information (Fig. S1). Representative sdAb genes were then cloned into a periplasmic expression vector, pet22b, for protein preparation and transformed into Rosetta (DE3) *E. coli* strain (EMD Millipore, Billerica, MA). The expression and purification procedure was performed according to the previously published protocol (Conway et al., 2010; Goldman et al., 2014). Following IMAC extraction, sdAbs were further purified from other protein contaminants or aggregates through gel filtration chromatography using a Superdex 75 10/300 GL column (GE Healthcare) on a BioLogic DuoFlow chromatography system (Bio-Rad). Monomeric sdAb concentration was determined using BCA protein kit (Pierce) or a NanoDrop 1000 instrument. Most reagents were purchased from Sigma-Aldrich (St Louis, MO) and enzymes were purchased from New England Biolab (Ipswich, MA) unless specified.

2.4. Fluorescence based melting assay

The melting temperature of each sdAb was measured by a fluorescent dye-based assay as outlined previously (Liu et al., 2013). This technique relies on the fluorescence enhancement of Sypro Orange (Sigma), as it interacts with the hydrophobic amino acids on a protein, which become accessible upon thermal unfolding. Each sdAb was diluted to a concentration of 500 µg/mL in a final volume of 20 µL PBS. The Sypro Orange dye (ThermoFisher) was added to each sample at a dilution of 1:1000. Samples were measured in triplicate using a StepOne Real-Time PCR machine (Applied Biosystems). The heating program was run in continuous mode from 25°C – 99°C at a heating rate of 1% (~2°C per minute). Data was recorded using the ROX filter and the melting point was determined to be the peak of the first derivative of the fluorescence intensity. All three replicates gave essentially identical values for the melting temperature.

2.5. Surface plasmon resonance

Surface plasmon resonance (SPR) kinetic measurements were performed using the ProteON XPR36 (Bio-Rad) following a similar protocol to those we have described previously (Anderson et al., 2012). For testing the kinetics of the anti-CD20 antibodies, a GLC chip was coated with Hoc, Hoc-CD20, and NeutrAvidin. For immobilization, the proteins were diluted to ~ 20 µg/mL in 300 µL of 10 mM acetate buffer, pH 5. The standard EDC coupling chemistry provided by the manufacturer was utilized to attach the antigens to the chip, with the exception that distilled water was maintained as the running buffer until after the coupling step. A biotinylated CD20 peptide purchased from GeneScript with the following sequence

SKISHFLKMESLNFIRAHTPYINIYNCEPANPSEKNSPSTQYCYSIQ and the N-terminal modification of Biotin-Ahx was bound to the NeutrAvidin spot by flowing the peptide over the surface at ~1 µg/ml for 5 minutes. All experiments were performed at 25 °C.

The binding of RTX and the sdAbs was tested by flowing six concentrations (three-fold dilutions from 1000 to 0 nM) at 100 µL/min for 90 s over the antigen-coated chip and then monitoring dissociation for 420 s. The chip was regenerated using 0.085% phosphoric acid for 36 s, prior to any additional testing. The ordering of the antibody concentrations in the flow channels over the chip was alternated from left-to-right to right-to-left between tests to minimize artifacts due to incomplete regeneration. The data is corrected for interspot signal and zero concentration, when appropriate. The results were analyzed using the accompanying ProteOn Manager 3.1 software using a global analysis Langmuir fit.

2.6. MagPlex assays: titer and monoclonal phage assay

MagPlex assays were performed essentially as described previously (Anderson et al., 2007). Briefly, MagPlex beads were coated with the desired protein (Hoc, Soc, CD20-Hoc, etc.) using the recommended two step EDC / sulfo-NHS chemistry. A biotin labeled goat anti-llama IgG was prepared by using a 10 fold molar excess of NHS-LC-LC-biotin after 30 minutes the excess biotin was removed by gel filtration on using a Zeba spin 7K desalting columns (ThermoFisher). The protein-coated MagPlex beads (~100/set) were mixed with various concentrations of purified llama IgG in the wells of a 96-well polystyrene round bottom microtiter plate. After 30 minutes the beads were washed by placing the plate on a 96f magnet (BioTek) and washing three times with PBS + 0.05% Tween (PBST). The beads were then incubated with the biotin labeled goat anti-llama IgG (1 µg/mL). After 30 minutes the beads were washed 3x and then, to complete the

fluorescent sandwich assay, the beads were incubated for 30 minutes with 2.5 µg/mL streptavidin conjugated phycoerythrin (SAPE, Columbia Biosciences). After a final wash the binding was measured on the MAPIX instrument (Luminex Corp.).

To evaluate the phage displaying sdAbs a similar procedure was followed. For this assay, 10 µL of amplified phage supernatant was added to 90 µL PBST in the microtiter plate. Then the MagPlex beads were added and incubated for 1 hour. After washing as above, the beads were incubated with a 1 µg/mL anti-M13 IgG (GE Healthcare), biotinylated as described above. Then the assay was completed as described above.

2.7. Immunostaining and Flow Cytometry

For immunostaining Human B lymphocyte cell line SU-DHL-4 (ATCC CRL2957), first RTX and representative anti-CD20 sdAbs were biotinylated as described in 2.6. Approximately 10^6 - 10^7 SU-DHL-4 cells were fixed by the addition of 4% paraformaldehyde at room temperature for 15 min. Fixed cells were then washed with PBS and resuspended in 3% Bovine Serum Albumin (BSA) in PBS and incubated with biotinylated sdAb (bt-sdAb) at different concentrations at 4 °C for overnight. Next day, cells were washed with PBS and incubated with 4 µg/mL of NA-PE (neutravidin-phycoerythrin) at 4 °C for 2 hrs. Cells were washed with PBS two times and resuspended in 250 µL of PBS and run on an Accuri C6 flow cytometer using standard lasers and filters for PE (FL-2). For each sample 10^5 to 4×10^5 events were collected in a gate corresponding to the cell population. Histogram for each sample was plotted with the events vs FL-2 and compared among the different samples.

2.8. Gel electrophoresis and western blotting

SU-DHL-4 cells were pelleted by centrifugation and washed with PBS three times. RIPA buffer (ThermoFisher) with protease inhibitors (Roche Life Science) was added to the cell pellet in the concentration of 5×10^5 cells/mL. Genomic DNA was sheared several times using a 26 gauge needle. Approximately 1×10^7 cells and 4.5 µg of the recombinant CD20-Hoc/ Hoc were loaded into each corresponding well in a 4-12% NuPAGE gel (ThermoFisher). The proteins were separated by electric current in the presence of MES-SDS buffer. The proteins in the gel were then transferred to a PVDF membrane at 60 V for 2 hrs using Tri-glycine (19.2 mM Tri-HCl and 192

mM Glycine) buffer in 20% methanol. The blot was then briefly washed with 1xTBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween 20) and blocked with 5% milk in TBST (TBSTM) for 1 hr at room temperature (RT). The membrane was then incubated with bt-RTX/ sdAb at the concentration 10 µg/mL in TBSTM for 1 hr at RT, followed by 15 min washing with TBSTM 3 times and subsequently incubated with HRP conjugated Streptavidin or HRP conjugated anti-His Ab at RT for 1 hr. The membrane was washed 3 time with TBST and incubated with chemiluminescent substrate (ThermoFisher) at RT for 5 min. The images were then collected by ChemiDoc™ XRS+ System (Bio-Rad) using Image lab software version 5.1.

3. Results

3.1. Preparation of recombinant Soc, Hoc, and CD20-Hoc fusion

The large extracellular loop of CD20 formed by residues 142 to 187 is a proven target for anti-CD20 antibodies, and thus was utilized for generating sdAbs. Hoc and Soc are outer capsid proteins of T4 phage. A fusion construct CD20-Hoc was prepared to act as both an immunogen for immunizing llamas, as well as a target to select phage displayed sdAbs and evaluate isolated antibodies. The DNA fragment encoding residues 142-187 of human CD20 (NCBI reference: NM_152866) was inserted at the N-terminus of the carrier protein, the T4 highly antigenic outer capsid protein (Hoc), through flanking XhoI sites (Fig. 1A and 1B). The fusion protein sequence was confirmed by sequencing (Fig. 1C). Initially, overexpression of Soc, Hoc and CD20-Hoc in *E.coli* tended to form aggregates. However, co-expression of a chaperone protein with recombinant Hoc and CD20-Hoc facilitated proper folding of recombinant proteins and significantly increased the yield of the soluble protein at 25 °C (Hayhurst and Harris, 1999). We were able to obtain 4-10 mg protein per liter of bacteria, sufficient for subsequent immunization, selection and testing.

We utilized SPR and a known anti-CD20 mAb, RTX, to confirm that the CD20-Hoc fusion protein was an appropriate surrogate for the full length protein for the generation of an anti-CD20 immune response. RTX bound both immobilized CD20-Hoc and NeutrAvidin bound biotin labeled CD20 peptide, but showed no binding to immobilized Hoc when evaluated by SPR (Fig. S2). The affinity of RTX to the biotin labeled CD20 peptide (dissociation constant; K_D) was determined to be 380 nM using an equilibrium analysis of the binding curves. This value is

two orders of magnitude higher than the 5.2 nM K_D that was determined for binding CD20 on cells. (Reff et al., 1994)

3.2. Llama immunization and construction of CD20 immunized phage display library

To raise heavy chain antibodies against CD20, two llamas were immunized with recombinant CD20-Hoc fusion as indicated in Fig. 1. Their purified polyclonal antibodies showed a substantial differential binding to CD20-Hoc versus Soc upon evaluation by a MagPlex direct binding assay (Fig. S3).

With the presence of an immune response towards CD20-Hoc confirmed, we proceeded with the construction of a phage displayed sdAb library. The DNA fragments encoding VHH domains containing 3 unique CDRs and 4 conserved framework regions (FRs) were obtained using PCR. Amplified fragments were purified and later cloned into a plasmid DNA for displaying VHH on the M13 phage tails as fusions with minor coat protein, pIII. In this study, a library of M13 phage-displayed sdAbs derived from llamas immunized with CD20-Hoc was developed and was approximated to have 7.5×10^6 unique colony forming units (CFU). Library complexity was estimated through the number of transformants, taking into account that partial sequencing of the library showed 75% of the representative clones had intact and unique sequences.

3.3. Biopanning, selection of CD20 binders and analysis of binder sequences

R0 phage amplified from the immune library was subjected to two rounds of biopanning using two methods as described in methods section: method 1 (M1) was the standard selection protocol, method 2 (M2) consisted of subtractive panning by pre-selecting on Hoc coated wells and including soluble Hoc during panning on immobilized CD20-Hoc. **M1 was expected to enrich binders to both Hoc and CD20, while M2 with Hoc subtraction was used to specifically enrich CD20 binders. The second methods was used to ensure the success of selecting CD20 specific binders. Our results indicated that** both methods were able to enrich potential binders against CD20-Hoc fusions by two orders of magnitude as indicated in Fig. 2. Monoclonal phages were selected based on results from both an ELISA assay (Table S1) and MagPlex assay (Fig S4) from round 2 (R2) of both panning methods. Forty-one clones in total (from both M1 and M2) were selected and sequenced **as indicated in Fig. S1. They all had unique sequences, but fell into distinct families based on similarity of their CDR3.** Clones

screened from the 1st method, without Hoc subtraction, are labeled as M1 followed by the clone name. Those from the second method, with Hoc subtraction, are labeled M2, followed by the clone name. Clone sequences were aligned and grouped by CDR3 homology (Fig S1). Based on CDR3 sequences, they were divided into five classes and two or three clones from each class along with M27G, the only member of class D, were cloned for protein expression and characterization of binding by SPR. The yields for most of the selected sdAb clones ranged from 3-10 mg per liter of bacteria, sufficient for further evaluation.

3.4. Surface plasmon resonance binding affinity measurements

A SPR based biosensor was used to examine the differential binding affinity of the sdAbs to CD20-Hoc versus Hoc coated surfaces (Table 1). SdAbs in three out of the five classes (Fig. S1) showed differential binding; the full sequences of the 25 members of classes A, C, or D are shown in Fig. 3. The 15 class A clones possess identical CDR3 residues (96-109), except M112D, which contains a mismatched Asn (N) at residue 101(N101) (Fig. 3A). Three clones, M28C, M28D and M29C show two mismatched sequences, N52 in CDR2 and G61 in FR 3 with the rest of clones. All class A clones were selected from M2 except M112D from M1; clones M28C, M24E and M112D were chosen for protein expression and for further measurement of binding affinity to CD20-Hoc and B-lymphoma cells. Class C contains 9 clones selected from both methods. They appear to have nearly identical CDR3 residues (96-105) except residues 99 and 102. M110C with V99 and the only member of the class to have T102 (instead of R102) and M110B with common G99 and R102 were selected for further analysis. M27G is a unique sequence and forms its own class (D); its CDR3 contains only 8 residues, 96-103.

Most of the clones selected from M2 panning showed differential binding to CD20-Hoc fusion versus Hoc (Table 1 and Fig. 4). The specificity and kinetics of binding of the sdAbs were evaluated by SPR. Binding affinity, K_D , for each sdAb was calculated and compared between surfaces coated with either CD20-Hoc or Hoc (Table 1). Class A sdAbs, M28C (**Fig. 4A and 4B**) and M24E (**data not shown**) with identical CDR3 showed good binding affinity (~ 10 nM K_D) to CD20-Hoc fusion (Fig 4A), with much higher affinities for CD20-Hoc relative to Hoc binding. Very interestingly, M112D with a mismatched N101 in CDR3 region also showed differential binding between the CD20-Hoc fusion (Fig. 4C) and Hoc (Fig. 4D), but had an off rate (k_d) ~ 2 -fold higher. These results suggest that the change of **D101 to N101** in CDR3 has a

significant impact on the binding to CD20. The binding affinity for class C sdAb, (M110C) show comparable binding affinity to CD20-Hoc fusion as class A sdAbs, but with much lower on and off rates (Fig. 4E); in addition, M110C had only a 8-fold differential in K_D for CD20-Hoc versus Hoc binding (Fig. 4F). The class D clone, M27G, also exhibits a similar K_D for CD20-Hoc fusion as the class A and C sdAbs, but like the class C sdAb has lower on and off rate than the class A clones. However, the K_D differential was 3 orders of magnitude for the CD20-Hoc versus Hoc binding, due to very poor Hoc binding (Fig. 4H). Interestingly, M27G was the only clone that showed binding to the Bt-CD20 peptide (not shown).

Class B and E clones, as represented by M13D and M110A respectively, bound to both CD20-Hoc and Hoc with very similar affinities, and were thus judged to be Hoc binding sdAbs (Table 1).

3.5. Measurement of T_m by fluorescence based melting assay

The thermostability of the selected sdAbs was compared by measuring their melting temperature, T_m , using a fluorescence based melting assay that employs the fluorescent dye Sypro Orange. When thermally unfolded the protein's exposed hydrophobic residues bind to Sypro Orange and increase the dye's quantum yield generating a sharp increase in high fluorescent intensity at the protein's T_m . The selected sdAbs exhibited a range of T_m s between 50 and 65 °C, as indicated in Table 2; except for M112D, whose T_m was below 50 °C. Besides the unique N101, M112D also possesses unique residues E15 in FR1 and G30 in CDR1 (Fig. 3A), which may negatively impact its thermostability compared to clones, M24E and M28C. M28C with a mismatched residue, N52, in CDR2, also shows a lower T_m compared to M24E. On the contrary, Class C sdAbs, M110B and M110C, with varied residues in CDRs and FR2, show very comparable T_m s.

3.6. Immunostaining and Flow Cytometry

To assess sdAb binding to full length CD20, the B-lymphoma cell line, SU-DHL-4, was used for immunostaining and flow cytometry measurements. It is known that CD20 is overexpressed in B lymphoma cells. To conduct immunostaining, purified sdAbs and RTX were first biotinylated. After binding to the cells the sdAbs were fluorescently tagged by the addition of NA-PE. Class A sdAbs, bt-M24E and bt-M28C, show increased fluorescent cell population (the right peak) in a concentration dependent manner as indicated in **Figs 5A and S5B respectively**. There was no

significant increased fluorescent cell population at concentrations between 5-10 $\mu\text{g/mL}$ (Fig. S5A), therefore, Ab concentrations higher than 10 $\mu\text{g/mL}$ were used for immunostaining as indicated in Fig. 5. Surprisingly, M112D with only one mismatched N101 has no binding to cells even at the highest Ab concentration, 30 $\mu\text{g/mL}$ (Fig. 5B), while class C sdAb, M110C, does show increased fluorescent cell population shift in a concentration dependent manner (Fig. 5C), same as M27G (Fig. 5D). Bt-RTX was used as a positive control for immunostaining and flow cytometry analysis **as indicated in S6A**. Competition assays by RTX were also performed (**Fig S6B and S6C**). There is 100% fluorescent cell population shift at the concentration of 10 $\mu\text{g/mL}$ of bt-RTX (blue line), but the binding could only be partially inhibited in the preincubation with 10 $\mu\text{g/mL}$ RTX (red line in Fig S3A). The presence of RTX slightly inhibited the binding of bt-M24E to cell surface CD20 (Fig.S6B), but did not inhibit the binding of bt-M27G to CD20 (Fig. S6C).

3.7. SdAb binding to CD20 in SU-DHL-4 cells using western blotting

Binding of bt-RTX to denatured full length CD20 in DHL-4 cells was confirmed by western blotting as indicated in Fig. S7. Our data indicate that even in the presence of 100 mM Dithiothreitol (DTT) in the loading buffer, CD20 still runs as a dimer in the size of 70 kD (Fig. S7E). M24E shows better binding signal to CD20 than bt-RTX and a weak signal in monomer as indicated by the arrow in Fig. S7A. The 53 kD band also appeared in the blots A, B and D. M24E representing class A clones also binds to CD20-Hoc, but does not bind to Hoc (Fig. S4A), while M27G and M110C bind much stronger to Hoc (lanes 3) than CD20-Hoc (lanes 2 in Figs. S7C and S7D). M112D does not bind to either CD20-Hoc or Hoc (Fig. S7B), but did show the binding to CD20 in the cell lysate.

4. Discussion

We have successfully prepared and expressed recombinant CD20-Hoc as an immunogen to immunize llama and generate an immune sdAb phage-display library. **Ideally the full length native CD20 antigen would be used to immunize animals. The full length of recombinant CD20, however, tends to mis-fold and aggregate in the overexpression system and is difficult to purify the native form as an immunogen. Further, immunization with full length CD20 does not guarantee the success of producing Abs specifically against extracellular CD20 loop region. Fortunately, the extra-membrane CD20 loop, the main**

antibody binding region, is soluble and easy to prepare as an immunogen. Moreover, CD20 loop fusion protein has a properly folded secondary loop structure to serve as an alternative for full length CD20 in detection assays (Anbouhi et al., 2012). Consistently, our result indicates that the fusion of CD20 loop with highly antigenic T4 Hoc (highly antigenic outer capsid protein) binds to RTX (Fig. S2), suggesting the fusion appears to have recognizable secondary structure. The fusion of the CD20 loop with the highly antigenic T4 Hoc, to generate CD20-Hoc proved immunogenic enabling the generation of antibodies against CD20 in the immunized animals.

Biopanning with Hoc subtraction was able to select binders specific for CD20 from the immune library. Our data indicates that the class A sdAbs, which possess the same unique CDR (Fig. 3; Fig S1) and was the dominant class selected mainly from M2, has the best binding specificity to the CD20 fusion and B lymphoma cells among all selected clones. It clearly shows the superiority of the subtractive panning method, one which has been effectively used to obtain specific binders against other targets (Conway et al., 2010). The mismatch residue in the CDR2 between M28C and M24E did not affect the binding to CD20 fusion and cells (Figs. 5A and S5B), however the mismatch, D to N, in the 2nd residue within the CDR3 region completely abolishes the binding to native CD20 on the cell surface (Fig. 5B) and denatured CD20 fusion (Fig. S7B), indicating that a change from negative charge of the acidic carboxyl group in Asp to an aliphatic amino acid, Asn, has a dramatic effect on the binding to the CD20 large loop. It is likely that the Asp residue is essential to the binding interaction. More mutagenesis studies are required to confirm this hypothesis. On the contrary, in class C clones, which were mostly selected using M1, two mismatch CDR3 residues, G to V and R to T in M110C do not affect the binding to the CD20-Hoc fusion or cells. Although M27G shows 3 orders stronger binding to CD20-Hoc than Hoc from SPR measurements (Table 1), it appeared to bind Hoc better than CD20-Hoc on the western blots. Class C clones (M110C) also showed similar results from the SPR measurements and western blotting as M27G. Our results suggest that both class C sdAbs and M27G did bind to the CD20 structure in Hoc fusion protein, but bound less well to the denatured CD20 or the native CD20 loops on SU-DHL-4 cells (Fig. 5C). It is likely that the conformation of CD20 fusion differs from the native CD20 on cell surface resulting in stronger binding by these sdAbs for the analog. In contrast, RTX bound less well on CD20-Hoc and denatured CD20 in cell lysates (Fig. S7E), but better to native CD20 on cells (Fig. S6A).

Site-directed mutagenesis and epitope mapping studies showed that the extracellular loop of human CD20 (hCD20) contained multiple epitopes for most Ab binding and A170 and P172 within the loop were critical to determine the secondary structure of the large loop for specific binding by RTX and some other anti-CD20 monoclonal Abs (Du et al., 2007; Polyak and Dean 2002). Our data show that RTX has some inhibition on M24E binding to DHL cells (Fig. S6B), suggesting that class A sdAbs may share some overlapping epitope sequence with RTX. While in contrast, the binding to cells by class C sdAbs and M27G were not affected by RTX inhibition, indicating that their binding epitope is different from RTX (Fig. S6C). Our preliminary data also suggest that class A sdAbs bind to a different epitope from class C sdAbs and M27G since their binding to cells were not affected by pre-incubation with class C sdAb or M24E (data not shown).

The native human CD20 does not exist as a monomer in the plasma membrane in B lymphoma cells. It forms a tetramer and associates with other proteins (Bubien et al., 1993). Although in the presence of reducing reagent, CD20 migrated on sodium dodecyl sulphate-polyacrylamide gel at 33,000 to 35,000 (Deans et al., 2002), it was shown previously that the phosphorylation of CD20 in malignant B lymphoma cells migrated slower on the gel (Tedder and Schlossman, 1988). Our western blot data indicate that CD20 exists mostly as cross-linked dimers in SU-DHL cells (Fig. **S7E**) as only a small portion of CD20 monomer was detected by class A sdAbs (**Fig. S7A and S7B**). An unidentified band migrating at 53,000 detected by CD20 sdAbs is possibly phosphorylated CD20.

The induction of homotypic CD20 aggregation triggered by monoclonal Ab binding has a direct effect on cell apoptosis. It is expected that divalent monoclonal Ab such as RTX strongly bind to CD20 and quickly trigger downstream effectors. Our data consistently showed that divalent RTX bound cells stronger than the monovalent sdAb (Fig. 5) and RTX bound denatured CD20 weaker than the class A sdAbs (Fig. S7). To improve the apparent binding affinity by increasing the avidity of class A CD20 sdAbs to cell surface CD20 and further trigger CD20-mediated apoptosis, sdAbs can be fused to streptavidin (SA) or Rhizavidin (RZ) to form tetrameric or dimeric sdAb fusions respectively for binding to CD20 oligomers in cells. Our previous studies have demonstrated that sdAb-SA and sdAb-RZ exhibit much better target binding affinity compared to nonfused sdAb (Walper et al., 2013; Liu et al., 2016). **Affinity maturation can be**

combined with multimerization in an effort to further increase the affinity of the sdAb for cell surface CD20.

Small sdAbs can reach targeted tumor cells penetrate tumor tissues better than full length Abs (Revets et al, 2005; Siontorou 2013). Even the size of multimeric sdAb fusions is also smaller than full length Abs and can reach tumor cells better. The stability of the sdAb structure also offers better robustness than conventional Abs in vivo. Recombinant sdAbs are soluble with properly folded structures and are easily produced in large quantities. SdAbs can be produced more economically than conventional Abs. All these properties make sdAb a great potential therapeutic Ab for B-cell lymphoma.

5. Conclusion

We utilized a fusion between the large loop from CD20 and Hoc (CD20-Hoc) as an immunogen and successfully immunized llama to generate VHH antibody against CD20. An immune sdAb display library was constructed and for the first time, sdAbs showing specific binding to CD20 in SU-DHL cells were selected. CD20 sdAbs showing specific binding to the CD20 fusion relative to Hoc were identified through subtractive panning and can be further modified to generate multimeric sdAb fusions for even better binding to CD20.

Acknowledgments

We thank to Dr. Jonathan M. Adelson for his encouragement of this work. This work was supported by NRL 6.2 base funding sponsored by Office Naval Research (ONR).

Contribution of Authors

JL designed, performed the experiments and wrote the manuscript; DZ performed the experiments; EG designed the experiments and edited the MS; GA designed and performed the experiments and wrote the MS.

References

1. Anbouhi, M.H., Abolhassani, M., Bouzari, S., Khanahmad, H., Aghasadeghi, M.R., Madadkar-Sobhani, A., Amanzadeh, A., Behdani, M. and Shokrgozar, M.A., 2012, Immunological evaluation of predicted linear B-cell epitopes of human CD20 antigen. *Biotechnol Appl Biochem* 59, 186-92.
2. Anderson, G.P., Ortiz-Vera, Y.A., Hayhurst, Andrew., Czarnecki J., Dabbs J., Vo, B., and Goldman Ellen R., 2008, Evaluation of llama anti-botulinum toxin heavy chain antibody. *The Botulinum J* 1, 100-115.
3. Anderson, G.P., Legler, P.M., Zabetakis, D. and Goldman, E.R., 2012, Comparison of immunoreactivity of staphylococcal enterotoxin B mutants for use as toxin surrogates. *Anal Chem* 84, 5198-203.
4. Anderson, G.P., Matney, R., Liu, J.L., Hayhurst, A. and Goldman, E.R., 2007, Multiplexed fluid array screening of phage displayed anti-ricin single domain antibodies for rapid assessment of specificity. *Biotechniques* 43, 806-11.
5. Bubien, J.K., Zhou, L.J., Bell, P.D., Frizzell, R.A. and Tedder, T.F., 1993, Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca^{2+} conductance found constitutively in B lymphocytes. *J Cell Biol* 121, 1121-32.
6. Cheson, B.D. and Leonard, J.P., 2008, Monoclonal antibody therapy for B-cell non-Hodgkin's lymphoma. *N Engl J Med* 359, 613-26.
7. Conway, J.O., Sherwood, L.J., Collazo, M.T., Garza, J.A. and Hayhurst, A., 2010, Llama single domain antibodies specific for the 7 botulinum neurotoxin serotypes as heptaplex immunoreagents. *PLoS One* 5, e8818.
8. Corpet, F., 1988, Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* 16, 10881-90.
9. Cragg, M.S., Walshe, C.A., Ivanov, A.O. and Glennie, M.J., 2005, The biology of CD20 and its potential as a target for mAb therapy. *Curr Dir Autoimmun* 8, 140-74.
10. Deans, J.P., Li, H. and Polyak, M.J., 2002, CD20-mediated apoptosis: signalling through lipid rafts. *Immunology* 107, 176-82.
11. Du, J., Wang, H., Zhong, C., Peng, B., Zhang, M., Li, B., Huo, S., Guo, Y. and Ding, J., 2007, Structural basis for recognition of CD20 by therapeutic antibody Rituximab. *J Biol Chem* 282, 15073-80.
12. Foon, K.A., 2000, Monoclonal antibody therapies for lymphomas. *Cancer J* 6, 273-8.
13. Ghahroudi, M.A., Desmyter, A., Wyns, L., Hamers, R. and Muyldermans, S., 1997, Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS Lett* 414, 521-6.
14. Goldman, E.R., Anderson, G.P., Liu, J.L., Delehanty, J.B., Sherwood, L.J., Osborn,

- L.E., Cummins, L.B. and Hayhurst, A., 2006, Facile generation of heat-stable antiviral and antitoxin single domain antibodies from a semisynthetic llama library. *Anal Chem* 78, 8245-55.
15. Goldman, E.R., Brozozog-Lee, P.A., Zabetakis, D., Turner, K.B., Walper, S.A., Liu, J.L. and Anderson, G.P., 2014, Negative tail fusions can improve ruggedness of single domain antibodies. *Protein Expr Purif* 95, 226-32.
 16. Griffiths, A.D., Malmqvist, M., Marks, J.D., Bye, J.M., Embleton, M.J., McCafferty, J., Baier, M., Holliger, K.P., Gorick, B.D. and Hughes-Jones, N.C., 1993, Human anti-self antibodies with high specificity from phage display libraries. *Embo J* 12, 725-34.
 17. Hayhurst, A., Happe, S., Mabry, R., Koch, Z., Iverson, B.L. and Georgiou, G., 2003, Isolation and expression of recombinant antibody fragments to the biological warfare pathogen *Brucella melitensis*. *J Immunol Methods* 276, 185-96.
 18. Hayhurst, A. and Harris, W.J., 1999, *Escherichia coli* skp chaperone coexpression improves solubility and phage display of single-chain antibody fragments. *Protein Expr Purif* 15, 336-43.
 19. Liu, J.L., Walper, S.A., Turner, K. B., Lee, P.A., Medintz, I. L., Susumuc, K., Oh, E., Zabetakis, Daniel., Goldman, E. R., and Anderson, G. P., 2016, Conjugation of biotin-coated luminescent quantum dots with single domain antibody-rhizavidin fusions. *Biotechnology Reports* 10, 50-65.
 20. Liu, J.L., Anderson, G.P., Delehanty, J.B., Baumann, R., Hayhurst, A. and Goldman, E.R., 2007, Selection of cholera toxin specific IgNAR single-domain antibodies from a naive shark library. *Mol Immunol* 44, 1775-83.
 21. Liu, J.L., Zabetakis, D., Goldman, E.R. and Anderson, G.P., 2013, Selection and evaluation of single domain antibodies toward MS2 phage and coat protein. *Mol Immunol* 53, 118-25.
 22. Muyldermans, S., 2013, Nanobodies: natural single-domain antibodies. *Annu Rev Biochem* 82, 775-97.
 23. Polyak, M.J. and Deans, J.P., 2002, Alanine-170 and proline-172 are critical determinants for extracellular CD20 epitopes; heterogeneity in the fine specificity of CD20 monoclonal antibodies is defined by additional requirements imposed by both amino acid sequence and quaternary structure. *Blood* 99, 3256-62.
 24. Press, O.W. 2000 Innovative new therapies for non-Hodgkin's lymphomas: monoclonal antibodies and immunoconjugates. In: P.C. Perry (Ed.) *American Society of Clinical Oncology Education Book*. American Society of Clinical Oncology, Alexandria, VA, pp. 328-337.
 25. Pruszyński, M., Koumariannou, E., Vaidyanathan, G., Revets, H., Devoogdt, N., Lahoutte, T. and Zalutsky, M.R., 2013, Targeting breast carcinoma with radioiodinated anti-HER2 Nanobody. *Nucl Med Biol* 40, 52-59.
 26. Reff, M.E., Carner, K., Chambers, K.S., Chinn, P.C., Leonard, J.E., Raab, R., Newman, R.A., Hanna, N. and Anderson, D.R., 1994, Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* 83, 435-45.
 27. Revets, H., De Baetselier, P., Muyldermans, S., 2005, Nanobodies as novel agents for cancer therapy. *Expert Opin Biol Ther.* 5(1):111-24.
 28. Schultz, J., Lin, Y., Sanderson, J., Zuo, Y., Stone, D., Mallett, R., Wilbert, S. and Axworthy, D., 2000, A tetravalent single-chain antibody-streptavidin fusion protein

- for pretargeted lymphoma therapy. *Cancer Res* 60, 6663-9.
29. Siontorou, C. G., 2013, Nanobodies as novel agents for disease diagnosis and therapy. *Int. J. Nanomedicine* 8:4215-27.
 30. Tedder, T.F. and Engel, P., 1994, CD20: a regulator of cell-cycle progression of B lymphocytes. *Immunol Today* 15, 450-4.
 31. Tedder, T.F. and Schlossman, S.F., 1988, Phosphorylation of the B1 (CD20) molecule by normal and malignant human B lymphocytes. *J Biol Chem* 263, 10009-15.
 32. Teeling, J.L., Mackus, W.J.M., Wiegman, L.J.J.M., van den Brakel, J.H.N., Beers, S.A., French, R.R., van Meerten, T., Ebeling, S., Vink, T., Slootstra, J.W., Parren, P.W.H.I., Glennie, M.J. and van de Winkel, J.G.J., 2006, The biological activity of human CD20 monoclonal antibodies is linked to unique epitopes on CD20. *J Immunol* 177, 362-71.
 33. Turner, K.B., Liu, J. L., Zabetakis, D., Brozozog-Lee, P.A., Anderson G. P., Goldman, E. R., 2015, Improving the biophysical properties of anti-ricin single-domain antibodies. *. Biotechnology Reports* 6, 27-35.
 34. Unciti-Broceta, J.D., Del Castillo, T., Soriano, M., Magez, S. and Garcia-Salcedo, J.A., 2013, Novel therapy based on camelid nanobodies. *Ther Deliv* 4, 1321-36.
 35. Vaneycken, I., Devoogdt, N., Van Gassen, N., Vincke, C., Xavier, C., Wernery, U., Muyldermans, S., Lahoutte, T. and Caveliers, V., 2011, Preclinical screening of anti-HER2 nanobodies for molecular imaging of breast cancer. *Faseb J* 25, 2433-46.
 36. Walper, S.A., Brozozog Lee, P.A., Goldman, E.R. and Anderson, G.P., 2013, Comparison of single domain antibody immobilization strategies evaluated by surface plasmon resonance. *J Immunol Methods* 388, 68-77.

Fig. 1. Sequences for CD20 peptide, fusion of CD20 and T4 HOC. A. The large loop region in Human CD20 includes the residues 142-187 from CD20 protein. Residues, A170 and P172, in red font are critical to form an epitope for Ab binding. B. CD20 peptide was cloned into N-terminus of T4 Hoc (highly antigenic T4 outer capsid protein) through NcoI- XhoI site. C. Amino acid sequence for CD20-Hoc fusion. CD20 peptide is in red font and Hoc is in blue font.

Fig. 2. Results for two panning methods. A. Method 1 (M1) is a standard panning procedure without subtracting recombinant Hoc and there is approximately 400 fold enrichment from R1 to R2. B. M2 is a subtractive panning procedure subtracted with Hoc and there is approximately 100 fold enrichment from R1 to R2.

Fig. 3. Consensus sequences for three classes of sdAbs binding to CD20. A. Class A binders with longest CDR3 sequences (a.a. 96-109). B. Class C bonders with a shorter CDR3 sequence (a.a. 96-105). C. Class D has CDR 3 sequence, QMPKYDNW (a.a. 96-103).

Fig. 4. SPR measurements of differential binding affinity of sdAbs to CD20-Hoc vs Hoc. CD20-Hoc binding kinetics are shown in A, C, E, G, while Hoc binding kinetics are shown in B, D, F, H.

Fig. 5. Analysis of immunostaining cells by flow cytometry. SU-DHL-4 cells immunostained with either bt-RTX or bt-sdAbs at different concentrations were measured by flow cytometer, Accuri C6. Histograms of cells stained with Neutravidin phycoerythrin (NA-PE) were used as a negative control for each bt-sdAb. A. Cells stained with bt-M24E at different concentrations show the right shift of histograms due to more cells binding to bt-M24E, while there is no change of histograms of cells stained with bt-M112D in B. Cells stained with bt-M110C (C) and bt-M27G (D) only show moderately shift of histograms at different concentrations of bt-sdAbs.

Table 1. Affinity measurements for anti-CD20 sdAbs by SPR.

Class	Clone	Hoc-CD Binding Affinity			Hoc Binding Affinity			Ratio
		k_a (1/Ms)	k_d (1/s)	K_D (M)	k_a (1/Ms)	k_d (1/s)	K_D (M)	Hoc K_D / Hoc-CD20 K_D
A	M28C	3.0×10^5	4.5×10^{-3}	1.5×10^{-8}	6.8×10^4	3.6×10^{-1}	5.3×10^{-6}	353 : 1
A	M24E	7.5×10^5	4.3×10^{-3}	5.7×10^{-9}	2.0×10^5	4.6×10^{-2}	2.3×10^{-7}	40 : 1
A	M112D	2.8×10^5	7.0×10^{-3}	2.6×10^{-8}	1.5×10^5	3.2×10^{-1}	2.1×10^{-6}	81 : 1
B	M13D	7.9×10^4	3.9×10^{-4}	5.0×10^{-9}	4.1×10^4	7.2×10^{-4}	1.8×10^{-8}	3 : 1
C	M110C	2.4×10^4	3.2×10^{-4}	1.4×10^{-8}	3.9×10^3	6.3×10^{-4}	1.7×10^{-7}	8 : 1
D	M27G	1.8×10^4	9.9×10^{-4}	5.8×10^{-8}	5.2×10^0	2.2×10^{-3}	4.4×10^{-4}	7586 : 1
E	M110A	1.3×10^5	1.9×10^{-4}	1.4×10^{-9}	1.3×10^5	2.3×10^4	1.8×10^{-9}	1 : 1

Table 2: SdAb melting point (T_m) measurement

Class	Name	T _m (°C)
A	M24E	56
A	M28C	52
A	M112D	49
C	M110B	64
C	M110C	62
D	M27G	62

Fig. 1.

A. Human CD20 peptide sequence:

¹⁴²KI SHF LKM ESL NFI RAH TPY INI YNC EPA NPS EKN SPS TQY CYS IQ¹⁸⁷.

B. Human CD20 and T4 Hoc protein fusion.

C. Protein sequence for CD20-Hoc fusion.

MGKISHFLKMESLNFIRAHTPYINIYNCEPANPSEKNSPSTQYCYSIQLEMTFTVDITPKTPTGVIDETKQFTAT
PSGQTGGGTITYAWSVDNVPQDGAETFSYVLKGPAQKTIKVATNTLSEGGPETAEATTTITVKNKTQTTTLAV
TPASPAAGVIGTPVQFTAALASQPDGASATYQWYVDDSQVGGETNSTFSYPTTSGVKRIKCVAQVTATDYDALS
VTSNEVSLTVNKKTMNPQVTLPPSINVQQDASATFTANVTGAPEEAQITYSWKKDSSPVEGSTNVYTVDTSSVG
SQTIEVTATVTAADYNPVTVTKTGNVTVTAKVAPEPEGELPYVHPLPHRSSAYIWCGWWVMDEIQKMTEEGKD
WKTDDPDSKYYLHRYTLQKMMKDYPEVDVQESRNGYIIHKTALETGIIYTYPAAALEHHHHHHH

Fig. 2.

A

B

Fig. 3.

Fig. 4.

Fig. 5