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Affinity selection of a camelized V_H domain antibody inhibitor of hepatitis C virus NS3 protease

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The HCV genome encodes, within the NS3 gene, a serine protease whose activity specifically cleaves the viral polyprotein precursor. Proteolytic processing of HCV polyprotein precursor by the viral NS3 proteinase is essential for virion maturation and designing specific inhibitors of this protease as possible anti-viral agents is a desirable and practical objective. With a view to studying both the function of HCV NS3 protease and to designing inhibitors of this enzyme, we directed our interest towards engineering macromolecular inhibitors of the viral protease catalytic activity. We describe here the affinity-selection and biochemical characterization of one inhibitor, cV_HE2, a 'camelized' variable domain antibody fragment, isolated from a phage displayed synthetic repertoire, which is a potent and selective inhibitor of proteolysis by the NS3 enzyme. In addition to being useful as a biological probe to study the function of HCV protease, this inhibitor can serve as a potential pharmacophore model to design anti-virals. Moreover, the results suggest a way of engineering improved human-derived small recognition units tailored for enzyme inhibition.

Keywords: anti-viral/camelized antibody/hepatitis C virus/phage display/protease inhibitor

Introduction

Hepatitis C virus (HCV) is commonly accepted to be the major etiological agent of both parenterally transmitted and sporadic non-A non-B hepatitis (Choo *et al.*, 1989; Kuo *et al.*, 1989). It is estimated that this virus infects 100–200 million people worldwide and can lead to chronic and potentially life-threatening infections; liver cirrhosis and primary liver cell carcinoma can result in premature death in a substantial number of infected individuals (Chien *et al.*, 1992). Protective immunotherapy for HCV infection is not available and interferon treatment is of only limited efficacy (Weiland, 1994). For these reasons, a thorough dissection of the HCV life cycle and the identification of targets for anti-viral therapies are important goals. The viral genome is ~9400 nucleotides long encoding a precursor polyprotein (Figure 1) of 3011 amino acids (Kato *et al.*, 1990; Choo *et al.*, 1991; Takamizawa *et al.*, 1991; Grakoui *et al.*, 1993). The enzymatic cleavage of the polyprotein precursor produces structural proteins and enzymes that are essential to the life cycle of HCV (Tomei *et al.*, 1993). These proteins are released from the precursor polyprotein by cellular proteases in association with membranes of the endoplasmic reticulum (Hijikata *et al.*, 1991), whereas all proteolytic cleavages downstream of the non-strucural gene 3 (NS3) are catalyzed by a serine protease contained within the N-terminal region of NS3 (Figure 1). Cleavage activity at three of the four sites affected by NS3 occurs in *trans*, whereas processing at the carboxy terminus of NS3 is a *cis* event (Tomei *et al.*, 1993). Substrate cleavage mediated by NS3 protease occurs between Cys/Ser or Cys/Ala and between Thr/Ser in *cis* (Pizzi *et al.*, 1994; Failla *et al.*, 1996).

The product of the NS3 gene is a multidomain protein of 70 kDa that, in addition to the protease domain at the N-terminus, contains an RNA helicase at its C-terminus (Kim *et al.*, 1995). It was demonstrated that a 20 kDa N-terminal fragment of NS3, in association with the viral polypeptide co-factor, NS4A, is capable of performing all cleavages in both *in vitro* translation and transfection experiments with an efficiency indistinguishable from that of the wild type enzyme (Failla *et al.*, 1995). The X-ray structure of the NS3 protease domain, devoid of co-factor (Love *et al.*, 1996) and with the bound NS4A peptide (Kim *et al.*, 1996), was determined. By analogy with data that have emerged from studies with flaviviruses (Chambers *et al.*, 1990), it was presumed that inhibition of the NS3 protease activity could lead to the production of non-infectious viral particles, and hence this enzyme has become one of the main targets for anti-viral drug design.

In recent years, protease inhibitors, both low molecular weight compounds and proteinaceous molecules, have emerged as very important pharmaceutical agents (Neurath, 1989). By displaying on filamentous phage mutants of natural proteinaceous inhibitors, new specificities have been selected with high potency (Roberts *et al.*, 1992; Dennis and Lazarus, 1994; Rottgen and Collins, 1995; Wang *et al.*, 1995; Markland *et al.*, 1996a,b). In this context, small protein scaffolds on to which functions can be engineered have emerged as a means of generating conformationally defined structures with potential as pharmacophores (Sollazzo *et al.*, 1995; Zhao *et al.*, 1995). In nature, immunoglobulins are unsurpassed as molecules for generating ligands for practically any target, especially as their variable regions can be displayed on the surface of filamentous phage, increasing their potential even further (reviewed by Winter *et al.*, 1994). Antibody variable domains can provide a very useful source of structural templates for designing low molecular weight lead compounds based on their complementarity-determining regions (CDR) (Dougall *et al.*, 1994; Sollazzo, 1995). In order to generate pharmacophore models, smaller sized antibody-derived fragments could be the molecules of choice as their binding activities can be focused on a smaller surface area.

Attempts were made to reduce the size of the minimum antibody fragment required for antigen binding. This led to the design of a 'minimized' V_H domain molecule, the *minibody* (Pessi *et al.*, 1993; Tramontano *et al.*, 1994) and the 'camelized' antibody (cV_H), a modified human V_H domain obtained through mimicking camel heavy chains for use as a small recognition unit (Davies and Riechmann, 1994; Riechmann, 1996).

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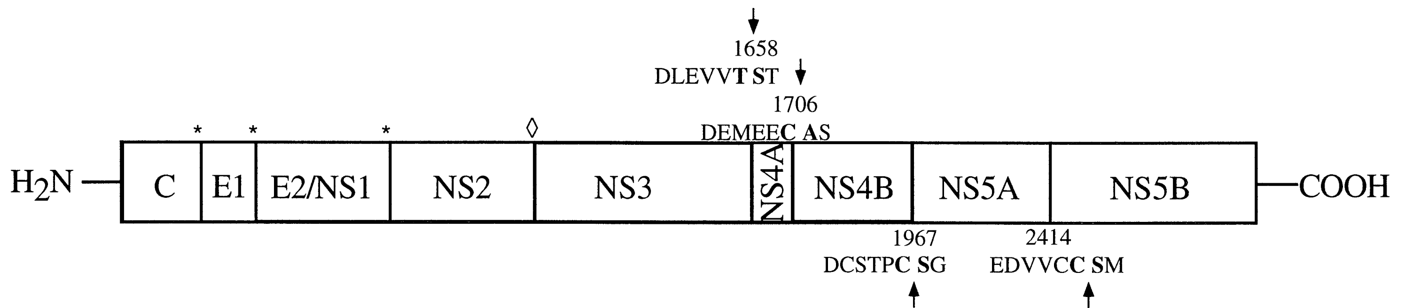

Fig. 1. HCV polyprotein schematic structure and processing. Cleavages mediated by cellular proteases are marked with asterisks. Cleavage between NS2 and NS3 (◇) is mediated by the NS2/NS3 protease activity. NS3 protease cleaves between NS3 and NS4A (*cis* cleavage), NS4A and NS4B, NS4B and NS5A and NS5A and NS5B (arrowheads). Amino acid number and substrate sequence at which cleavage occurs are indicated.

Molecules specific for hapten, protein and peptide ligands with affinities in the micro-nanomolar range were isolated from a repertoire of phage-displayed cV_H with randomized CDR3 (Davies and Riechmann, 1995). These results have been further validated by determining the X-ray structures of the natural Camelidae molecules (Desmyter *et al.*, 1996; Spinelli *et al.*, 1996), which provided the ultimate experimental evidence that V_H-derived molecules represent the smallest natural immunoglobulin based recognition unit and open up new possibilities for generating and engineering macromolecules with highly specific binding properties.

From the analysis of the X-ray structure of NS3 protease, it was predicted that the design of low molecular weight inhibitors would be a very challenging task owing to the relatively featureless appearance of the substrate binding groove (Kim *et al.*, 1996). With a view to studying both the function of HCV protease and the future design of small molecule inhibitors of its enzymatic activity, we sought to engineer macromolecules that would bind NS3 protease and to study their effects on the catalytic activity of this protease and ultimately on the viral life cycle. We describe here the isolation from a repertoire of a camelized human antibody domain of one variant that binds to the HCV NS3 protease domain and inhibits selectively and competitively its enzymatic activity. This macromolecular inhibitor may serve as a probe for further characterizing the role this enzyme plays in HCV replication and as a basis for the development of anti-viral compounds. Also, the results reported here may provide clues for engineering improved small recognition unit enzyme inhibitors.

Materials and methods

Microbiological and recombinant DNA techniques

Microbiological and recombinant DNA methods were carried out according to standard protocols (Ausubel *et al.*, 1994) or as recommended by suppliers. Oligonucleotides were synthesized using an Applied Biosystems (Foster City, CA) 380B synthesizer. Phage manipulation and *Escherichia coli* electroporation were carried out as described previously (Martin *et al.*, 1994; Davies and Riechmann, 1995). Nucleotide sequences were determined using Sequenase (United States Biochemical, Cleveland, OH) according to the supplier's recommendations. The design of cV_H wild type has been described previously (Davies and Riechmann, 1994, 1995).

Construction, expression and partial purification of NS3-myc protease

The choice of fusing the myc epitope to the C-terminus of NS3 protease 20 kDa fragment was dictated by the assumption that modification of the N-terminus could interfere with interaction with NS4A co-factor (Failla *et al.*, 1995; Kim *et al.*, 1996). To construct NS3-myc, plasmid pT7-NS3 (1027–1206) encoding the HCV BK strain NS3 protease domain from residue 1027 to 1206, was used as a template for PCR amplification. Fusion of the myc epitope EFEQKLISQQDLG to the C-terminus of the protease domain was obtained by using oligo-myc and NS3up as PCR primers (oligo-myc: GCTAGCCCAGGTCTTCTTCAGAGATCAGTTTCTGCTC-GAATTCCCGCATAGTAGTTTCCATGGA; NS3up: GCATA-CATATGGCGCCCATCACGGCC). The PCR fragment was digested with *Nde*I and cloned into pT7–7 vector digested with *Nde*I and *Sma*I. The resulting pT7-NS3-myc was fully sequenced to ensure that no mutations had been introduced by PCR. To induce expression of the protease, *E.coli* BL21 cells were transformed with pT7NS3-myc and grown at 37°C in LB medium to a density of 0.9 OD; 400 μM IPTG were added and cells were grown for a further 4 h at 23°C. Cells were then harvested by centrifugation and pellets were resuspended in 25 mM sodium phosphate buffer, pH 6.5, 50% glycerol, 10 mM DTT, 0.5% CHAPS, 1 mM EDTA and disrupted using a standard French press. Homogenates were clarified by centrifugation at 120 000 *g* for 1 h and loaded on an HR 26/10 S-Sepharose column equilibrated with 25 mM sodium phosphate, pH 6.5, 3 mM DTT, 0.5% CHAPS, 10% glycerol (equilibration buffer) operating at 2 ml/min. The protease was eluted with equilibration buffer containing 1 M NaCl and dialyzed 1:100 for 8 h at 4°C against equilibration buffer. After this step the protease was 60% pure as judged by Coomassie-stained SDS–PAGE. The protease-containing solution was made 50% in glycerol content and kept at –20°C until use. The protease was immobilized on anti-myc coated Dynabeads (see below) and extensively washed with SLB. In order to check whether the immobilized protein was still enzymatically active, an aliquot of protease-bound Dynabeads was incubated for 6 h in SLB buffer containing 100 μM substrate peptide Ac-DEMEECASHLPYK. Cleavage of this peptide was verified by subsequent HPLC analysis of the solution.

NS3 protease assays

Assays were performed in 50 mM Tris, pH 7.5, 1% CHAPS, 15% glycerol, 0.1 mM DTT using 20 nM recombinant NS3 protease purified from *E.coli* as described previously (Steinkuhler *et al.*, 1996a). The protease was preincubated for 15 min with a 14-mer peptide corresponding to the central domain of the protease co-factor NS4A with the sequence GSVVIVGRIILSGR (Tomei *et al.*, 1996). To 60 μl of assay mix, up to 6 μl of cV_HE2 solutions (or corresponding amounts

of buffer) were added and incubated for a further 30 min at 25°C. Reactions were started by adding 40 μM substrate peptide Ac-DEMEECASHLPYK-NH_2 and stopped by adding 40 μl of 1% TFA at $<$20% conversion. Samples were analyzed by HPLC using a Merck–Hitachi chromatograph equipped with an autosampler, column oven and fluorescence detector. Samples of 45–90 μl were injected on to a reversed-phase HPLC cartridge column (LiChrospher C18, 5 μm, 0.4×7.5 cm, Merck) equilibrated with 90% solvent A (H_2O–0.1% TFA) and 10% solvent B (acetonitrile–0.08% TFA) and operating at a flow rate of 2.5 ml/min. A 10–40% gradient of solvent B at 5%/min was used to separate cleavage fragments. Peaks were detected by monitoring tyrosine fluorescence (excitation at 260 nm, emission at 305 nm). Cleavage products were quantified by integrating chromatograms with respect to samples in which 100% conversion was achieved by 12 h of incubation in the presence of 2 μM protease.

IC_{50} values were calculated by fitting inhibition data to Equation 1 using Kaleidagraph software:

$$\text{Activity (\%)} = (\text{maximum activity})/\{1 + ([I]/IC_{50})^S\} \quad (1)$$

where [I] is the cV_HE2 inhibitor concentration, maximum activity is that of the enzyme in the absence of inhibitor and *S* is the slope factor of the curve.

Reversibility of inhibition was assessed by dilution experiments. Briefly, NS3 protease was preincubated with cV_HE2 at a concentration of 4–5×IC_{50} as described above. After 30 min, half the sample was diluted 10-fold and in both samples activity was determined and compared with the activity of a sample incubated with a buffer blank and a sample incubated from the beginning with a 10-fold diluted inhibitor concentration. Inhibition was defined as reversible if more than 75% of activity was recovered upon dilution. Inhibition mechanisms were determined by performing substrate titration experiments using concentrations of substrate peptide between 15 and 250 μM (K_m/3.3 and 5×K_m) in the absence and presence of 125 and 250 nM of cV_HE2. Initial rates of cleavage were determined on samples with $<$20% conversion. Kinetic parameters were calculated from least-squares fit of initial rates as a function of substrate concentration with the help of Kaleidagraph or Sigmaplot software, assuming Michaelis–Menten kinetics. K_i and K_{ii} values were calculated by re-fitting the data to a modified Michaelis–Menten equation:

$$V = V_{max}S/\{K_m\ (1 + [I]/K_i\} + S\ \{1 + [I]/K_{ii})\} \quad (2)$$

Affinity selection

The library used for the selection was a kind gift from Dr L.Riechmann (MRC, Cambridge, UK). Briefly, the library was constructed by inserting randomized (NNG/C) oligonucleotides in the region corresponding to CDR3 of prototype cV_H between residues 98 and 103 (Brookhaven Protein Data Bank, 1VHP) shown in Figure 2. The library contained a total of ~10^8 transformants having insertions ranging from 5 to 12 residues in length (for details, see: Davies and Riechmann, 1995). Dynabeads M-280 sheep anti-mouse antibody (Dynal) were coated with 90 pmol of anti-myc mAb 9E10 according to the supplier's instructions. A saturating amount of myc-tagged NS3, 400 pmol in selection buffer (SLB: 15% glycerol, 0.1 mM DTT, 50 mM NaCl, 0.5% CHAPS, 10 mM Tris, pH 7.5,) was used for immobilization on to the anti-myc mAb 9E10. After a 2 h incubation at room temperature, the unbound NS3 was eliminated by five washes (100 μl, 1 min each) with SLB. The beads were then blocked with 3% (w/v) *E.coli* bacterial extract in SLB, which was more efficient than non-fat milk or BSA as a scavenger reagent, and washed once again before use. Then ~10^{11} TU (transducting units) of the cV_H library in 100 μl SLB were added to the beads and affinity-selected for 3 h at 23°C in the presence of 3% *E.coli* bacterial extract and 10^{11} UV-killed f1 phage particles as a blocking agent. Subsequently, the beads were washed 10 times and resuspended in 100 μl of SLB. The suspension was loaded on to 700 μl of a 30% sucrose cushion and the beads were collected by applying a magnetic field (Dynal, MPC). This step was repeated a second time before acidic elution of the bound phage with 0.1 M Tris–glycine buffer (pH 2.2). Following neutralization of pH and phage amplification in TG1 cells, the selection was repeated four times using the same input of purified phage as in the first round. A depletion step on to the anti-myc mAb 9E10-coated beads was carried out after each enrichment cycle to counter-select for phage interacting with the matrix devoid of protease. As a measure of the efficacy of this counter-selection, we estimate that ~50% of input phage population is recovered after the depletion step.

Expression and purification of cV_H

The V_H encoding expression plasmids were transformed in TG1 *E.coli* strain bearing the plasmid pDMI1 which expresses *Lac*I (kind gift from Dr Bujiard, University of Heidelberg). Cells were grown in fortified SB medium (Power *et al.*, 1992) in a bench-top fermenter (MFS, SAVI, Italy) at 27°C; the stirring rate was set at 500 r.p.m., the pH was kept between 7.0 and 7.5 and the air flow was fixed at 0.5 Nlt/min (l/min of nitrogen equivalents). When the culture A_{600} reached 2.0, expression was induced by addition of 500 μM of IPTG and the air flow was increased to 1 Nlt/min, culture was continued for 6 h (corresponding to a final A_{600} of 5.0) and cells were collected by centrifugation. The pellet was immediately resuspended in 50 ml (1/20 of the original volume) in 30 mM Tris, pH 7.4, 1 mM EDTA, 20% sucrose and left on ice for 30 min. Cells were centrifuged (20 min at 2500 *g*) and supernatant 2 was kept. The pellet was resuspended in 50 ml of pre-cooled 5 mM $MgSO_4$ and incubated for another 30 min at 4°C. The solution was centrifuged and the supernatant was pooled with supernatant 2, filtered and purified using IMAC (Hochuli *et al.*, 1987).

Gel filtration chromatography

Gel filtration experiments were run on a Pharmacia FPLC system, with a Superdex-75 analytical column equilibrated in TBST containing 10% glycerol and 0.1 mM DTT. The calibration run was done with a Pharmacia Biotech LMW gel filtration calibration kit, which includes the following markers: dextran blue, bovine serum albumin, ovalbumin, chymotrypsinogen and RNase corresponding to 200, 67, 43, 25 and 13.7 kDa, respectively.

In vitro translation of NS3 and substrate

DNA fragments derived from HCV-BK strain cDNA were inserted downstream of the 5′ untranslated region of encephalomyocarditis virus and under the T7 promoter in the pCite-1 vector (Novagen) in the appropriate translational reading frame and followed by a termination codon. The plasmids pCiteNS3–4AΔcut and pCiteNS5ABΔC51, expressing the HCV proteins NS3-4A with a mutated cleavage site and NS5AB from residue 1965 to residue 3010, respectively, have been described previously (Steinkuhler *et al.*, 1996a,b; Tomei *et al.*, 1996).

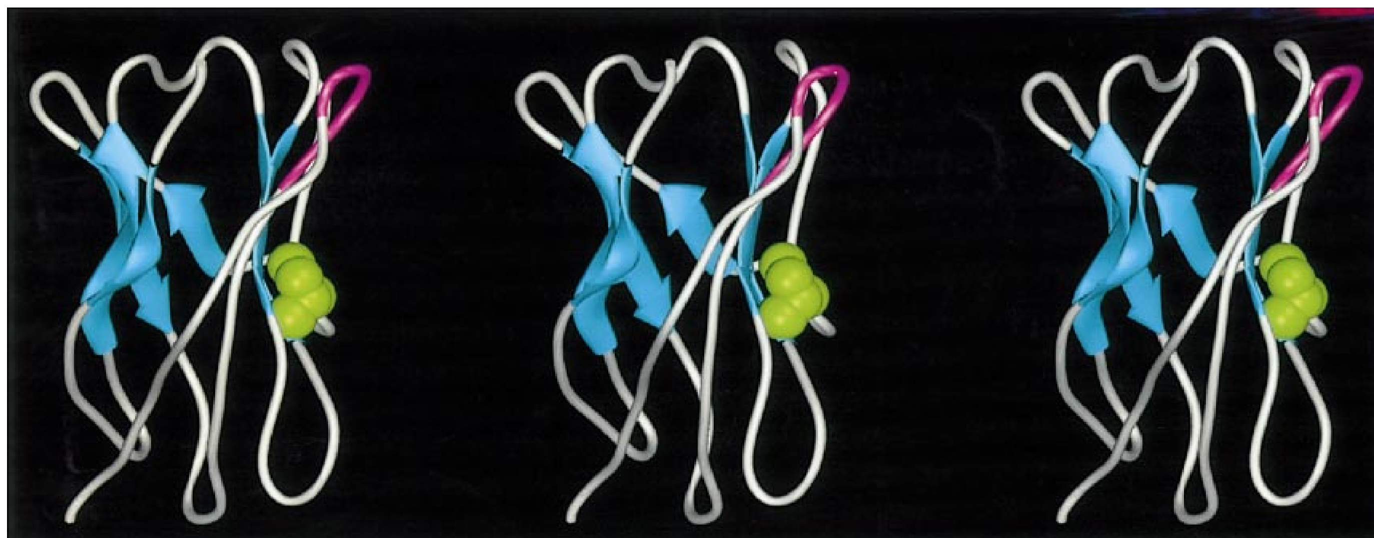

Fig. 2. NMR structure of cV_H prototype molecule. Stereo drawing of the camelized VH-P8 domain NMR structure (Riechmann *et al.*, 1996), PDB entry 1VHP (Bernstein *et al.*, 1977), showing the close spatial proximity of CDR3 (magenta) and residue Ile 47 (green). The figure was drawn using RIBBONS (Carson, 1987).

In vitro transcription was done with T7 RNA polymerase (Promega). The transcripts were translated for 1 h at 30°C in the presence of [^{35}S]methionine using RNA-dependent rabbit reticulocyte lysate (Promega). Cleavage of labelled precursor was assessed by SDS–PAGE on 10% gels and exposed on the Phosphorimager from Molecular Dynamics.

Elastase and kallikrein assays

The pancreatic porcine elastase (PPE), its substrate (Me-o-Suc-Ala-Ala-Pro-Val-pNA) and the pancreatic porcine kallikrein (PPK) were purchased from Calbiochem. The PPK substrate Chromozym (D-Pro-Phe-Arg-pNA) was purchased from Boehringer Mannheim. The PPE assay was performed in a 96-well plate, in 50 mM TES, pH 7.5, 500 mM NaCl, 0.05% Triton X-100, 5% DMSO buffer; the enzyme was added to the final concentration of 0.5 µg/ml and substrate to 500 µM. Samples were tested in a 100 µl final volume and after 75 min of incubation at room temperature under constant agitation, the OD was read at 405 nm. The PPK assay was performed in 120 mM Tris, pH 8.0, 0.01% Triton X-100, 5% DMSO; the concentration of the protease and its substrate were 50 nM and 100 µM, respectively. After incubation for 1 h at 23°C under agitation the OD was recorded at 405 nm.

Results

In order to carry out affinity selection of randomized CDR3 variants of cV_H domains displayed on filamentous phage, it was fundamental to engineer the target NS3 protease 20 kDa minimal domain that can be expressed in *E.coli* and purified in large quantities (Steinkuhler *et al.*, 1996a) to allow its immobilization on to a solid-phase matrix in a bioactive conformation. To this end we fused a myc-tag peptide (EFEQKLISQQDLG) to the C-terminus of HCV NS3 domain, thus achieving an enzyme variant which retains catalytic activity when immobilized through the anti-myc mAb 9E10 (data not shown). The NS3 affinity matrix was prepared by saturating anti-mouse-coated magnetic beads with a complex of myc-tagged NS3 protease and the tag-specific mAb 9E10. As a source of ligands we used the cV_H library in which the CDR3 of prototype cV_H domain (Figure 2), randomized both in sequence and length (5–12 residues) and displayed as fusion with the pIII protein of *fdCAT* (Davies and Riechman, 1995). Four rounds of selection were carried out and subtractive steps were included between rounds. A depletion step consists of incubating the amplified library with the matrix, including all reagents but devoid of NS3 20 kDa fragment. Because NS3 protease requires detergent and kosmotropic agents for optimal activity (Steinkuhler *et al.*, 1996a), affinity-selection steps were carried out for 4 h at room temperature, in the presence of 15% glycerol and 1% CHAPS detergent, under mild reducing conditions (0.1 mM DTT and in a nitrogen atmosphere), to maintain adequate conditions for the enzymatic activity but avoiding disruption of the V_H disulfide linkage. Under these buffer conditions the protease is active for 3–4 h, judged adequate to carry out the affinity selection.

Following selection, 42 phage clones from the fourth round were cultured, concentrated by PEG precipitation, their ssDNA extracted and the CDR3 amino acid sequence was determined by DNA sequencing (Table I). Some of these putative NS3 ligands were present as multiple isolates; particularly striking was clone cV_HE2 that represented the major component (20/42) of the phage population. Its CDR3, considerably rich in proline residues, shows a suggestive similarity to other isolates, albeit the lengths of their CDR3 differ greatly. Because the library was constructed using randomized oligonucleotides according to the scheme NNG/C, the relatively high percentage of Pro residues observed here is not due to a bias in the repertoire, as suggested also by the sequence of other isolates recovered from a different study (Davies and Riechmann, 1995). We decided to study some of these clones further with the aim of identifying protease inhibitors. To this end, the DNA fragments encoding the variable region of the isolated cV_H of some *bona fide* NS3 ligands were excised from the phage RF dsDNA by digestion with *Xho*I and *Bst*EII and subcloned into pUCV_H-myc vector, suitable for expression in the periplasmic space of *E.coli* (Davies and Riechmann, 1995). Next, the cV_H proteins bearing the myc epitope and the $(His)_6$ tag genetically fused to their C-termini were purified by IMAC (Hochuli *et al.*, 1987) before testing. As a preliminary screen for inhibition activity, we used the *in vitro* translation assay

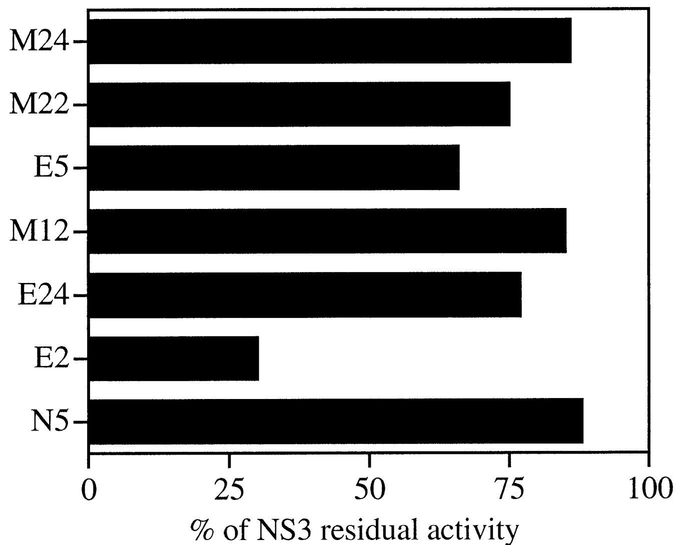

Fig. 3. *In vitro* translated 20 kDa NS3 inhibition assay. *E.coli* expressed and IMAC-purified cV_H proteins (reported on the *y*-axis) were tested at 1.5 μM in triplicate samples for their ability to inhibit *in vitro* translated 20 kDa NS3 protease by monitoring the percentage conversion (*x*-axis) of the *in vitro* translated NS5AB substrate.

Table I. Amino acid sequence of CDR3 from affinity-selected cV_H molecules

Name	CDR3 sequence	Number of isolates/42
E2	EPRIPRPPS	20
E4	STTLSTSPITK	6
E5	APIHPRALTPS	1
E17	PRAPQI	1
E24	RPRAPQV	4
M2	PKPPQYY	1
M3	AQVDSPTSFTP	1
M4	ADHVNEAWLS	1
M5	SKCSYHIPPYTS	1
M12	SKIVDDVSFVP	1
M17	HSLSSPQTGVT	1
M20	HPASAKFPSTT	1
M22	NAPGNEPLGDRS	1
M23	GYDYGAW	1
M24	EPRHARARRS	1
N5[a]	KSLPD	

[a]N5 is a non-selected cV_H.

(Steinkühler *et al.*, 1996b) in which the *in vitro* transcribed RNAs encoding the 20 kDa NS3 fragment and one of the substrates, NS5AB, are incubated in the presence of fixed amounts of cV_H proteins. The purified proteins encoded by several constructs were tested at 1.5 μM in triplicate samples and only cV_HE2 was revealed as an inhibitor of the protease activity (Figure 3). At this concentration, most of the molecules tested showed little or no activity (0–30% inhibition), within the range of non-specific inhibition given by the non-selected, negative control cV_HN5 and therefore were not pursued further. On the other hand, cV_HE2 appeared to be a relatively potent inhibitor, deserving additional characterization.

In order to determine the apparent molecular weight of cV_HE2 under NS3 protease assay buffer conditions, we carried out gel filtration FPLC in this buffer, except that the glycerol concentration was lowered to 10% (the maximum compatible with chromatography). The protein migrates as a dimeric species with an apparent molecular weight of 25 kDa (Figure

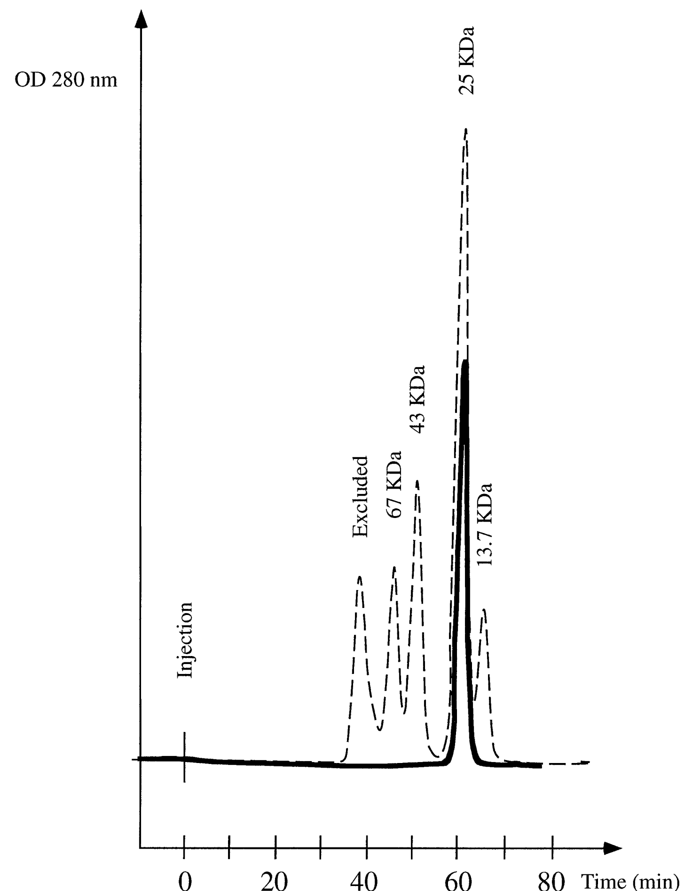

Fig. 4. Gel filtration chromatography. The molecular weight of the purified cV_HE2-tagged protein was determined by FPLC using a Superdex-75 column in NS3 assay buffer containing 10% glycerol and 0.5% CHAPS. The molecular weight standards are indicated.

4), whereas other selected and unselected molecules elute as monomeric species, thereby suggesting that the CDR3 sequence of cV_HE2 affects the physical state of this molecule (see Discussion). The FPLC elution profile did not change even in the absence of glycerol and CHAPS.

An accurate estimate of the potency of cV_HE2 inhibitor was carried out through titration experiments by monitoring the residual enzyme activity as a function of cV_HE2 concentration using a small fluorescent substrate at a concentration of 40 μM. It is worth noting that the IC_{50} of cV_HE2 was not affected by the co-factor NS4A and was estimated to be 300 nM (Figure 5). Because cV_HE2 activity is not inhibited by NS4A peptide and *vice versa*, it appears that the selected ligand can bind both forms of the enzyme, both as a free species and as a heterodimer complex. It was also important to establish the reversibility of the inhibition activity, assessed by an assay in which we pre-incubated the enzyme with 1.5 μM concentration of the cV_HE2 inhibitor (five times its IC_{50} value) followed by a 10-fold dilution and determination of the residual enzymatic activity (Figure 6). The results were consistent with reversible inhibition as we recovered more than 80% of the enzyme activity upon dilution of the complex. To establish the mechanism of action of the cV_HE2 inhibitor, substrate titration experiments were performed in the absence and in the presence of 125 and 250 nM of cV_HE2 (Figure 7). By fitting the experimental data to a modified Michaelis–Menten equation (Equation 2), the dissociation constants of the enzyme inhibitor

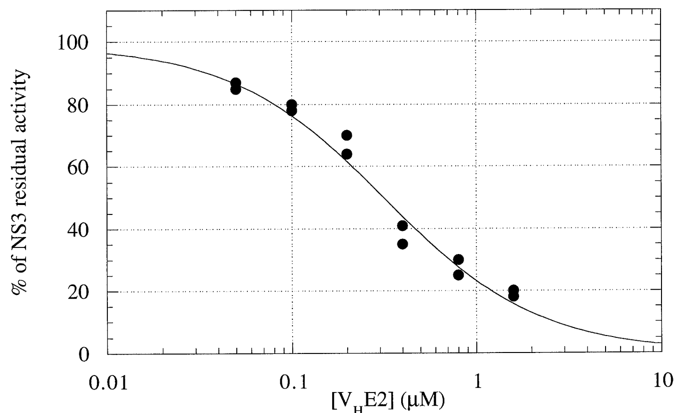

Fig. 5. Determination of cV_HE2 IC_{50}. Recombinant NS3 20 kDa fragment protease (20 nM) was incubated in the presence of increasing amounts of purified cV_HE2 inhibitor (duplicate), 40 μM substrate and 10 μM NS4A co-factor. Percentage of residual activity (*y*-axis) was determined as function of inhibitor concentration (*x*-axis) and the IC_{50} value derived.

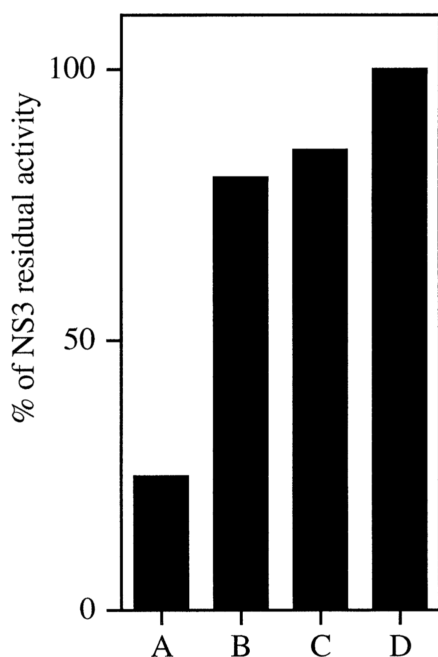

Fig. 6. Reversibility assay. Recombinant NS3 20 kDa fragment protease, substrate and co-factor (as in Figure 3) were incubated in the presence of different amounts of inhibitor for 30 min (in duplicate). The percentage of enzyme residual activity is reported on the *y*-axis: (**A**) 1.5 μM cV_HE2; (**B**) 1.5 μM cV_HE2, after incubation diluted 10-fold and activity determined; (**C**) 0.15 μM inhibitor; (**D**) as in (B) except that the enzyme was incubated in the same amount of cV_HE2 buffer.

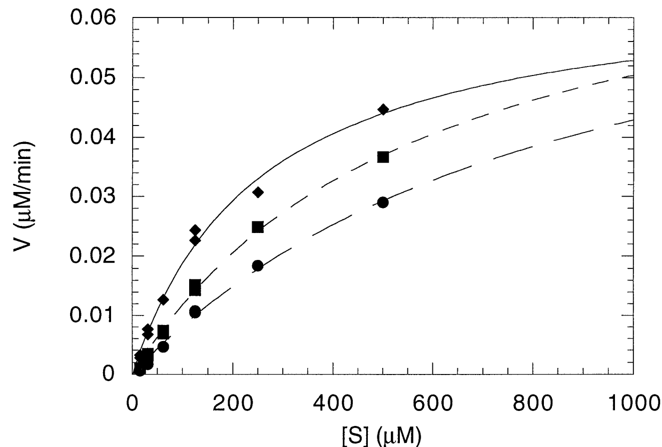

Fig. 7. Determination of the inhibition mechanism. To determine the mechanism of inhibition of cV_HE2, substrate titration curves were recorded in the absence and in the presence of two different concentrations of inhibitor: 250 nM (circles), 125 nM (squares), no inhibitor (diamonds), and the values of K_i (enzyme–inhibitor complex dissociation constant) and K_{ii} (ternary enzyme–inhibitor–substrate complex dissociation constant) derived by fitting the experimental data to a modified Michaelis–Menten equation.

complex (K_i) and of the ternary enzyme–inhibitor–substrate complex (K_{ii}) were determined. We obtained K_i = 150 nM and a very high value for K_{ii}. This indicates that the cV_HE2 inhibitor binds to the free enzyme but has virtually no affinity for the enzyme–substrate complex, as expected for a competitive inhibitor. Probably the selected molecule binds to the enzyme active site or to a nearby epitope, thus hampering the substrate entry, but in any case its binding site is far from the NS4A binding site. We also determined that cV_HE2 was not cleaved by NS3 after overnight incubations with high concentrations (30 μM) of NS3 (data not shown), demonstrating that the observed inhibition of NS3 by cV_HE2 is not due to cleavage of a competitor substrate. Next, we checked selectivity by testing cV_HE2 on two commercially available serine protease assays, namely porcine elastase and kallikrein. Neither of these enzymes was inhibited to any measurable extent upon incubation with a concentration of cV_HE2 corresponding to a 5–30-fold excess of the inhibitor/substrate ratio (data not shown), indicating a substantial level of selectivity.

Finally, because of the lack of cell-based replication systems for HCV, in order to determine if cV_HE2 was active on the entire (70 kDa) NS3 gene product (including the helicase domain) and on the natural substrate, we exploited the *in vitro* translation assay described previously (Tomei *et al.*, 1993) as a surrogate for a biological assay. In this experiment (Figure 8), cV_HE2 inhibits 70 kDa-borne proteolytic activity with an apparent IC_{50} of ~1 μM, 3.3-fold above the value determined by the activity titration assay with the (13-mer) synthetic substrate.

Discussion

Because of the importance of HCV as a human pathogen and the lack of an effective treatment (Weiland, 1994), there is a crucial need to develop inhibitors of viral metabolism. Proteases are fundamental components of physiological regulation and are involved in the pathophysiology of a number of disease states (Neurath, 1989). They are essential to the life cycle of many parasitic pathogens and to processing viral precursor proteins (Korant, 1988; Krausslich and Wimmer, 1988). These observations have led to an ever increasing interest in proteases as potential therapeutic targets. Previous studies have indicated that the HCV NS3 protease activity is required for processing the non-structural proteins NS3, NS4 and NS5 in addition to acting *cis* at its C-terminus to release itself from the polyprotein precursor, in a similar fashion to the viral protease of flaviviruses and pestiviruses (Miller and Purcell, 1990). Proteolytic events mediated by the NS3 protease are likely to be absolute requirements for the generation of an active viral replication apparatus. Because NS3 is crucial for the HCV polyprotein processing and its flaviviral analog has been shown to be essential for viral replication (Chambers *et al.*, 1990), the NS3 protease has become the focus of intensive study to develop anti-HCV drugs, as emphasized by two recent reports on the

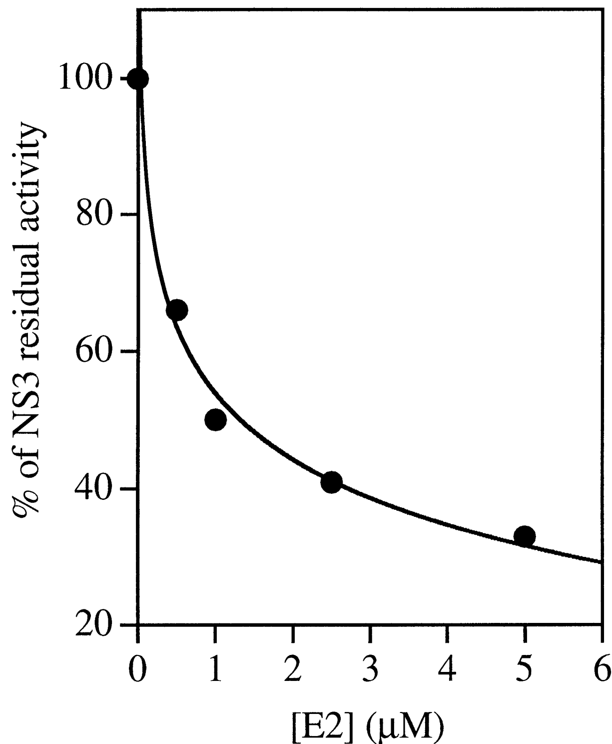

Fig. 8. *In vitro* translated 70 kDa NS3 inhibition assay. Full-length NS3 (70 kDa fragment) and NS5AB substrate genes were transcribed *in vitro* using T7 polymerase and translated in reticulocyte lysate. Different concentrations of purified cV_HE2 protein (*x*-axis) were pre-incubated (in duplicate samples) with the protease and NS4A, and subsequently the substrate was added and incubated further, allowing conversion to occur. The percentage conversion (*y*-axis) was determined by densitometric analysis of the radiolabeled product after separation by SDS–PAGE.

structural determination of NS3 protease domain (Kim *et al.*, 1996; Love *et al.*, 1996). In particular, it was argued that the absence of some conserved loops, in the NS3 protease, renders its substrate binding site relatively shapeless, thus it was anticipated that the making of substrate-based small molecule inhibitors will be a daunting task (Kim *et al.*, 1996). For these reasons, we anticipate that a deeper understanding of the NS3 surface properties and the availability of biological tools for studying it will have a substantial impact on the development of effective inhibitors. Here we report the affinity selection and characterization of cV_HE2, a camelized V_H domain (Davies and Riechmann, 1994), that is a relatively potent and selective competitive inhibitor of the NS3 protease activity.

The Camelidae is the only taxonomic family known to possess functional heavy-chain antibodies, devoid of light chains (Hamers-Casterman *et al.*, 1993). V_H domains lacking their V_L partners have been bacterially expressed and shown to be able to bind antigen specifically (Ward *et al.*, 1989). Camelization of a human V_H domain (V_H-P1) to mimic camelid heavy chains was achieved by introducing the mutations G44E, L45R and W47G (Davies and Riechmann, 1994, 1996) that are crucial for reducing the affinity for the cognate V_L domain (Chothia *et al.*, 1985). Camelizing mutations also made the structural determination of this cV_H by NMR spectroscopy possible (Riechmann, 1996), except that residue 47 was mutated in isoleucine, which yields better expression and higher stability compared with the G47 containing V_H domains (Davies and Riechmann, 1994; 1996). The solution structure of the cV_H showed well defined regions of β-structure and less well defined connecting loops and turns (Riechmann, 1996). The general topology of two pleated β-sheets and the conformation of the hypervariable loops H1 and H2 were shown to be very similar to those of V_L-associated V_H domains (Figure 2).

Usually, the G47I mutation does not affect antigen binding or specificity (Davies and Riechmann, 1995, 1996); however for cV_HE2 we observed a 20-fold reduction in potency which is not dependent on dimerization. The cV_HE2 protein forms dimeric species independently from the nature of its residue 47 (G or I). In the light of recent structural data, it is conceivable that in the absence of cognate V_L some CDR3 structures can be affected by the nature of residue 47, which in turn may promote dimerization. In fact, the NMR structure of the parental cV_H, which has a shorter CDR3 than cV_HE2 (Figure 2), demonstrated that it is a stable monomeric protein (Riechmann, 1996). In addition, that dimerization may be dependent on the nature of CDR3 is also supported by the 1.85 Å X-ray structures of antigen-free llama V_H domain (Spinelli *et al.*, 1996) and by the 2.5 Å crystal structure of a camel V_H in complex with its antigen, lysozyme, as reported recently (Desmyter *et al.*, 1996). Compared with human and mouse V_H domains, there are no major backbone rearrangements in the V_H framework, but the architecture of the region of V_H that interacts with a V_L in a conventional F_v is different from any previously seen. The camel antibody has a very long CDR3, one half of which contacts the V_H region which in conventional immunoglobulins interacts with a V_L, whereas the other half protrudes from the antigen binding site and penetrates into the lysozyme active site.

The CDR3 segment that penetrates deeply into the active site of lysozyme provides most of the binding energy; unfortunately, this is unlikely to be the case for the NS3/cV_HE2 interaction, as suggested by the analysis of the X-ray structure of the NS3 specificity pocket (Kim *et al.*, 1996). Nonetheless, cV_HE2 may still be a useful tool for guiding the synthesis of CDR3 peptido-mimetics as shown successfully, albeit with an expected loss in potency (Smythe and von Itzsein, 1994). In the absence of structural data, there are two considerations that can be made about the mechanism of inhibition for cV_HE2: (i) the inhibitor binds NS3 both as a free enzyme and as a heterodimeric complex with NS4A; (ii) the mechanism of action is that of a competitive inhibitor. It is therefore likely that cV_HE2 binds to the enzyme active site or to a nearby epitope, thus hampering substrate entry, but in any case its binding site is far from the NS4A binding site. It should also be emphasized that the inhibitor is not cleaved by NS3, which can be a consequence of the absence of a suitable Cys residue within the CDR3 sequence or a unfavorable geometry (as a substrate) of the CDR backbone structure. Taking these results together, it is tempting to propose that to design candidate molecules (good building blocks) for the selection of more general enzyme inhibitors, it would be wise to engineer cV_H constructs with a long CDR3, part of which could be randomized to fit the target enzyme active site cleft whereas an invariant component (which would act as an additional framework) could be designed based on the natural camel antibody (Desmyter *et al.*, 1996), to shield the former V_L interface, thus avoiding dimerization. In this respect it may also be relevant to point out that by using a minibody scaffold (Pessi *et al.*, 1993) having shorter CDRs (six residues) for the selection of NS3 inhibitors, we were able to recover a ligand of comparable potency but showing a non-competitive inhibition

mechanism (N.Dimasi, F.Martin, C.Volpari, N.Brunetti, G.Biasiol, S.Altamura, R.Cortese, R.De Francesco, C.Steinkuhler and M.Sollazzo, in preparation).

This study confirms previous findings with selected cV_H ligands specific for hapten, protein and peptide whose affinities are in the micro- to nanomolar range (Davies and Riechmann, 1995). On the other hand, by displaying on filamentous phage variants of natural proteinaceous inhibitors such as BPTI (Roberts *et al.*, 1992), Ecotin (Wang *et al.*, 1995), APPI (Dennis and Lazarus, 1994), hPSTI (Rottgen and Collins, 1995), LACI-D1 (Markland *et al.*, 1996a,b), protease inhibitors have been remodelled and new specificities selected with K_is in the low nanomolar range. However, is our experience that selection of hPSTI variants (Kazal-type inhibitor) using NS3 as a target has yielded competitive inhibitors of modest (micromolar K_i) potency (N.Dimasi, F.Martin, C.Volpari, N.Brunetti, G.Biasiol, S.Altamura, R.Cortese, R.De Francesco, C.Steinkuhler and M.Sollazzo, in preparation). Probably, more potent cV_H inhibitors will be achieved by a stepwise approach of affinity maturation, since the complexity of the repertoire explored (10^7–10^8) represents only a subset of all the possible combinations of a 6–12 amino acid long CDR3. In order to improve the potency of our macromolecule lead, cV_HE2, it is conceivable to identify by Ala scanning the residues within its CDR3 that are crucial for activity and to optimize them by subsequent cycles of randomization/selection (possibly in the context of I47 framework format to ensure higher levels of expression). Once a more potent compound is obtained, it may be possible to proceed with the synthesis of the smallest cyclic peptide that conserves significant levels of bioactivity.

These easy to generate and engineer minimal antigen recognition units serve as potential pharmacophoric models to drive low molecular weight compound design (reviewed by Sollazzo *et al.*, 1995) and in addition provide useful biological probes in cell based assays and animal models to validate NS3 protease as pharmacological targets for such a clinically important pathogen.

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