Discovery of an allosteric mechanism for the regulation of HCV NS3 protein function

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Supplementary Methods

Cloning, expression and purification of HCV NS3/4a
The full length genotype 1b NS3/4a construct comprises residues NS4a21-32NS33-631 and cloned into a pET 28 vector via NdeI and XhoI restriction sites. The sequences of the cloned coding regions for the different genotypes analogous to the construct described above were derived from Genbank entries as follow: genotype 1a (H77c) GI2327070; genotype 1b (BK) GI329770; genotype 2a (JHF1) GI40714444; genotype 3a GI91983642; genotype 4a GI89519404; genotype 5a GI3660725; genotype 6 GI324330488. The plasmid was freshly transformed into BL21 (DE3) or Rosetta 2 (DE3) E. coli cells and grown at 37°C in Terrific Broth supplemented with 50ug/ml of kanamycin and 100Μ M ZnCl₂ or ZnSO₄, and induced with IPTG after lowering the temperature to 18°C. Cells were suspended in lysis buffer at 4°C (50mM HEPES pH 7.5, 300mM NaCl, 10% Glycerol, 0.1% n-octyl-β-D-glucopyranoside, protease inhibitor cocktail (Calbiochem)) lysed by sonication and clarified by centrifugation. The protein was captured by IMAC chromatography (Qiagen) and further purified using ion exchange chromatography (GE Healthcare). The final polish was done using a superdex-200 gel filtration column (GE Healthcare). Fractions were analysed by SDS PAGE (Novex) pooled and concentrated to a final concentration of ~6mg/ml. Analysis by mass spectrometry showed the mass of the purified wildtype protein to be 70663D. The expected mass was 70794D indicating the loss of the N-terminal methionine, which was verified by N-terminal sequencing. A representative gel illustrating the purity level achieved is shown in supplementary figure 5.

NS3/4a protease Assays
The protease activity of the full length NS3/4a was measured using a FRET-based assay utilizing a peptide substrate derived from the NS4A/B cleavage site (Anaspec) and labelled at one end with a quencher (QXL520) and at the other with a fluorophore (5-FAMsp). Full length NS3/4a was incubated with test compounds and peptide substrate in 50 mM Tris pH8.0, 20 mM DTT, 1% CHAPS, 10% glycerol and 5% DMSO. For isolated protease domain assays, the assay buffer contained 50mM Tris pH8, 20mM DTT, 1% CHAPS, 35% glycerol and 15μM NS4a peptide (KGSVVIVGRIILSGRK). The reaction was followed by monitoring the change in fluorescence on a Molecular Devices Gemini plate reader for 30 minutes at room temperature. Initial rates were calculated from the progress curves using SoftMax Pro (Molecular Devices). The IC₅₀ value was then calculated from replicate curves using Prism GraphPad software.
Protein stability and susceptibility
The stability of the HCV NS3/4a protein was tested by incubating samples of the protein at a concentration of 16.5μM at RT in the presence and absence of 12 fold molar excess of compound 5 for up to 17h 30min. Identical NS3/4a samples were incubated over the same time course with sequencing grade trypsin and chymotrypsin at 1:60 protease to NS3/4a ratios using buffer B 50mM Hepes pH 7.6, 150mM NaCl, 10% glycerol and D 50mM Hepes pH 7.6, 30mM NaCl, 10% glycerol. Controls to rule out inhibition of the proteases by compound 5 were run alongside, using lysozyme as the substrate. Samples were taken at t=0, 10, 20, 45, 90, 180 and after overnight incubation t=17 hours 30min and run on SDS 4-20% gradient polyacrylamide gels (Novex).

Size Exclusion Chromatography
Analytical size exclusion runs were performed on a Superdex 200 analytical column (GE Healthcare) at 4°C under varying buffer conditions. The NS3/4a protein at a concentration of 6mg/ml was ultracentrifuged prior to injection on to the column equilibrated in one of the following buffers: (A) 50mM HEPES, pH7.6, 1M NaCl, 10% glycerol, 1mM TCEP; (B) 50mM HEPES, pH7.6, 0.15M NaCl, 10% glycerol, 1mM TCEP (C) 50mM HEPES, pH7.6, 0.15M NaCl, 1mM TCEP or (E) 50mM HEPES, pH7.6, 0.15M NaCl, 1mM TCEP, 1uM Compound 3. The column calibrated using the 1.35-670kD molecular weight standards from BioRad. Flow rate of the column was adjusted for the presence of glycerol in the buffers. Representative chromatograms of the elution profiles are shown in supplementary figure 4.

Sedimentation Velocity Analysis
Sedimentation Velocity Experiments were done at CAMIS, New Hampshire, USA (C. May, T. Laue). NS3/4a protein was ultracentrifuged prior to buffer exchange into the appropriate buffer using Microspin columns (GE Healthcare). The protein was diluted using the same buffer to achieve a final concentration of 0.5mg/ml. Choice of buffer conditions was aimed to mimic crystallisation, physiological and bioassay conditions, respectively: (A) 50mM HEPES, pH7.6, 1M NaCl, 10% glycerol; (B) 50mM HEPES, pH7.6, 0.1M NaCl, 10% glycerol or (D) 50mM HEPES, pH7.6, 0.03M NaCl. Experiments were performed at 25 °C, in a Beckman Optima XL-I analytical ultracentrifuge. Spin Analytical charcoal epon 12 mm double-sector centerpieces were used in an eight-hole AN 50 Ti rotor at 42,000 rpm. Radial absorption scans were measured at λ = 280 nm. Sedfit 12.0 was used for data analysis, with a 68% confidence limit. Sedimentation coefficients were corrected to standard s_{20,w} conditions using the experimental buffer density and buffer viscosity as calculated with SEDNTERP. Results of this analysis are shown on supplementary figure 5.

Dynamic Light Scattering
Fixed angle light scattering experiments were performed on an Avid Nano W130i instrument (Avid Nano, High Wycombe, UK). The NS3/4a protein was ultracentrifuged prior to buffer exchange into the same buffers as specified for sedimentation velocity. The final protein concentration was 5mg/ml. The experiments were performed at room temperature using the disposable BladeCells from AvidNano. The data was analysed using system software i-Size version 1.3.0.3 which allowed for correction to solvent
viscosity from glycerol or ionic strength. A negligible degree of error will have been introduced by not correcting for both simultaneously. Repeating the measurement but analysing the data using the alternative solvent correction showed not effect to the margin of error.

**Replicon Assay**

Direct determination of HCV replicon RNA levels in HCV replicon bearing huh-7 cells was done by seeding 100,000 cells/well in a 6 well tissue culture plates and allowing them to attach overnight before compound addition at a final DMSO concentration of 0.1%. At 72 hours post compound addition RNA was extracted using a Qiagen RNeasy kit (Qiagen) according to manufacturer’s instructions. All samples were normalized for total RNA concentration. Quantitative RT-PCR was carried out using the following HCV NS5B gene specific primers: HCV5BF: CTCCATGGCCTTAGCGCATTT and HCV5BR: AAAAAACAGGATGGGCCTATTGG in a one-step reaction using the Quantitect SYBR Green RT-PCR kit (Qiagen) following manufacturer’s instructions. Briefly 2 ng of sample RNA was combined with the NS5B primers listed above at a final concentration of 1µM and an equal volume of 2x Quantitect SYBR Green RT-PCR Master Mix. Reactions were transferred to a thin walled 96 well plate and the RT reaction was carried out using the MX3005p (Stratagene) instrument at 50°C for 30 minutes, followed by a denaturation step at 94°C for 15 min. The PCR amplification was conducted in 45 cycles, each of which was 94°C for 15 s followed by 59°C for 30 seconds, then 72°C for 2 minutes. Amplification of HPRT RNA, as an internal RNA normalization control, for each sample was determined in separate reactions. The amount of input RNA from the untreated control sample was varied in order to generate a standard curve by which the relative levels of replicon RNA from each treated sample could be expressed as fold changes relative to the untreated control sample.

**Western blot analysis of polyprotein processing**

Cell lysates from replicon bearing Huh-7 cells were treated for 24 hours with the stated compounds at 10 x EC50 concentrations. Samples were resolved on 4-12% NuPage gels (Life Technologies) and immunoblotted with antibodies specific for (A) HCV NS5b (ab65410, Abcam) or (B) GAPDH (Chemicon Intl.). Untreated cell lysates were used as controls for cell lysates that were treated for different time intervals with compound 6, telaprevir or GS-7977 respectively. Molecular weight markers ran alongside (Rainbow markers, GE Healthcare) HCV NS5b and GAPDH were followed by infrared dye labelled anti-rabbit or anti-mouse antibodies (Licor Bioscience, Lincoln, NE, USA). Blots were scanned to detect infrared fluorescence on the Odyssey Infrared Imaging System (Licor Bioscience).

**Cytotoxicity Assay**

In this assay 4 x 10^3 huh-7 cells persistently infected with the HCV subgenomic replicon construct pFKI3889luc-ubi-neo/NS3-3'/ET were plated /well in a 96 well tissue culture plate and allowed to attach overnight in DMEM medium supplemented with 10% FBS 1% NEAA, and 250 µg/ml gentamicin. The medium was replaced with fresh medium as described above lacking gentamicin. Semilog dilutions of the compound in medium were added to triplicate wells to give a 0.1% DMSO final concentration. Plates were incubated
at 37°C in 5% CO₂ and air for 72 h. Following the 72 h incubation, the test compound’s CC₅₀ value was determined by adding Alamar Blue™ (Biosource International, Camarillo, CA, USA) and incubating for 6 h. The plate was read at 535 nm (excitation) and 590 nm (emission) on a SpectraMax Gemini reader (Molecular Devices) to determine the number of viable cells by measuring the conversion of rezasurin (Alamar blue) to resorufin in response to mitochondrial activity.

Antiviral Resistance Selection

Compound 5 resistant mutations in the subgenomic replicon (pFK13889luc-ubi-neo/NS3-3’/ET) were isolated by culturing Huh-7 cells persistently infected with this subgenomic replicon construct in the presence of medium containing 500µg/L G418 and 3µM compound 5 for 11 days, followed by 10µM of compound 5 for a further 28 days. Individual G418 resistant colonies were isolated and resistance to compound 5 was confirmed by determining their EC₅₀ by Luciferase activity. RNA was isolated from each clonal population of cells using a Qiagen RNasearch kit (Qiagen) according to manufacturer’s instructions. The RNA for each sample was reverse transcribed by priming 1 µg of the isolated colony RNA with the NS4AR primer (listed below) in a reaction using GoScript reverse transcriptase reagents (Promega) as described in manufacturer’s instructions. The resulting first strand cDNA was denatured at 95°C for 15 min, followed by PCR amplification of the corresponding NS3 protein using the following primers:

NS3F: ATGGCGCTATTACCGCCTAC
NS4AR: CTCCGCTTTGCTTGGTGGCTG

The PCR amplification was conducted in 30 cycles (94°C for 15 s followed by 55°C for 30 s, then 72°C for 1 min). Amplified PCR products were cloned into the pCR4-TOPO vector using manufacturer’s instructions for the TopoTA Cloning Kit for Sequencing (Invitrogen). Sequencing of the cloned PCR products was performed using vector specific primers.

Validation of cellular target: Compound 5 dose-dependently inhibited HCV NS3 target function at 0.4µM in a genotype 1b HCV replicon bearing Huh-7 cell line as determined by luminesce activity from the replicon encoded luciferase gene (Suppl Fig. 6). Replication of the replicon construct and therefore luciferase expression levels are known to be dependent on HCV NS3 protease activity, amongst other viral protein activities. Compound 5 was also demonstrated to bind full length HCV NS3/4a by ITC (Table 1) and direct structural determination (Fig. 3c-e). Furthermore compound 5 was demonstrated to inhibit the protease activity of full length NS3/4a in a FRET-based assay (Table 1). Notably selection pressure placed upon cells persistently infected with the GT1b replicon by compound 5, resulted in the of development resistance mutations that mapped to regions of HCV NS3/4a shown by x-ray structural analyses to affect the compound 5 binding site (Fig 3f). At concentrations of compound 5 that rapidly lead to a greater than 3 log decline in replicon RNA levels the health of the cell measured as measured by an Alamar blue assay was unaffected.

Compound Synthesis and characterization
Compounds 3-6 were prepared as follows:

Compound 3 - 2,4-difluoro-3-phenoxy-benzylamine hydrochloride.

48% HBr (10 mL) was added to 2,4 difluoro-3-methoxybenzylamine (1 g, 5.78 mmol) and heated to 145 °C for 1 hour, mixture concentrated and triturated with ethyl acetate to afford 3-aminomethyl-2,6-difluoro-phenol hydrobromide (12 g). ^1H NMR (400 MHz, DMSO-d6): 10.39 (1H, s), 8.17 (3H, s), 7.12 (1H, t), 6.97 (1H, q), 4.03 (2H, s). MS: [M+H]^+ 160

A solution of di-tert-butylicarbonate (10.91 g, 0.05 mol) in tetrahydrofuran (60 mL) was added dropwise over 1 hour to an ice cold mixture of 3-aminomethyl-2,6-difluoro-phenol hydrobromide (12 g, 0.05 mol), tetrahydrofuran (60 mL), water (120 mL) and 6M sodium hydroxide (21 mL, 0.125 mol). The mixture was warmed to RT, acidified with 5% citric acid (240 mL) and extracted with ethyl acetate (2 x 120 mL). The combined organic phase was washed with sat. brine (120 mL), dried over magnesium sulfate, filtered and concentrated. The residue was triturated with petrol, filtered and dried to give (2,4-difluoro-3-hydroxy-benzyl)carbamic acid tert-butyl ester (13.9 g).

^1H NMR (400 MHz, CDCl3): 6.93-6.76 (2H, m), 4.89 (1H, bs), 4.34 (2H, s), 1.47 (9H, s).

A mixture of (2,4-difluoro-3-hydroxy-benzyl)carbamic acid tert-butyl ester (1 g; 3.8 mmol), 2-trimethylsilylphenyltrifluoromethane sulfonate (1.38 g; 1.2 equivalents) and cesium fluoride (1.76 g; 3 equivalents) in acetonitrile (25 mL) was stirred at room temperature for 6 hours. The reaction mixture was diluted with ethyl acetate (25 mL), washed with brine then dried over sodium sulfate, filtered and evaporated. The crude material was purified by flash column chromatography using gradient elution from 0 to 25% ethyl acetate in 40 / 60 petroleum ether. Product containing fractions were combined and evaporated to give 625 mg of (2,4-difluoro-3-phenoxy-benzyl)carbamic acid tert-butyl ester as a pale yellow solid.

^1H NMR (400 MHz, DMSO-d6): 7.49-7.39 (1H, m), 7.39-7.33 (2H, m), 7.33-7.19 (2H, m), 7.11 (1H, t), 6.95 (2H, d), 4.17 (2H, d), 1.50-1.15 (9H, m).

(2,4-difluoro-3-phenoxy-benzyl)carbamic acid tert-butyl ester (625 mg) was dissolved in 4M HCl in 1,4-dioxiane then stirred at room temperature overnight. The reaction mixture was evaporated and the residue triturated with diethyl ether (10 mL). The resulting solid was collected by filtration, washed with diethyl ether (5 mL) and dried under vacuum to
give 480mg of the hydrochloride salt of compound 3 as a white solid. \(^1\)H NMR (400 MHz, DMSO-d6): 8.68 (3H, s), 7.68-7.56 (1H, m), 7.46-7.32 (3H, m), 7.13 (1H, t), 6.99 (2H, d), 4.08 (2H, s). \(^1^3\)CNMR (100MHz, DMSO-d6): 130.47 (2CH), 125.64(CH), 123.57 (CH), 115.21 (CH), 112.65 (2CH), 37.47 (2CH\(_2\)). \(^1^9\)FNMR (376 MHz, DMSO-d6): -129.7 (1F), -132.7 (1F). HRMS: calculated m/z = 236.088146; measured m/z = 236.087496.

**Compound 4 - (R)-1-(4-chloro-2-fluoro-3-phenoxy-phenyl)-propylamine.hydrochloride.**

6-Chloro-2-fluoro-3-methylphenol (35 g, 0.218 mol), cesium fluoride (100 g, 0.654 mol) and acetonitrile (350 mL) were combined, stirring at room temperature under nitrogen. 2-(Trimethylsilyl)phenyl triflate (65 g, 0.218 mol) in acetonitrile (100 mL) was added over 20 minutes, followed by acetonitrile (250 mL). The resulting mixture was stirred at room temperature overnight. The reaction was quenched with 10% aqueous potassium hydroxide (350 mL) and extracted with petrol (7 x 700 mL). The combined organics were dried (magnesium sulfate) and concentrated in vacuo at 40 °C to give 1-chloro-3-fluoro-4-methyl-2-phenoxybenzene (44.5 g, 0.188 mol). \(^1\)H NMR (400 MHz, CDCl\(_3\)): 7.36-7.26 (2H, m), 7.18 (1H, dd), 7.13-6.97 (2H, m), 6.92 (2H, d), 2.31 (3H, d).

1-Chloro-3-fluoro-4-methyl-2-phenoxybenzene (44.5 g, 0.188 mol), N-bromosuccinimide (100.4 g, 0.564 mol), azobisisobutyronitrile (2.2 g, 0.013 mol) and carbon tetrachloride (445 mL) were stirred under nitrogen and heated to 80 °C overnight.
Further N-bromosuccinimide (20 g, 0.112 mol) and azobisisobutyronitrile (2.2 g, 0.013 mol) were added. Heating was continued for a further 6 hours, when the reaction was complete by $^1$H NMR. Heating was removed and the reaction mixture was cooled to room temperature. Water (440 mL) was added and the phases were separated. The aqueous was extracted with dichloromethane (2 x 220 mL) and the combined organics were dried (magnesium sulfate) and concentrated in vacuo at 40 °C to give 1-chloro-4-dibromomethyl-3-fluoro-2-phenoxybenzene (98.3 g). The material was used directly without purification. $^1$H NMR (400 MHz, CDCl$_3$): 7.68 (1H, dd), 7.43-7.31 (3H, m), 7.11 (1H, t), 6.92 (2H, d), 6.88 (1H, s).

1-Chloro-4-dibromomethyl-3-fluoro-2-phenoxybenzene (98.3 g), isopropanol (740 mL), silver nitrate (64 g, 0.376 mol) and water (150 mL) were combined. The resulting mixture was stirred for 2 hours and then filtered. The filtrate was concentrated in vacuo at 40 °C and water (375 mL) was added to the residue. The mixture was extracted with dichloromethane (2 x 270 mL) and the combined organics were dried (magnesium sulfate) and concentrated in vacuo at 40 °C. The residue was chromatographed on a silica pad, eluting with a gradient of 0-5% ethyl acetate / petrol to give 4-chloro-2-fluoro-3-phenoxybenzaldehyde (31 g, 0.123 mol). $^1$H NMR (400 MHz, CDCl$_3$): 10.32 (1H, s), 7.73 (1H, dd), 7.43 (1H, dd), 7.40-7.31 (2H, m), 7.17-7.07 (1H, m), 6.94 (2H, d).

4-Chloro-2-fluoro-3-phenoxybenzaldehyde (37.8 g), (R)-(+)2-methyl-2-propanesulfinamide (19.1 g, 0.158 mol), titanium(IV) ethoxide (68.8 g, 0.301 mol) and dichloromethane (565 mL) were combined. The resulting mixture was stirred overnight under nitrogen. The solution was diluted with dichloromethane (565 mL) and solid sodium sulfate decahydrate (380 g) was added with vigorous stirring for 1 hour. The slurry was filtered and the filtrate was concentrated in vacuo at 40 °C. The residue was chromatographed on a silica pad, eluting with a gradient of 0-20% ethyl acetate / petrol to give (R)-2-methylpropane-2-sulfonic acid 1-(4-chloro-2-fluoro-3-phenoxyphenyl)meth-(E)-ylideneamide (26.8 g, 0.076 mol). $^1$H NMR (400 MHz, DMSO-d$_6$): 8.65 (1H, s), 8.47 (1H, d), 7.95 (1H, dd), 7.66 (1H, dd), 7.43-7.32 (2H, m), 7.31-7.21 (1H, m), 6.98 (2H, d), 1.21 (9H, s).

A solution of ethylmagnesium bromide (50 mL, 0.15 mol) was added over 35 minutes to a solution of (R)-2-methylpropane-2-sulfinic acid 1-(4-chloro-2-fluoro-3-phenoxyphenyl)meth-(E)-ylideneamide (26.5 g) in tetrahydrofuran (530 mL) at -70 °C. After 3 hours stirring at -70 °C, the mixture was quenched with saturated ammonium chloride (270 mL). Water (270 mL) was added and the phases were separated. The aqueous was extracted with ethyl acetate (2 x 270 mL) and the combined organics were washed with saturated brine (270 mL), dried (magnesium sulfate) and concentrated in vacuo at 40 °C. The residue was chromatographed on a silica pad, eluting with a gradient of 20-60% ethyl acetate / petrol to give (R)-2-methylpropane-2-sulfinic acid [(R)-1-(4-chloro-2-fluoro-3-phenoxyphenyl)propyl]amide (11.9 g, 0.031 mol). $^1$H NMR (400 MHz, DMSO-d$_6$): 7.55-7.44 (2H, m), 7.36 (2H, dd), 7.10 (1H, t), 6.88 (2H, d), 5.75 (1H, d), 4.36 (1H, q), 1.93-1.80 (1H, m), 1.75-1.63 (1H, m), 1.11 (9H, s), 0.86 (3H, t).

4M Hydrogen chloride in 1,4-dioxane (24 mL) was added to a solution of (R)-2-methylpropane-2-sulfinic acid [(R)-1-(4-chloro-2-fluoro-3-phenoxyphenyl)propyl]amide (11.9 g, 0.031 mol) in methanol (120 mL). After stirring for 1 hour, the solution was concentrated in vacuo at 40 °C. The residue was slurried in 3:1 petrol / ether (120 mL), filtered and dried in vacuo at 40 °C to give the hydrochloride salt of compound 4 (9.3 g).
as a white solid. \(^1\)H NMR (400 MHz, DMSO-d6): 8.58 (2H, s), 7.70-7.55 (2H, m), 7.38 (2H, dd), 7.12 (1H, t), 6.91 (2H, d), 4.41 (1H, s), 2.07-1.95 (1H, m), 1.92-1.79 (1H, m), 0.82 (3H, t). [M+H-NH3]\(^+\) = 263. HRMS: calculated m/z = 280.089895; measured m/z = 280.089363.

**Compound 5** - {((S)-3-[{(R)-1-(4-chloro-2-fluoro-3-phenoxy-phenyl)-propylamino]-butyramide)}}.hydrochloride.

\[
\begin{array}{c}
\text{H}_2\text{N} \\
\text{O} \\
\text{Cl} \\
\text{H} \\
\text{F}
\end{array} \xrightarrow{} 
\begin{array}{c}
\text{H}_3\text{N} \\
\text{O} \\
\text{Cl} \\
\text{F}
\end{array} 
\]

Triethylamine (0.04 mL, 0.25 mmol) was added to a mixture of (R)-1-(4-chloro-2-fluoro-3-phenoxy-phenyl)propylamine hydrochloride (80 mg, 0.25 mmol) and acetoacetamide (26 mg, 0.25 mmol) in DCE (3 mL), followed by glacial acetic acid (0.04 mL, 0.5 mmol) and sodium triacetoxyborohydride (164 mg, 0.5 mmol). The resulting mixture was stirred at room temperature for 24 hours, poured into saturated sodium hydrogen carbonate and extracted into DCM. The organic fraction was dried over sodium sulfate, filtered and concentrated. The diastereomers were separated by column chromatography. Elution with 0-10% methanol in DCM afforded the (R,R) isomer which was subsequently converted to the hydrochloride salt (35 mg). Further elution provided the (S,R) isomer compound 5 which was subsequently converted to the hydrochloride salt (3 mg). \(^1\)HNMR (400 MHz, Me-d3-OD): 7.62-7.44 (2H, m), 7.40-7.29 (2H, m), 7.11 (1H, t), 6.90 (2H, d), 4.65 (1H, dd), 3.54-3.39 (1H, m), 2.71-2.53 (2H, m), 2.27-2.01 (2H, m), 1.37 (3H, d), 0.91 (3H, t). \(^{13}\)CNMR (100MHz, DMSO): 130.55 (2CH), 127.17(CH), 126.72 (CH), 123.56 (CH), 115.02 (2CH), 53.6 (CH), 49.5 (CH), 38.8 (CH\(_2\)), 36.91 (CH\(_2\)), 16.0 (CH\(_3\)), 10.3 (CH\(_3\)) \(^{19}\)FNMR (376 MHz, DMSO-d6): -129.3 (1F). MS: [M+H]\(^+\) = 365. HRMS: calculated m/z = 365.142659; measured m/z = 365.141428.

**Compound 6** – (S)-N-(2-amino-ethyl)-3-[{(R)-1-(4-chloro-2-fluoro-3-phenoxy-phenyl)-propylamino]-butyramide} dihydrochloride.
A solution of \((R)-1-(4\text{-chloro}-2\text{-fluoro}-3\text{-phenoxy}-\text{phenyl})\)propylamine (350 mg, 1.25 mmol) and methyl crotonate (0.13 mL, 1.25 mmol) in methanol (3 mL) was heated to 80 °C for 2x2 hours under microwave irradiation. Methyl crotonate (0.13 mL, 1.25 mmol) was added and the reaction further heated to 130 °C for 3 hours under microwave irradiation, before being concentrated. The residue was purified by column chromatography, eluting with 30-40% ethyl acetate in petrol to give the product (245 mg) as a mixture of diastereomers.  

\[\text{Me Crotonate, MeOH, MW} \quad \text{LiOH, MeOH} \]

1. BocNHCH$_2$CH$_2$NH$_2$, HATU, HOAt, DMF
2. HCl, Dioxane

A solution of \((R)-1-(4\text{-chloro}-2\text{-fluoro}-3\text{-phenoxy}-\text{phenyl})\)propylaminobutyric acid methyl ester (743 mg, 1.96 mmol) and lithium hydroxide (2.74 mL of a 1M aqueous solution, 2.74 mmol) in methanol (10 mL) was stirred at room temperature overnight, then concentrated. A 100 mg (0.27 mmol) portion of the residue was dissolved in DMF (2 mL) then treated with tert-butyl N-(2-aminoethyl)carbamate (52 mg, 0.32 mmol) followed by 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (122 mg, 0.32 mmol). The reaction mixture was stirred for 1 hour at room temperature before more 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (122 mg, 0.32 mmol) was added and the reaction stirred 1 hour further. The mixture was concentrated, and then partitioned between water and chloroform. The organic fractions were dried over sodium sulfate, filtered and concentrated. The crude product was dissolved in 1,4-dioxane (2 mL) and HCl (5 mL of a 4M solution in 1,4-dioxane) was added. The resulting solution was stirred for 1.5 hours, and then concentrated. The residue purified by preparative HPLC and subsequent HCl salt formation afforded the \((R,R)\) isomer (26 mg) as a white solid. Further elution and subsequent HCl salt formation afforded the \((S,R)\) isomer compound 6 (24 mg) also as a white solid.  

\[\text{1H NMR (400 MHz, Me-d3-OD): 7.57 (2H, d), 7.35 (2H, dd), 7.11 (1H, t), 6.90 (2H, d), 4.67 (1H, dd), 3.55-3.42 (3H, m), 3.07 (2H, t), 2.80-2.61 (2H, m), 2.31-2.05 (2H, m), 1.45-1.35 (3H, m), 0.91 (3H, t).} \]

\[\text{13C NMR (100MHz, DMSO): 130.55 (2CH), 127.25 (CH), 126.82 (CH), 123.57 (CH), 115.03 (2CH), 53.29 (CH), 49.28 (CH), 38.98 (2CH2), 36.91 (CH2), 26.25 (CH2), 15.9 (CH3), 10.3 (CH3).} \]

\[\text{19F NMR (376 MHz, DMSO-d6): -129.5 (1F).} \]

\[\text{MS: [M+H]$^+$ 408. HRMS: calculated m/z = 408.184857; measured m/z = 408.184873.} \]
Compound 7 – {((R)-3-[(S)-1-(4-chloro-2-fluoro-3-phenoxy-phenyl)-propylamino]-butyramide)}.hydrochloride.

Compound 7 was prepared in an analogous manner to compound 5 but starting from (S)-1-(4-chloro-2-fluoro-3-phenoxy-phenyl)propylamine hydrochloride.

1H NMR (400 MHz, Me-d3-OD): 7.57 (1H, d), 7.52-7.43 (1H, t), 7.35 (2H, t), 7.11 (1H, t), 6.90 (2H, d), 4.64 (1H, m), 3.44 (1H, m), 2.68-2.53 (2H, m), 2.22-2.04 (2H, m), 1.37 (3H, d), 0.92 (3H, t). MS: [M+H]$^+$ 365.

**Supplementary Results**
**Supplementary table 1.** Genotypic conservation of the allosteric site

Sequence analysis of the allosteric site across the consensus sequences of the most prevalent genotypes. A high degree of sequence conservation (75-96%) is observed, suggesting the likelihood of the functional relevance of the site.

| Genotypes | 1a | 1b | 1c | 2a | 2b | 3a | 3b | 4a | 5a | 6a | 6b |
|-----------|----|----|----|----|----|----|----|----|----|----|----|
| A | A | A | A | A | A | A | A | A | A | A | A |
| R | R123 | T | T | R | R | R | R | R | R | R | R |
| R | R156 | R | R | R | R | I | R | R | R | R | R |
| N | N | S | G | N | N | N | N | N | N | N | N |
| D | D | D | D | D | D | D | D | D | D | D | D |
| D | D | D | D | D | D | D | D | D | D | D | D |
| D | D | D | D | D | D | D | D | D | D | D | D |
| D | D | D | D | D | D | D | D | D | D | D | D |
| C | C | C | C | C | C | C | C | C | C | C | C |
| O | O | Q | Q | Q | Q | Q | Q | Q | Q | Q | Q |
| O | O | G | G | Q | Q | Q | K | Q | Q | Q | Q |
| E | E | E | E | E | E | E | E | E | E | E | E |
| G | G | G | G | G | G | G | G | G | G | G | G |
| G | G | G | G | G | G | G | G | G | G | G | G |
| H | H | H | H | H | H | H | H | H | H | H | H |
| L | L | L | M | L | L | F | L | M | L | L | L |
| L | L | L | L | L | L | L | L | L | L | L | L |
| L | L | L | L | L | L | L | L | L | L | L | L |
| M | M | M | M | M | M | M | M | M | M | M | M |
| F | F | F | F | F | F | F | F | F | F | F | F |
| P | P | P | P | P | P | P | P | P | P | P | P |
| S | S | S | S | S | S | S | S | S | S | S | S |
| Y | Y | Y | Y | Y | Y | Y | Y | F | Y | Y | Y |
| I | I | I | I | I | I | V | I | I | I | I | I |
| V | V | V | V | V | V | V | V | V | V | V | V |
| V | V | V | V | V | V | V | V | V | V | V | V |
| V | V | V | V | V | V | V | V | V | V | V | V |
| V | V | V | V | V | V | V | V | V | V | V | V |
| Y | V830 | V | M | M | T | T | Y | I | I | I | I |
| Y | V78 | V | A | A | V | V | V | V | V | V | V |

| Conservation (%) | 93 | 100 | 100 | 75 | 75 | 86 | 93 |
|------------------|----|-----|-----|----|----|----|----|
| 75               | 93 | 100 | 100 | 75 | 75 | 86 | 93 |
| 86               | 86 | 86 | 86 | 86 | 86 | 86 | 86 |
| 93               | 93 | 93 | 93 | 93 | 93 | 93 | 93 |
The distance each residue is from the binding footprint of compound 5 has been categorized as follows: Below 5 Å – 16 residues (57%); 5-7 Å - 9 residues (32%); 7-9 Å – 3 residues (11%). A small number of more distant residues have been included as they either interact directly with a residue crucial for binding or they interact through a network of conserved water molecules.

Amino acid residues are colour coded dependent on the side-chain properties: Red – acidic; green – basic; blue – polar; orange – aromatic; pale yellow – lipophilic; yellow – cysteine; mauve – glycine or proline.

| Cmpd | GT1a [μM] | GT1b [μM] | GT2a [μM] | GT3a [μM] | GT4 [μM] | GT5 [μM] | GT6 [μM] |
|------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 6    | 48% @0.01 | 66% @0.01 | 44% @100  | 60% @0.03 | 50% @0.1  | 46% @0.01 | 65% @0.03 |

**Supplementary table 2:** *In vitro* protease inhibition of compound 6 across genotypes

| Compound | 1 WT | 2 WT | 3 WT | 4 WT | 5 WT | 6 WT | V630L |
|----------|------|------|------|------|------|------|-------|
| **Data collection** | | | | | | | |
| Beamline | ESRF ID23-1 | ESRF ID23-1 | ESRF ID29 | DLS I03 | ESRF ID23-1 | DLS I03 | DLS I03 |
| Wavelength | 0.9762 | 1.0 | 1.0726 | 0.9763 | 0.979 | 0.9763 | 0.9795 |
| Space group | P212121 | P212121 | P212121 | P212121 | P212121 | P212121 | P212121 |
| α, β, γ (°) | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 |
| Resolution (Å) | 87.6-2.4 (2.46-2.40)* | 87.0-2.40 (2.46-2.40) | 87.0-2.10 (2.15-2.10) | 91.13-2.50 (2.56-2.50) | 141-2.50 (2.56-2.50) | 91.13-2.18 (2.24-2.18) | 91.5-2.50 (2.56-2.50) |
| Rsym or Rmerge | 0.071 (0.371) | 0.079 (0.444) | 0.083 (0.403) | 0.086 (0.706) | 0.066 (0.537) | 0.097 (0.781) | 0.114 (0.843) |
| I / σI | 10.3 (2.7) | 6.6 (2.0) | 5.7 (2.2) | 9.3 (1.8) | 10.6 (1.8) | 13.5 (1.7) | 12.2 (2.4) |
| Completeness (%) | 98.7 (100.0) | 98.4 (99.4) | 99.5 (99.5) | 98.6 (99.6) | 97.8 (94.7) | 99.0 (90.9) | 94.0 (95.9) |
| Redundancy | 3.0 (3.1) | 3.0 (2.8) | 3.1 (3.2) | 3.2 (3.2) | 3.0 (2.7) | 4.8 (4.0) | 5.6 (5.6) |

**Refinement**

| | Compound 1 WT | Compound 2 WT | Compound 3 WT | Compound 4 WT | Compound 5 WT | Compound 6 WT | Compound 5 V630L |
|---|---|---|---|---|---|---|---|
| Resolution (Å) | 87.6-2.4 | 87.0-2.45 | 87.0-2.14 | 87.1-2.5 | 141-2.5 | 91.1-2.2 | 91-2.5 |
| No. reflections | 49185 | 49603 | 75027 | 46983 | 45471 | 68234 | 41403 |
| Rwork / Rfree | 18.7/25.7 | 19.7/26.9 | 19.3/26.1 | 18.6/27.5 | 18.3/26.4 | 18.9/24.5 | 16.7/25.1 |
| No. non H atoms | Protein | 9627 | 9612 | 9612 | 9608 | 9608 | 9608 | 9987 |
| Ligand/ion | 10 | 15 | 2 x 17 | 2 x 19 | 2 x 25 | 2 x 28 | 2 x 25 |
| Water | 346 | 325 | 859 | 470 | 245 | 355 | 377 |
| B-factors | Protein | 40.8 | 42.9 | 33.6 | 47.9 | 52.4 | 40.0 | 43.8 |
| Ligand/ion | 70.4 | 54.9 | 29.0 | 29.7 | 26.1 | 22.1 | 24.5 |
Supplementary table 3: X-ray data collection, refinement and validation statistics

| Compd | $K_d$ (µM) | Δ$G$ (Kcal/mol) | Δ$H$ (Kcal/mol) | -TΔS (Kcal/mol) |
|-------|------------|----------------|----------------|----------------|
| 3     | 29         | -6.2           | -7.9           | 1.7            |
| 5     | 0.06       | -9.9           | -15.7          | 5.8            |
| 6     | 0.022      | -10.4          | -14.6          | 4.2            |
| 7     | 24         | -6.3           | 10.8           | 4.5            |

Supplementary table 4 Thermodynamic parameters for select compounds binding to HCV NS3.

Improvements in compound affinity are largely driven by a more favourable enthalpic contribution. In contrast, the entropic contribution tends to be unfavourable but is not greatly affected by increasing compound affinity. It is tempting to interpret this thermodynamic data as being consistent with our tunnel binding model: compounds bind to and stabilise a closed conformation of HCV NS3 with a concomitant entropic penalty that has to be overcome enthalpically in order to improve compound affinity. Whilst our data is consistent with this model it is not definitive.

Supplementary Table 5 – Cytotoxicity profile of compound 5.

| Compd | Huh7 [µM] | HCT116 [µM] | HepG2 [µM] | MRC5 [µM] |
|-------|------------|-------------|------------|-----------|
| 5     | >30        | >30         | >30        | >30       |

Supplementary Table 6 - In vitro selectivity data for compound 5 over other serine proteases.

| Compd | Trypsin [µM] | Urokinase [µM] |
|-------|--------------|----------------|
| 5     | 11% at 300   | Inactive at 300 |
Supplementary Figures

Supplementary figure 1 Polyprotein processing and western blot analysis
Cell lysates from replicon bearing Huh-7 cells were treated for 24 hours with the stated compounds at 10 x EC_{50} concentrations. Samples were immunoblotted with antibodies specific for (A) HCV NS5b or (B) GAPDH. Untreated cell lysates were used as a control and loaded in the first lane, followed by cell lysates treated with compound 6, telaprevir or GS-7977 respectively. Molecular weight markers are shown as labelled. Panel A shows the amount of processed viral protein present in the cell culture in the presence of our allosteric inhibitor, the active site inhibitor telaprevir or the HCV polymerase inhibitor GS7977. The strongest band on the blot, running below the 76kD molecular weight marker is consistent with the NS5b protein, with a molecular weight of 66kD. The bands at around150kD are consistent with the molecular weight expected for unprocessed NS4a-NS5b protein (~153kD) and NS4b-NS5b (~149kD) can be gleaned in the control sample and increases in intensity in the samples treated with compound 6 and telaprevir, yet is absent in the nucleoside analogue GS7977 samples. The band running above 102kD may be an artefact. The nature of this experiment does not allow us to unambiguously separate the effect of the compounds on the inhibition of viral replication.
from that of polyprotein processing, despite the absence of the bands in the GS7977 sample. Panel B is a guide for the normalisation of sample loading.

**Supplementary Figure 2** Titration Curves of the wildtype (A) and V630L mutant (B) replicons.

Clonal populations of Huh-7 cells bearing replicons with the V630L mutation were selected and identified following selective pressure from compound 5. EC$_{50}$ values were determined in cells carrying WT vs the V630L mutant replicon using the active site protease inhibitor telaprevir as a control. Triplicate wells were analysed using a 10 point dilution series to generate the curves shown. Three independent experiments were run with representative graphs for one such experiment shown here with error bars representing the standard deviation from the means for each point. The graphs show no change within experimental error in the EC$_{50}$ for telaprevir against the WT and V630L mutant replicon. The EC$_{50}$ values for compound 5 show a 30 fold drop in the V630L mutant compared to WT. This result confirms that the cell based antiviral activity of compound 5 is mediated by the compound binding at the newly discovered allosteric site on the HCV NS3/4a protein.
Supplementary Figure 3 Sedimentation Velocity Analysis

Hydrodynamic analysis of the WT protein using sedimentation velocity shows the calculated distribution of species $c(s)$ vs the corrected sedimentation coefficient $s_{20,w}$ of the protein. At high ionic strength (1M NaCl) and in the presence of 10% V:V glycerol (buffer A, black curve) the protein shows a sedimentation coefficient value of $s_{20,w} = 4.98$, consistent with it being present predominantly as a dimer. A very small amount of monomer can be seen at lower $s$ value. This agrees with the SAXS data of Schiering, reporting mostly closed dimer and some monomer under similar conditions. Fixed angle dynamic light scattering (DLS) (Avid Nano W301i) experiment under these conditions resulted in a mean hydrodynamic radius of $R_h = 5.7\text{nm}$ suggestive of a dimer. At the lower ionic strength of 0.15M NaCl and 10% V:V glycerol (buffer B, red curve) a redistribution of species is observed with a predominant species at $s_{20,w} = 3.85$, a value consistent to that of a monomer. The mean hydrodynamic radius by DLS under these conditions was of $R_h = 5.07\text{nm}$. Further lowering of the ionic strength to 0.03M NaCl while keeping the 10% V:V glycerol constant causes the peak to decrease by another ~3% to $s_{20,w} = 3.72$. This further drop in sedimentation coefficient value indicates a higher frictional coefficient for the protein, which indicates that the protein remains a monomer, but that it now populates a more extended conformation. DLS indicated an average hydrodynamid radius of $R_h = 5\text{nm}$, consistent with a more extended conformation. These observations are consistent with the studies reported recently. The NS3/4a protein has been shown to have the highest in vitro catalytic activity under no salt.

\[
s_{20,w} = 3.718 \pm 0.030
\]
\[
s_{20,w} = 3.854 \pm 0.151
\]
\[
s_{20,w} = 4.977 \pm 0.106
\]
conditions, suggesting that the extended conformation is required for function. 
Analysis of protein samples by SDS page incubated under the same conditions for
extended periods of time gave no evidence of proteolytic degradation, thus confirming
that the signals observed were due to different conformations of the HCV NS3/4a
protein.

Supplementary Figure 4 Size Exclusion Chromatography
A. Superposition of the chromatograms of the WT protein using an analytical superdex
200 column (GE Healthcare) in 50mM HEPES, pH7.6, 1M NaCl, 10% glycerol, 1mM
TCEP blue curve, 50mM HEPES, pH7.6, 0.15M NaCl, 10% glycerol, 1mM TCEP, green
curve and 50mM HEPES, pH7.6, 0.15M NaCl, 1mM TCEP red curve. At 1M NaCl the
protein appears as a homogeneous sharp peak of molecular weight consistent with a
monomeric species. At 0.15M NaCl and 10% glycerol, the peak begins to broaden
suggesting more conformational heterogeneity. Removal of the glycerol (red curve)
shows the emergence of a shoulder moving ahead of the main peak, suggesting a
different conformation of the protein. Based on the calibration of the column they faster
moving species is not likely to be a dimer, but rather an extended monomer. No dimers
are observed under high ionic strength conditions (dimers were seen to be predominant
by sedimentation velocity) which suggest that the interactions are weak enough to be
disrupted by the frictional flow present in the column.
B. The chromatograms of the WT protein in 0.15M NaCl and in the absence of glycerol
reproducibly showed the presence of a faster moving shoulder ahead of the main peak as
shown by the blue curve. The red curve shows the effect of the presence of 1uM of compound 5 (10-fold excess over IC$_{50}$) to the elution of the WT protein under otherwise identical conditions. The shoulder is less obvious consistent with a more homogeneous closed conformation of the protein.

**Supplementary Figure 5** Graph of observed thermodynamic trends for 230 compounds binding to HCV NS3/4a

Enthalpic ($\Delta H$) and entropic ($-T\Delta S$) values are plotted against the free energy of binding ($\Delta G$). The graph clearly shows that enthalpy is the major driving force for improving compound potency and that improving potency correlates well with a more favourable enthalpy and a less favourable entropy. Similar thermodynamic trends have been observed for a large and varied number of in-house targets, indicating that such trends are typical of Astex drug design and not necessarily target related.
Supplementary Figure 6 SDS PAGE analysis and proteolytic susceptibility

A. WT and mutant proteins were purified as describe in the materials and methods. Lanes 1 and 6 show Molecular weight markers (Rainbow markers, GE Healthcare); lane 2 is WT, lanes 3 and 4 are two different site directed mutants (T631A and T631C) of the full length NS3/4a not discussed in the text. Lane 5 is empty. The gel was stained with Coomassie blue and is representative of the quality of protein used for crystallisation and characterisation. Mass Spectrometry analysis of protein resulted in a mass of 70663D (expected 70794D) and indicated the loss of the N-terminal methionine, which was confirmed by peptide sequencing. Aggregation was observed upon incubation of the protein at concentrations exceeding 25uM at room temperature for periods of times exceeding 2 or more hours.
B. WT protein at 16.5uM concentration was incubated at room temperature in the presence and absence 12 molar excess of compound 5 for up to 17.5 hours. No evidence of proteolytic degradation was observed in either sample during the duration of the experiments. Identical samples were subjected to proteolysis using sequencing grade trypsin and chymotrypsin at a ratio of 1:60 in both cases. Inhibition of either protease by compound 5 was ruled out by using lysozyme as the substrate in a parallel experiment. The gel shows the samples from the trypsin susceptibility experiment taken at t=0min (lanes 2, 3,4), 10min (lanes 5,6), 20min (lanes 7,8), 45min (lanes 9,10), 90min (lanes 11,12), 180 min (lanes 13,14) and after overnight incubation t=17.5 hours (lanes 15, 17) and run on SDS 4-20% gradient polyacrylamide gels (Novex). Lane 1 are the molecular weight markers, lanes 2 and 17 show the t=0 and t=17.5h samples of the HCV NS3/4a protein alone respectively. No evidence of auto-degradation was observed over the entire sampling period in the NS3/4a samples regardless of whether compound 5 was present or not (lanes 2 and 17). The presence of the allosteric compound 5 in the NS3/4a protein samples delays but does not prevent the fragmentation of the HCV NS3/4a protein by either trypsin or chymotrypsin. This result is consistent with the model in which compound 5 binds at the interface between the protease and helicase domains and stabilises the closed, more compact conformation of the protein, thus protecting it from proteolytic degradation.
**Supplementary Figure 7** Isothermal titration calorimetry

WT protein was dialysed overnight at 4°C against 50mM HEPES, 300mM NaCl, 10% glycerol and 1mM TCEP at pH 7.4. Final protein concentration ranged between 7-20μM. Compound 5 was prepared in 100% DMSO, with a final experimental DMSO concentration between 1 and 3%. The figure shows a typical curve in which compound 5 was titrated into the sample cell containing the WT protein. The compound bound to the NS3/4a protein in 1:1 stoichiometry (N=0.9) with an affinity of 60nM and confirms that the protein in solution can adopt the closed conformation observed in the crystal structure.
### Compound 5

![Chemical structure of Compound 5](image)

| Category                        | Parameter             | Description                                                                 |
|---------------------------------|-----------------------|----------------------------------------------------------------------------|
| **Compound**                    | Additional names      | (S)-3-[(R)-1-(4-chloro-2-fluoro-3-phenoxy-phenyl)-propylamino]-butyramide    |
|                                 | Citation              | Synthesis procedures are available in the supplementary materials.           |
|                                 | Chemical descriptors  | Compounds 5 and 6 can be provided for use in academic laboratories, contact andrew.woodhead@astx.com. |
|                                 | Chemical compound page|                                                                             |
|                                 | Entries in chemical databases |                                                                             |
| **Availability**                |                       |                                                                             |
| **Additional comments**         |                       | None                                                                       |
| **In vitro profiling**          | **Target**            | The hepatitis C virus full length NS3 protein                               |
|                                 | **Potency**           | \(K_d = 62 \text{ nM (as measured by ITC); IC}_{50} = 100 \text{ nM (as measured in the fluorescence quench protease assay)}\) against full length NS3 protein. Inactive against the NS3 protease domain alone. See table 1. |
|                                 | **Mechanism of Inhibition** | Reversible allosteric inhibitor of NS3 function |
|                                 | **Selectivity**       | No activity against trypsin or urokinase, see supplementary table 6.        |
|                                 | **Potential reactivity** | None anticipated                                                                 |
|                                 | **SAR**               | During fragment screening, small molecules were identified binding in a relatively hydrophobic binding site, with residues M485, V524, C525, Q526 and V630 from the helicase domain and H57, V78, D79, D81 and R155 from the protease domain making key van der Waals contacts. Compound 5 was designed to bind more efficiently in this... |
site by improving vdW contacts, stabilising the bound conformation and picking up direct or water mediated hydrogen bonding interactions with residues L517, C525, D527 and E628. See table 1. PDB code to be inserted

| Structure of protein-ligand complex | None |
|-------------------------------------|------|

| Cellular profiling | Validation of cellular target | Dose dependent inhibition of viral replication in the cell based genotype 1b sub-genomic replicon $EC_{50} = 0.4 \, \mu M$; $CC_{50} > 10 \, \mu M$. See table 1. The emergence of antiviral resistance was demonstrated in the replicon assay. Mutants were localised to the allosteric binding site. No cytotoxicity was observed in Huh7, HCT116, MRC5, HepG2 cell lines up to 30 \, \mu M (highest concentration tested), see supplementary table 5. |
|-------------------------------------|------|
| Validation of cellular specificity | Additional comments | None |