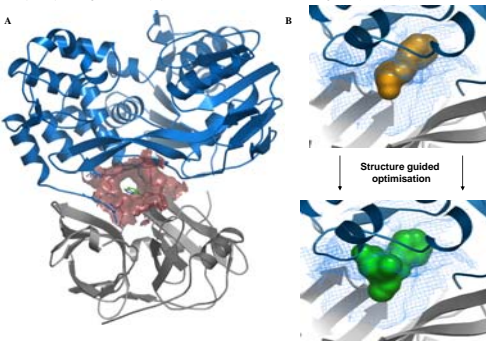


## BACKGROUND

- The Hepatitis C virus NS3 is a multi-functional protein containing a serine protease at the N-terminus and a helicase / nucleoside triphosphatase at the C-terminus. These functions are indispensable for viral replication.
- The crystal structure of the full length NS3 (1.2) shows that the protein adopts a compact conformation, with the protease active site situated at the interface between the two functional domains and occupied by the helicase (Figure 1).
- The closed conformation captured in the crystal structure is described in the literature as 'a snapshot' of the product of the cis-cleavage performed by the protease. The protein must be able to adopt an open conformation to allow binding of subsequent substrates to the protease active site. Recent data show that an extended conformation is also essential for helicase function (3).
- X-ray crystallographic screening of the full length NS3/4a protein lead to the discovery of a novel allosteric binding site (Figure 1). (For more detail please see Poster 352).
- The novel site is distinct from the active site and is located at the interface between the protease and helicase domains of the NS3/4a protein.
- Compounds binding at the novel site inhibit the catalytic function of the protein via the stabilisation of an auto-inhibited form (Model 1).
- Here we report the use of the cell based sub-genomic replicon system to demonstrate that compounds binding at this novel site inhibit viral replication and represent a new therapeutic approach for the treatment of HCV infection.

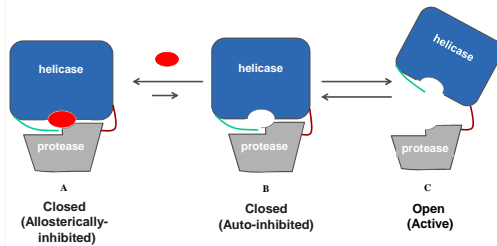
## METHODS

Huh-7 cells persistently infected with a subgenomic genotype 1b HCV-RNA construct containing a firefly Luciferase reporter gene (pFK389Luc-ubi-neo/NS3-3'ET) (Bartenschlager), were used to determine the cell based antiviral activity of compounds. HCV replication was quantified by luciferase activity as an indirect readout of HCV RNA load. Serial dilutions of the compounds were added to the cells and incubated for 72 hours for  $EC_{50}$  and  $CC_{50}$  determinations. Cytotoxicity was measured with Alamar Blue. Luciferase readouts were confirmed by quantitative RT-PCR using an NS5B primer. Resistant mutations against compound 1 were elicited by culturing cells in the presence of high concentrations of compound for an extended period of time. RNA from emerging colonies was isolated and sequenced. Identified mutations were incorporated into the wild-type enzyme by site directed mutagenesis for confirmation. *In vitro* affinities of the compounds were measured directly by isothermal titration calorimetry (ITC) and in a fluorescence-based (QXL-520) protease activity assay utilising a substrate peptide derived from the NS4A/B cleavage site (Anaspec).



**Figure 1:** X-ray crystal structures of the NS3/4a protein highlighting the novel allosteric site and bound inhibitors

A. Ribbon diagram of the full length NS3/4a protein. The protease domain is coloured in grey, helicase in blue. The novel allosteric site is represented as a red surface, with a bound fragment hit shown in green.  
B. Magnified view of the crystal structures of ligand bound complexes with a fragment hit (top) and optimized compound 1 (bottom) shown as surface representations.



## Model 1: Proposed mode of action of allosteric inhibitors

Colour key : red oval - allosteric inhibitor; green line - C-terminus of the helicase domain; brown line - flexible linker between the protease and helicase domains.

- Closed conformation - Allosteric inhibition can be achieved by a small molecule binding at the protease-helicase interface.
- Closed conformation - product of cis-cleavage (NS3 / NS4a), with the C-terminus occupying the protease active site.
- Open conformation - required for proteolytic activity and also reportedly required for helicase activity. This is the conformation which is inhibited by the peptidomimetic active site inhibitors such as Telaprevir and Boceprevir.

## RESULTS

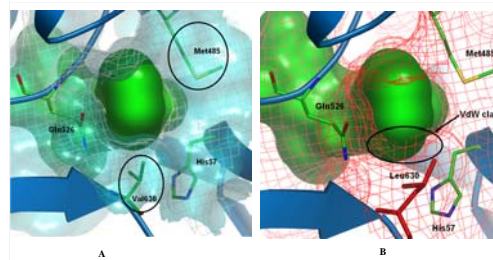
- The physiological relevance of the site was tested using tool compounds with sufficient *in vitro* affinity for the full length NS3/4a protein.
- Their ability to inhibit viral replication in the GT1b cell based sub-genomic replicon system supported the model, but did not rule out alternative modes of action.
- Enantiomers (compounds 1 and 2) with different *in vitro* potencies against the full length enzyme demonstrated that only the compound with the correct stereochemistry resulted in an antiviral effect in the cell based replicon system (Table 1).

Cpd	$K_d$ ITC ( $\mu$ M)	FL $IC_{50}$ ( $\mu$ M)	PD $IC_{50}$ ( $\mu$ M)	$EC_{50}$ ( $\mu$ M)	$CC_{50}$ ( $\mu$ M)
1	0.062	0.11	Inactive	0.33	>10
2	24	59% @ 30	ND	>10	>10

**Table 1:** Comparison of the activity of stereoisomers

$K_d$  is the dissociation constant of the compound to the full length protein as measured by Isothermal Titration Calorimetry (ITC).  $IC_{50}$  reflect the inhibition of protease activity of the full length (FL) and protease domain (PD) proteins respectively. Assay conditions for FL and PD are optimised for maximum signal and differ from each other.

- Further evidence that the observed inhibition by the compounds was mediated via binding at the novel allosteric site was achieved by applying selective pressure on the sub-genomic replicon with compound 1 and allowing resistance mutations to emerge.
- The experiment was repeated using compound 1 at increasing concentrations (10-30  $\times$   $EC_{50}$ ) for 39 days and following slightly different protocols in an effort to ensure that any identified mutations were reproducible and representative.
- The sequence of the generated resistant replicons mapped to mutations at positions M485V/T and V630L/F individually, both on the helicase domain.



## Figure 2: X-ray crystal structures highlighting the replicon resistance mutations

A. Magnified view of the wild type allosteric binding site. The surface representation of compound 1 is shown in green. Key amino acid residue have been labelled. The two residues observed to undergo mutation due to selective pressure from compound 1 are circled in black.  
B. X-ray crystal structure of the V630L mutant. Leu630 is highlighted in red. The larger side-chain in the mutant protein causes a Van der Waals clash with the ligand.

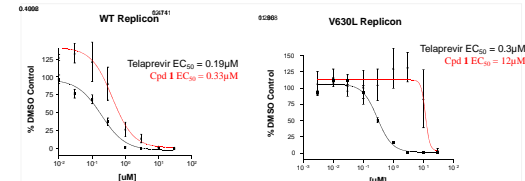
- The point mutations M485V and V630L were introduced independently into the full length protein construct and crystal structures solved to high resolution (Figure 2).
- The effect of the mutations on the  $IC_{50}$  of compound 1 were determined resulting in a 40-50 fold drop. A significantly reduced effect was observed for more potent optimised compounds such as 3 and as anticipated, no effect was observed for the active site inhibitor Telaprevir (Table 2).
- The observed reduction in activity for compound 1 is due to the binding pocket being partially occluded by the larger residue.

	1	3	4	Telaprevir
FL $IC_{50}$ $\mu$ M	0.11	66% @ 0.01	0.057	0.23
FL $K_d$ $\mu$ M	0.064	0.022	0.015	N/A
PD $IC_{50}$ $\mu$ M	Inactive	Inactive	N/A	0.07
M485V $IC_{50}$ $\mu$ M	2.3	46% @ 0.01	N/A	0.18
V630L $IC_{50}$ $\mu$ M	44% @ 3	71% @ 0.03	N/A	1.4
$EC_{50}$ $\mu$ M	0.33	0.008	0.006	0.3
$CC_{50}$ $\mu$ M	>10	>3	>10	>10
MW (Da)	~360	~410	~370	680
clog P	4.3	4.3	3.9	5.4

**Table 2:**  $IC_{50}$  values of compound 1 and representative later analogues against full length WT, mutant and protease domain (PD) only enzymes

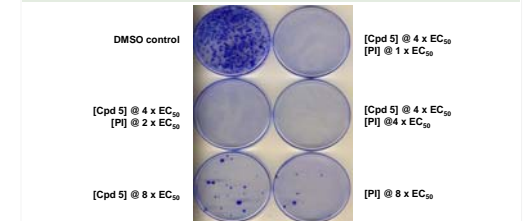
The compound used to raise mutants (1) shows a ~40X drop in potency when tested *in vitro* in the protease assay against the mutant proteins. Later, more potent compounds from alternative series (3,4), show no drop in affinity. The mutations show no effect on Telaprevir.

- The single V630L mutation was introduced back into the replicon, and the effect of the mutation on the  $EC_{50}$  of compound 1 and active site protease inhibitors were measured.
- As expected, the  $EC_{50}$ 's for compound 1 increased by ~40X, whereas for the active site inhibitor Telaprevir the activity remained unaffected (Figure 3).



**Figure 3:** Replicon titration curves for WT and V630L mutant

- Administration of an allosteric inhibitor in combination with representative examples of clinical development compounds in the replicon colony forming assay shows a marked decrease in the number of emerging resistance mutants (Figure 4).



**Figure 4:** Combination study of allosteric inhibitor with a PI in clinical development

Cpd 5 - representative allosteric inhibitor. PI - clinical protease inhibitor

## CONCLUSIONS

- Compounds that bind at the allosteric site are active in the Genotype 1b sub-genomic replicon system
- Mutants raised in the replicon system by selective pressure from an early compound map to the site and validate the approach
- NS3 allosteric inhibitors administered in combination with other classes of DAA's suppress the emergence of drug resistance mutations in replicon colony forming assays
- NS3 allosteric inhibitors represent a potential new therapeutic approach for the treatment of HCV infection

## References

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- Appleby, T.C., et al. 2011. Visualizing ATP-dependent RNA translocation by the NS3 helicase from HCV. J Mol Biol. 405(5):1139-1153.
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