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BRIEF COMMUNICATION

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Selective irreversible inhibition of a protease by targeting a noncatalytic cysteine

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Designing selective inhibitors of proteases has proven problematic, in part because pharmacophores that confer potency exploit the conserved catalytic apparatus. We developed a fundamentally different approach by designing irreversible inhibitors that target noncatalytic cysteines that are structurally unique to a target in a protein family. We have successfully applied this approach to the important therapeutic target HCV protease, which has broad implications for the design of other selective protease inhibitors.

The fundamental challenge in designing protease inhibitors is to achieve potency without sacrificing selectivity. This problem arises frequently because the typical protease inhibitor achieves potency through covalent interactions with the catalytic apparatus, yet such pharmacophores also confer affinity for other proteases in the same mechanistic family[1]. This is a significant challenge for protease drug design, because over 500 proteases exist in the human genome. Achieving selectivity while targeting the catalytic machinery is thus particularly difficult[2].

Covalent irreversible drugs that form persistent, nonlabile covalent bonds yield unique therapeutic benefits including rapid onset of inhibition, greater potency, longer duration of drug action and potent and persistent activity against mutations that would otherwise lead to drug resistance[3]. There are many examples of drugs that work through irreversible covalent bonding that have proven to be safe and successful therapies for a wide variety of indications[4]. Despite their prevalence, to date covalent drugs have largely been discovered serendipitously, and general methods to facilitate their deliberate discovery and design have yet to be described.

HCV NS3/4A viral protease (HCVP) activity is essential for viral replication[5] and has been recently validated as a clinical target[6–15]. Protease inhibitors such as telaprevir exemplify the challenges of covalent targeting of the catalytic binding site; upon binding to HCVP, the α-ketoamide forms a reversible covalent linkage with the catalytic serine that is conserved within proteases[6,10]. Indeed, telaprevir inhibits some host serine proteases at concentrations that may be achieved in a therapeutic setting[14,16].

The aim of this study was to achieve potent inhibition of viral proteases through covalent bond formation without compromising selectivity of the inhibitors. Our new design strategy used structural bioinformatics to create a structural alignment between viral proteases and host proteases to identify nucleophilic amino acids in the binding site that were unique to the viral proteases. Our structural alignment revealed that current covalent inhibitors such as telaprevir target a catalytic residue that is common across the protease family and therefore susceptible to selectivity issues. In contrast, we identified a nucleophilic amino acid, cysteine 159 (Cys159), in the substrate-binding site that could be targeted for covalent bonding. Importantly, Cys159 is strictly conserved across all 919 HCV NS3 sequences in a database of all known HCVP sequences, including all HCVP subtypes and genotypes sequenced to date (**Supplementary Fig. 1**), allowing design of a pan-genotype HCVP inhibitor. Although HCVP shows structural similarity with host proteases, Cys159 is structurally unique to HCVP and therefore was an ideal target for achieving selectivity between HCVP and host proteases.

Structure-based drug design was used to create a peptidomimetic inhibitor (**1**) (**Fig. 1a**), designed to form canonical reversible interactions with the S2-S1-S1′ pockets of HCVP similar to those observed with other reversible peptidomimetic inhibitors[12,17,18]. Further molecular modeling was used to evaluate structures that positioned a low-reactivity Michael acceptor close enough to Cys159 to form a covalent bond (**2**) (**Fig. 1a**). **2** was prepared by installation of an acrylamide using a simple glycine linker. Linking

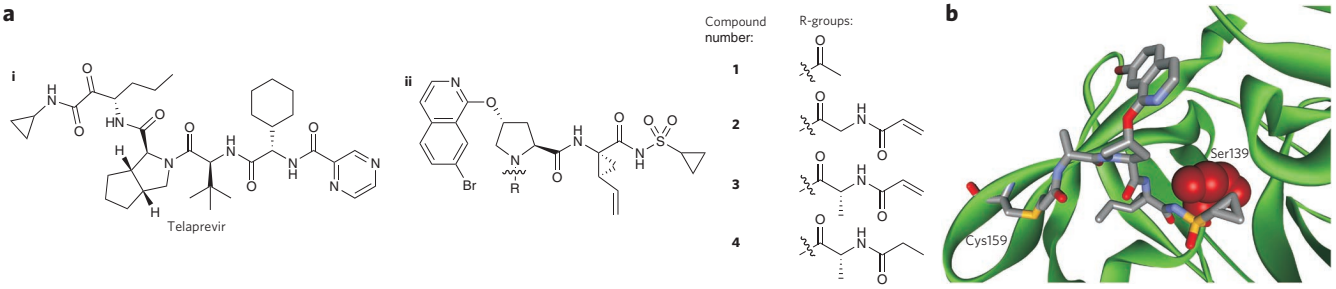

Figure 1 | Compounds rationally designed to covalently bond to a conserved HCVP cysteine. (**a**) Two-dimensional chemical structures of (i) telaprevir and (ii) targeted designed irreversible covalent inhibitors. (**b**) The structure of **3** bound to HCVP as determined using X-ray crystallography. The 2.8 Å complex shows **3** is covalently linked to the side chain of Cys159, which is clearly confirmed by the electron density. The catalytic Ser139 is shown as red spheres.

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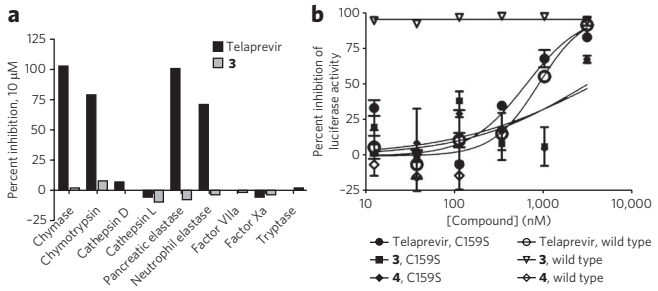

Figure 2 | Covalent NS3/4A inhibitor selectively modifies the target cysteine and gains potency through covalent modification. (**a**) Biochemical enzyme activity was assessed in the presence of 10 μM of **3** or telaprevir (MDS Pharma Services). **3** showed no inhibition of the human proteases tested, whereas telaprevir showed strong inhibition of several human proteases. (**b**) Wild-type Huh-7 cells or Huh-7 cells containing a C159S mutant replicon were grown in DMEM at 37 °C and incubated with telaprevir, **3** or **4**. After 72 h of compound incubation, luciferase activity was assessed. Luciferase activity was greatly reduced when **3** was incubated with wild-type replicon cells but was not inhibited when **3** was incubated with C159S replicon cells, indicating specificity of interaction with the target cysteine in a replicon setting. **4** did not inhibit luciferase activity either in wild-type replicon or C159S replicon, demonstrating that the covalent modification capability greatly enhances potency for this class of compounds. As expected, telaprevir is equally potent in wild-type and C159S replicon cells.

the electrophilic acrylamide via a D-alanine linker provides the more conformationally constrained inhibitor **3** (see **Supplementary Methods**). Our expectation was that propanamide **4**, the reversible congener of **3**, would bind weakly to the HCVP, as potent inhibitors reported to date typically possess functionality that forms extensive nonbonding interactions with the S3 and S4 pockets.

As predicted, **1** and **4** showed weak inhibition of the wild-type HCVP (half-maximal inhibitory concentration (IC_{50}) of **1** = 2,458 nM, **4** IC_{50} = 1,147 nM) whereas **2** and **3** were very potent inhibitors (**2** IC_{50} = 4 nM, **3** IC_{50} = 2 nM) (**Supplementary Table 1**). To further support the importance of covalency in conferring activity of **3**, we tested the activity of **3** against a mutant NS3 protein in which the target cysteine is changed to a serine (C159S). The C159S protease is comparable in enzymatic activity to wild-type protease (**Supplementary Fig. 2**); however, mutation of the amino acid required for bond formation results in a sharp decrease in potency of the covalent inhibitor (IC_{50} = 1,782 nM; **Supplementary Table 1**). In further support of the mechanism, **3** shifted the mass of HCVP by 685 Da, consistent with the formation of a covalent complex between **3** and the protease, but was unable to covalently bond to HCVP with the C159S mutation (**Supplementary Fig. 3**). We also confirmed with X-ray crystallography that **3** was covalently linked to the side chain of Cys159 (**Fig. 1b**, **Supplementary Fig. 4** and **Supplementary Table 2**).

The selectivity of **3** was further demonstrated using a panel of host proteases. As expected, **3** showed no notable inhibition of host proteases, whereas telaprevir inhibited multiple host proteases, when each inhibitor was tested at 10 μM (**Fig. 2a**). Moreover, **3** showed no significant nonspecific reactivity toward glutathione (**Supplementary Fig. 5**). These data highlight the value of covalent bonding to a noncatalytic residue as a means of achieving HCVP selectivity while minimizing the potential for nonspecific reactivity with other thiols such as glutathione.

Huh-7 wild-type (1b) replicon cells were used to demonstrate that **3** can potently inhibit HCVP activity in cells, leading to decreased replication of viral RNA. Luciferase activity was greatly reduced in cells treated with **3** (half-maximal effective concentration (EC_{50}) = 6 nM) (**Fig. 2b** and **Supplementary Table 1**). In contrast, the reversible congener, **4**, did not inhibit luciferase activity (EC_{50} > 3000 nM), demonstrating that covalent bonding greatly enhances potency for this class of compounds. Importantly, **3** does not inhibit proliferation of Huh-7 wild-type replicon cells, nor does it affect growth of other cell lines tested (**Supplementary Table 3**), strongly suggesting that replicon inhibition is because of specific viral protease inhibition. **3** was inactive in the C159S mutant replicon cells (EC_{50} > 3,000 nM) and, as expected, the activity of **4** and telaprevir were unchanged by the C159S mutation, as they are not dependent on the cysteine for their mechanism of action (EC_{50} > 3,000 nM, EC_{50} = 623nM, respectively) (**Fig. 2b** and **Supplementary Table 1**). Of note, the C159S mutant replicon cells showed fitness similar to that of wild-type replicon cells (**Supplementary Fig. 6**).

Numerous NS3 mutations have been reported that render HCVP resistant to the current protease inhibitors. Thus, activity against drug-resistant clinical mutations is important for effective antiviral therapeutics[19]. **3** was able to inhibit and bond to HCVP proteins of clinically relevant NS3 variants (**Supplementary Table 1** and **Supplementary Fig. 7**). Furthermore, the selectivity conferred by Cys159 also allows for binding and inhibition of HCVP from multiple genotypes (**Supplementary Table 1** and **Supplementary Fig. 8**), suggesting that this approach will lead to potent and selective pan-genotype HCVP inhibitors.

To demonstrate direct inhibition of HCVP activity using our irreversible covalent drug, we developed an assay using the internal self-cleavage activity of HCVP[20] (**Supplementary Fig. 9**). We confirmed the necessity of HCVP activity in the proteolytic cleavage of NS3/4A by expressing wild-type HCVP or a protease-dead mutant, NS3/4A-S139A (**Supplementary Fig. 10**). This autoproteolytic cleavage activity was used to directly measure HCVP activity in replicon cells in the presence and absence of the covalent inhibitor. When HCVP activity is inhibited, self-cleavage is abolished, leaving only the full-length holoenzyme. **3** demonstrated inhibition of HCVP internal self-cleavage activity, and the inhibition was sustained for 8–24 h after compound removal. In contrast, HCVP self-cleavage activity had completely returned by 30 min after removal of telaprevir (**Supplementary Fig. 11a**,**b**).

A unique advantage of an irreversible covalent molecule is that it allows the investigation of target occupancy in a time- and dose-dependent manner. We designed a biotinylated irreversible covalent probe that bonds to NS3/4A protease (**Supplementary Scheme 4**), enabling the quantitative analysis of NS3 occupancy with **3**, and found that inhibitory activity and NS3-occupancy closely correlate (**Fig. 3a**,**b**). Following treatment with **3**, there is little or no free NS3 available to bind to the biotinylated probe for at least 8 h after **3** has been removed (**Fig. 3b**). This indicates that essentially all of the NS3 protein was bound by **3**, and newly synthesized protein is being detected at 8–24 h. Return of self-cleavage activity is concomitant with the detection of newly synthesized protease. The biotinylated covalent probe compound is also an indicator of the selectivity of **3**, as the two compounds share structural similarities and electrophiles. Only the full-length NS3 protein and NS3/4A cleavage products were detected as having been labeled by the biotinylated probe, indicating that it is specific for NS3/4A under these conditions (**Fig. 3c**).

A targeted covalent design approach[21] has been applied to kinases, several of which are currently in clinical testing with encouraging evidence of efficacy and safety[22]. This study describes the first successful example of applying targeted covalent inhibition to the protease family. Our data indicate that the electrophile on **3** must be brought into close proximity to a nucleophilic thiol via specific affinity-driven binding to enable covalent bond formation between the small molecule and the targeted HCVP . This strategy enables us to selectively inhibit HCVP and minimize the potential for toxicity through reactivity with off-target proteins. **3** is an

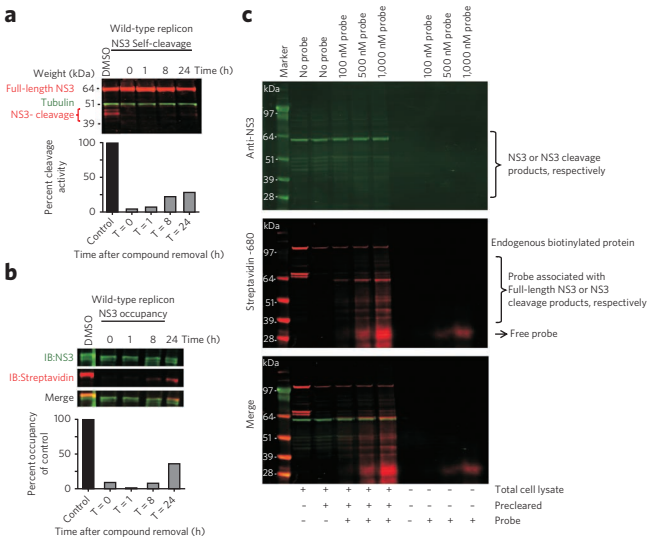

Figure 3 | Sustained inhibition of NS3/4A self-cleavage activity correlates with target occupancy, and the covalent compound shows a high degree of selectivity in cell lysates. Huh-7 wild-type replicon cells were incubated overnight with **3**, at a concentration of 1,000 nM. Compound was removed, and cells were washed in compound-free medium. Samples were taken at indicated times after compound removal. (**a**) Total cell lysates were analyzed by immunoblotting using an anti-NS3 antibody. (**b**) Lysates shown in **a** were immunoprecipitated (IP) with an anti-NS3 antibody and separated by SDS-PAGE. The three panels show immunoblots (IB) (top) anti-goat Alexa-800 to detect the primary anti-NS3 antibody; (middle) streptavidin–Alexa-680 for biotinylated proteins; (bottom) merged image of both channels to show colocalization of biotin and NS3. Occupancy of NS3 with **3** leads to exclusion of the biotinylated probe until the 24-h time point. The top panel shows detection of NS3 protein that was immunoprecipitated from the lysates. The bottom panel is the merged image from both detection methods, showing colocalization of the biotinylated band in the 24-h lane with the NS3 protein. (**c**) Wild-type Huh-7 replicon cell lysate was precleared with streptavidin beads and incubated ± probe compound at the indicated concentrations for 1 h at room temperature ($n = 2$). Total cell lysates were separated by SDS-PAGE and then underwent immunoblot analysis. The biotinylated probe is selective for NS3 full-length and NS3 cleavage products, with almost no other cellular proteins bound to the probe. Full-length NS3 is stable during the 2-h room temperature manipulations for preclearing and incubation with probe compound, whereas the cleavage products are partially degraded.

excellent prototype HCVP inhibitor but has a number of important limitations as a drug candidate; these properties have been optimized in our current development compounds, AVL-181 and AVL-192, which have excellent pharmacokinetics and bind potently to wild-type HCVP as well as to multiple other genotypes and mutant forms of HCVP, including C159S, but only covalently modify when Cys159 is present[23]. The successful design of a highly selective targeted covalent inhibitor of HCVP suggests that this approach can be broadly applied to other protease family members and indeed to a wide range of protein families.

Accession codes. Protein Data Bank: The atomic coordinates and the structure factor for compound 3 bound to HCV protease have been deposited with the accession code 3OYP.

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Author contributions

M.H., T.S.M., M.P.S., M.T.L., H.B., R.M.K. and P.C. performed experiments; D.N., L.Q., J.S. and R.C.P. designed and generated chemical compounds; Z.Z. and J.S. performed molecular modeling; M.N., W.F.W., R.C.P. and J.S. supervised the project; M.H., M.N., R.C.P. and J.S. wrote the manuscript.

Competing financial interests

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturechemicalbiology/.

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