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Discovery of a novel HCV helicase inhibitor by a de novo drug design approach

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ABSTRACT

Herein we report a successful application of a computer-aided design approach to identify a novel HCV helicase inhibitor. A de novo drug design methodology was used to generate an initial set of structures that could potentially bind to a putative binding site. Further structure refinement was carried out through docking a series of focused virtual libraries. The most promising compound was synthesised and it exhibited a submicromolar inhibition of the HCV helicase.

HCV infection is the second most common chronic viral infection in the world with global prevalence. An estimated 180 million people are chronically infected with this hepatitis C virus (HCV) and thus at increased risk of developing serious life threatening liver diseases including cirrhosis that may progress to hepatocellular carcinoma.[1,2]

There is no prophylactic or therapeutic vaccine available against HCV and the development of such a vaccine is not expected to occur soon. Current standard therapy, that is, the combination of pegylated interferon with ribavirin is only effective in about 60% of the patients and is associated with important side-effects.[3] Several drugs that target various stages of the replication cycle of HCV are currently in preclinical or clinical development; these include NS3 protease inhibitors, nucleoside and non-nucleoside polymerase inhibitors and cyclophylin binding compounds.[4,5]

So far the NS3 helicase has not been extensively explored as a target for inhibition of HCV replication.[6–9]

We devised a rational approach for the design of selective inhibitors of the HCV NS3 helicase. Several crystal structures of the HCV NS3 have been reported, that is, as individual domains (protease and helicase) as well as the full length protein. The structure reported by Kim[10] was co-crystallised with a strand of DNA bound to the helicase domain (PDB 1A1V). It was decided to use this structure as the starting point for the de novo design of novel potential inhibitors that could compete for the nucleic acid binding site. The first step was to define a potential binding site for a small molecule; we focused therefore on the area around Arg393. This residue is conserved in all HCV isolates and is also known to play a key role in activity of the enzyme since mutation R393A has a detrimental effect on the unwinding activity of the enzyme.[11] Targeting this specific amino acid represented a considerable challenge: after removing the nucleic acid from the structure it became apparent that the area around Arg393 was completely exposed to the solvent, making it more difficult for a small molecule to bind tightly to it without being replaced by water or the nucleic acid itself. However, on further inspection it was observed that the sulfur atom of Cys431 (a residue not involved directly in the nucleic acid binding and that is situated ~10 Å away from Arg393) had interacted to form an adduct with a molecule of mercaptoethanol present in the crystallisation water. This observation may suggest that Cys431 is accessible to a small molecule and that it is sufficiently reactive to establish a covalent bond with an appropriate compound. These observations led us to formulate the hypothesis that a molecule that would (i) interact with Arg393 and (ii) at the same time form a covalent bond with Cys431 through a chemically reactive group may have the potential to inhibit helicase activity. Such a covalent binder would be very difficult to displace by water or the incoming nucleic acid.

In the initial stages of inhibitor design, the de novo software package LigBuilder was used.[12] Programs of this type normally require the user to define an initial 'seed' in a binding site, after which the computer builds a series of molecules by adding to the growing structures the most suitable fragments taken from a given library.[13] The major drawback of this approach is that very often the proposed structures are highly complex and not synthetically

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accessible. Indeed this is the problem we faced when we examined the results obtained using the fragment library available in the software (Fig. 1, Structure **1**).

It is evident that the software attempted to fill the complete space in the large pocket generating a series of structures with complex chirality. To overcome this problem, the fragment library was reduced to include only residues that, when combined, were less likely to generate chiral centres. Also, a virtual wall of dummy atoms (atoms with no physical property) was created to reduce the site size and to force the software in designing a molecule that would indeed be interacting with the desired residues. The compounds thus generated were considerably simpler. Among them structure **2** was able to interact with Arg393 and also with another arginine located on the opposite side of the putative binding site, namely Arg481. Furthermore it was located close to Cys431 and, although it did not posses any functional group able to react with the amino acid, this represented an appropriate basic scaffold for further functionalization. At this point we employed a different approach to optimise our in silico design process. A series of virtual libraries were generated,[14] varying the linker between the two aromatic rings whilst replacing one of the carboxylic acid groups with a Michael's acceptor, which are known to react efficiently with natural thiols. These libraries were then docked[15] into the binding site and scored based on their ability to interact with the two arginines and to place the vinyl ketone in close proximity to Cys431. Our best structure (Fig. 1, Structure **3**) presented the reactive position of the Michael's acceptor ~5 Å away from the sulfur atom of the cysteine.

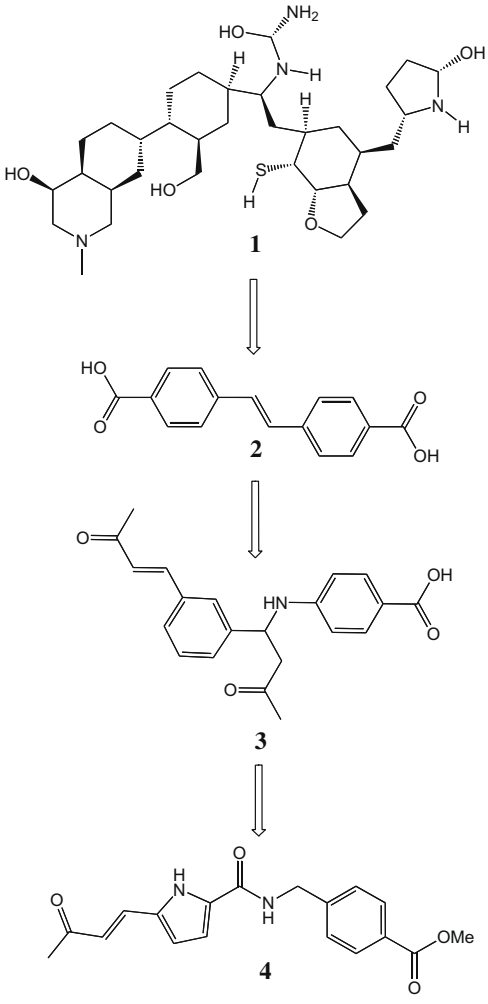


Figure 1. Evolution in the design of a novel HCV helicase inhibitor.

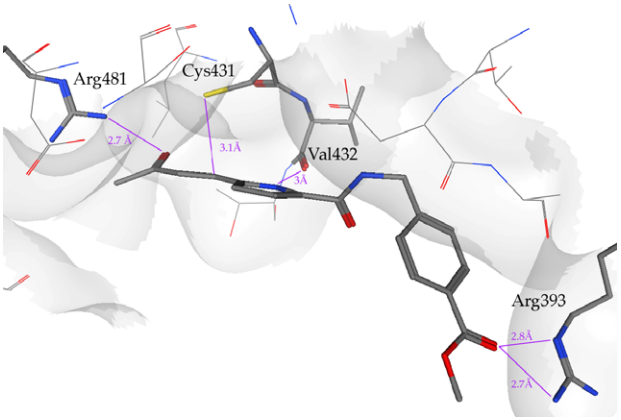
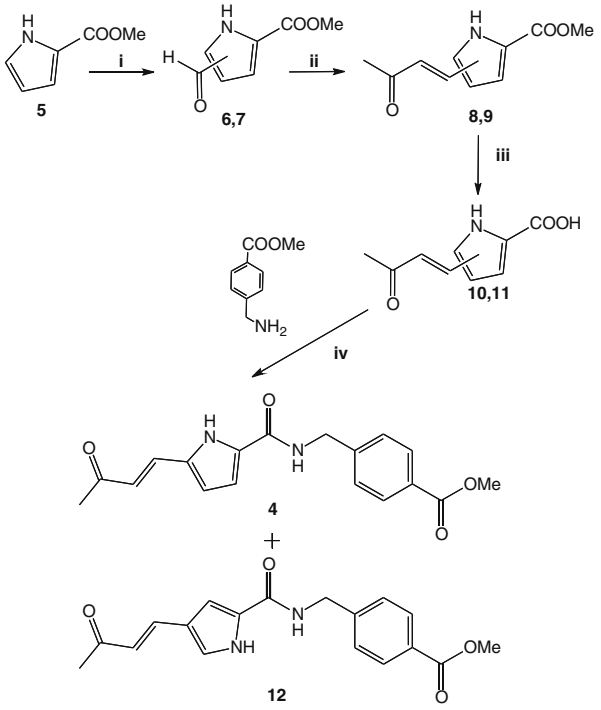


Figure 2. Proposed binding of compound **4** in the HCV helicase.

It also presented a side chain that might increase the interaction points with the enzyme. A molecular dynamic simulation was then performed on the ligand/protein complex to evaluate whether the binding of this compound was stable.[16] The 1 ns simulation revealed a potentially relatively stable interaction; however a slow drifting of the compound away from Cys431 (6–7 Å) was also observed. This was probably due to the steric hindrance of the aromatic ring. Therefore the replacement of the benzene with a smaller heterocycle was investigated. In addition, the linker side chain did not provide any apparent advantage in the MD simulation and was, therefore, removed in subsequent molecules. Finally, a pyrrolo derivative with a slightly longer linker (Fig. 1, Structure **4**) was identified as a potential inhibitor. Compound **4** presented all the key interactions (Fig. 2): hydrogen bond between the ester moiety and Arg393; hydrogen bond between the carbonyl group of the vinyl ketone and Arg481; distance between the sulfur atom of Cys431 and the reactive position of the Michael's acceptor of <4 Å, which remained stable in a MD simulation. This binding conformation is further stabilised by a hydrogen bond between the pyrrole NH and the carbonyl group of Val432. Encouraged by these results, compound **4** was synthesised and evaluated in the helicase assay.

The synthesis of this molecule is depicted in Scheme 1. Formylation of methyl pyrrole-2-carboxylate was carried out using DMF and $POCl_3$ mixture to yield 2 isomers, the 5- and 4- formyl derivatives (**6** and **7**, respectively) in a 2:1 ratio.[17] The two isomers were separated and were used in an aldolic condensation reaction with acetone in water and presence of pyrrolidine 30 mol % to afford **8** and **9**, respectively. This was followed by saponification reaction to yield the free carboxylic acid derivatives **10** and **11**, respectively. The later compounds were coupled with methyl 4-(aminomethyl) benzoate[18] in the presence of 1-ethyl 3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDCI) and dimethyl aminopyridine (DMAP) according to the parallel synthesis protocol of Boger[19] to get compounds **4** and **12**, respectively.

Compounds **4** and **12** were evaluated in a strand-displacement enzymatic assay based on the method of Hicham against purified, recombinant HCV helicase.[20] Compound **4** showed an IC_{50} of 0.26 μM, while the regioisomer **12** did not show any activity at a concentration as high as 100 μM. This is consistent with the model prediction, where the 2–4 substituted pyrrole does not dock with the Michael acceptor placed in proximity to Cys431, thus making the formation of the covalent bond less likely. To establish the importance of the cysteine residue to the binding of **4** we have performed the enzymatic assay in presence of the thioreactive agent NEM (*N*-ethylmaleimide) and we have observed the loss of inhibitory activity of the compound when added to the enzyme pre-incubated with NEM. Not surprisingly, the enzyme preserved its helicase activity in these conditions, probably because NEM, as



Scheme 1. Reagents and conditions: (i) DMF, $POCl_3$ reflux 15 m; (ii) acetone, pyrrolidine 30%, H_2O; (iii) KOH, MeOH reflux 15 h; (iv) EDCI, DMAP, rt, on.

mercaptoethanol, is not big enough to reach the nucleic acid binding site. To further highlight the role of the Michael acceptor in the biological activity of compound **4**, the mono-substitute pyrrolo analogue with the vinyl ketone moiety missing was also prepared directly from **5** using the same methodology described above and it did not shown any inhibition of the HCV helicase in our assay. It should also be noted that in the original crystal structure used, two other cysteine residues beside Cys431 appeared to have reacted with mercaptoethanol (Cys279 and Cys499), but these are placed on the enzyme surface well away from the nucleic acid binding site (>20 Å), thus, making their involvement in the activity of these compounds less probable. The active molecule was also evaluated for a potential inhibitory effect on HCV subgenomic replicon replication as reported earlier.[21] Compound **4** proved however rather cytostatic to the hepatoma cells (EC_{50} 3 μg/ml; CC_{50} 10 μg/ml), which was expected, given the fact that the vinyl ketone group is a known toxicophore. It is worth noting that the ester analogues were prepared and evaluated for biological activity. It is possible to speculate from the model that the free acid might have a better interaction with Arg393. However, it should also be taken into consideration that the increased polarity of the latter compounds would reduce the cellular permeation, thus making them less attractive for the development of future derivatives. Indeed, the free acid analogue is also currently being prepared also with the aim of obtaining a co-crystallised ligand/protein complex, which might give us the definitive proof that compound **4** binds to the helicase as predicted by the model.

In conclusion, we have used a de novo approach to design a novel HCV helicase inhibitor[22] that could target a newly identified pocket on the enzyme. These results are based on an in silico model. It is our aim and priority to obtain a crystal structure of the complex to validate our approach. This in turn may allow us to rationally optimise these compounds and to obtain a novel class of potent anti-HCV inhibitors.

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22. *General procedure for preparing **4** and **12***: To a solution of the appropriate pyrrole-2-carboxylic acid derivative (0.001 mol) in dry DCM, 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCl) (0.002 mol) and dimethyl aminopyridine (DMAP) (0.002 mol) were added. Methyl 4-(aminomethyl)benzoate (0.001 mol) in dry THF was stirred for 3 min under N_2 atmosphere then was added to the above mentioned DCM mixture. The resulting mixture was stirred at room temperature for 48 h under N_2. The resulting mixture was evaporated under reduced pressure. The crude product was chromatographed using ethyl acetate as eluent to afford **4**, **12**; Characterization of compound **4**. Off-white solid, mp 208–209 °C. ^{1}H NMR (DMSO-d_6): δ 12.09 (br s, 1H), 8.90 (t, J = 6.05 Hz, 1H), 7.94 (d, J = 8.3 Hz, 2H), 7.44 (m, 3H), 6.93 (dd, J = 2.05, 3.7 Hz, 1H), 6.75 (d, J = 16.3 Hz, 1H), 6.69 (dd, J = 2.2, 3.6 Hz, 1H), 4.54 (d, J = 5.95 Hz, 2H), 3.84 (s, 3H), 2.24 (s, 3H). ^{13}C NMR (DMSO-d_6): δ 27.17 (CH_3). δ 41.78 (CH_2). δ 52.05 (CH_3). δ 128.08, 129.79, 131.14, 145.36, 160.08, 166.08, 197.45 (7C, quaternary C). δ 111.99, 113.76, 123.88, 127.34, 129.26, 132.79 (6C, CH). Anal. Calcd for $C_{18}H_{18}N_2O_4$: C, 66.25; H, 5.56; N, 8.58. Found: C, 66.01; H, 5.68; N, 8.33.