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Discovery of novel HCV polymerase inhibitors using pharmacophore-based virtual screening

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ABSTRACT

We report the use of pharmacophore-based virtual screening as an efficient tool for the discovery of novel HCV polymerase inhibitors. A three-dimensional pharmacophore model for the HCV-796 binding site, NNI site IV inhibitor, to the enzyme was built by means of the structure-based focusing module in Cerius2 program. Using these models as a query for virtual screening, we produced a successful example of using pharmacophore-based virtual screening to identify novel compounds with HCV replicon assay through inhibition of HCV polymerization. Among the hit compounds, compounds **1** and **2** showed 56% and 48% inhibition of NS5B polymerization activity at 20 μM, respectively. In addition, compound **1** also exhibited replicon activity with EC_{50} value of 2.16 μM. Following up the initial hit, we obtained derivatives of compound **1** and evaluated polymerization inhibition activity and HCV replicon assay. These results provide information necessary for the development of more potent NS5B inhibitors.

Hepatitis C virus (HCV) is a positive-strand RNA virus that was first identified in 1989.[1] Six major genotypes and many subtypes have been described as belonging to this member of the *Flaviviridae* family. It is estimated that more than 170 million people worldwide are chronically infected with HCV.[2] Chronic hepatitis C shows no symptoms with most people, but once established, it develops liver cirrhosis and hepatocellular carcinoma (HCC) within the next decade.[3,4] The current treatment, a combination of interferon alpha and the nucleoside analog Ribavirin, has limited efficacy and severe side effects since less than 50% of patients infected with the predominant genotype 1 of the virus show a sustained virological response, which is defined as undetectable viral load six months after the end of treatment.[5] Meanwhile, the success rate is higher in patients infected with HCV genotype 2 or 3 of the virus (80%).[6,7] Therefore, there is an urgent need for the development of more effective and better tolerated anti-HCV agents.

Many researchers have taken several different approaches to addressing the urgent medical need for treating HCV. Major research efforts have been focused on the identification of agents that inhibit specific steps in the life cycle of the virus. These 'HCV-targeted drugs' include small-molecule, orally bioavailable inhibitors of the HCV enzymes as well as nucleoside analog agents which attack the viral RNA. In addition, agents that can modulate the host immune response are being investigated for their ability to control and eliminate HCV infection. In spite of the hurdles posed by the lack of readily available in vivo models of viral infection, several compounds have just started to show promising results in early-phase clinical trials.[8]

The HCV polymerase (NS5B) is a critical enzyme in the lifecycle of the hepatitis C virus. The main functional role of NS5B in the virus-lifecycle is to assemble the replicase complex at the endoplasmic reticulum membrane and amplify the genetic material through RNA-dependent RNA polymerase (RdRp) activity.[9] The HCV NS5B polymerase inhibitors are classified as nucleoside inhibitors and non-nucleoside inhibitors (NNIs).[10,11] Nucleoside analogs bind at the active site, and NNIs bind to one of four previously identified sites, that is, NNI-I, NNI-II, NNI-III[12] and NNI-IV.[13] Examples of antiviral which have progressed into clinical development are such nucleoside inhibitors (NM-283, R1626 and R7128), and the JTK-109, VCH-759, GSK625433, and HCV-796 for NNI-I, NNI-II, NNI-III, and NNI-IV, respectively.[10, 14–17] Despite the dramatic progress achieved in the field, in terms of both cellular potency and clinical efficacy, the development of polymerase antiviral has suffered a high attrition rate due to toxicity issues. These failures foreground the need to develop other chemical scaffolds which are able to inhibit HCV replication.

Here, we report discovering a novel class of HCV polymerase inhibitors for NNI-IV site. In this study, we identified a potential allosteric site of HCV NS5B, located in the HCV-796 binding site.

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Targeted to this NNI-IV site, the hit compounds fit for this pharmacophore were virtually screened with the aid of the commercially available chemical library (http://www.asinex.com). Through the virtual screening, the compounds library containing 233,554 compounds was filtered to 18 chemicals. The inhibitory activities of the selected compounds were evaluated by the in vitro RdRp activity, and the HCV replicon assay.

For pharmacophore-based virtual screening of HCV NS5B inhibitors targeting NNI-IV, we investigated the HCV-796 binding pockets. The structure used for HCV NS5B was 3FQL,[18] obtained from the Brookhaven protein data bank (http://www.rcsb.org). The co-crystal structures of NS5B-Con1 (genotype 1b) with HCV-796 revealed a deep hydrophobic binding pocket at the palm region of NS5B (NNI-IV binding site). This pocket includes such residues as Pro197, Arg200, Leu204, Cys316, Ser365, Cys366, Ser368, Leu384, Met414, Tyr415 and Tyr448 (Fig. 1A). Initially, a pharmacophore map was generated by the use of Structure Based Focusing (SBF) module in Cerius 2 (Accelrys, San Diego, USA).[19] Pharmacophore features, like hydrogen donor/acceptor and lipophilic interactions, are illustrated in Figure 1B below. The hydrogen bond donor feature is located with side chain of Ser365, near the primer-grip, far apart from the catalytic site of HCV NS5B. In addition, the hydrogen acceptor feature is located with side chain of Arg200. The pharmacophore model was used as a search query for identifying inhibitors from the commercially available multi-conformer 3-D database comprising 233,554 chemicals (http://www.asinex.com). We carried out virtual screening of these compounds suitable for HCV NS5B NNI-IV site, using the CATALYST catSearch utility.[20] The compounds that either exhibit unfavorable interactions with the binding site or adopt unrealistic conformations on pharmacophore mapping were removed through visual inspection.

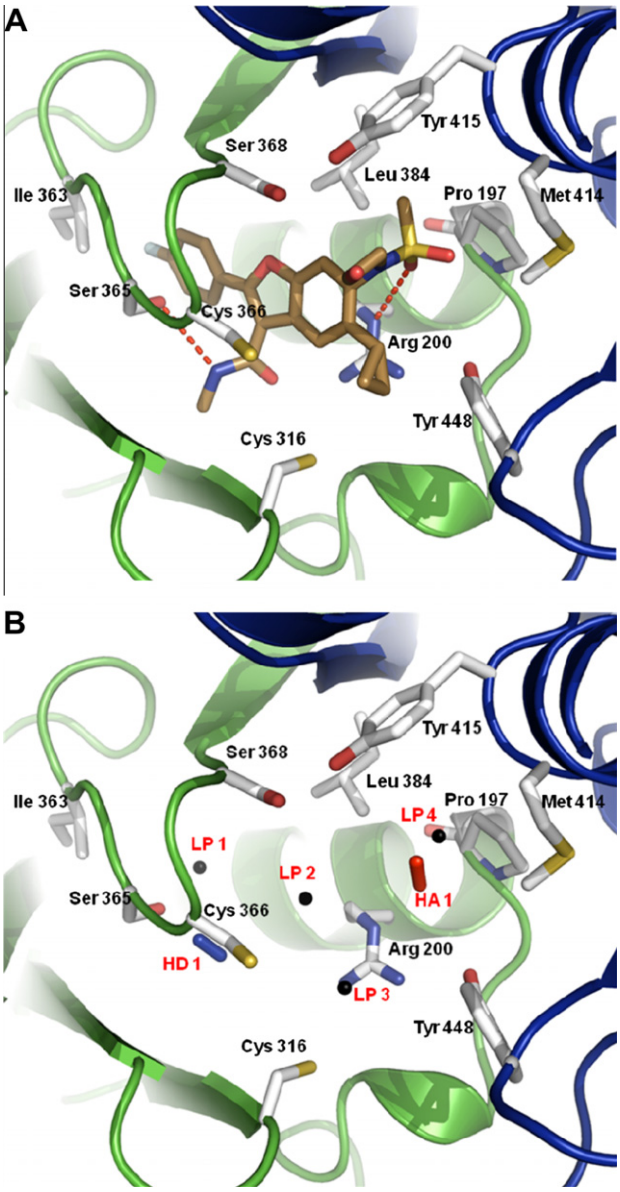


Figure 1. The co-crystal structure of HCV-796 with HCV NS5B polymerase and detailed map of proposed pharmacophore. Red dot lines in (A) indicate the hydrogen bond interactions. The hydrogen bond interactions of HCV-796 involve the side chain of Ser365 and Arg200 residues. The contact residues in the pharmacophore map (B) are displayed with the HCV-796 binding site. The HD (blue stick) and HA (red stick) denote the hydrogen bond donor and hydrogen bond acceptor, respectively. The LP1, LP2, LP3 and LP4 (black circles) represent lipophilic interaction sites of ligand.

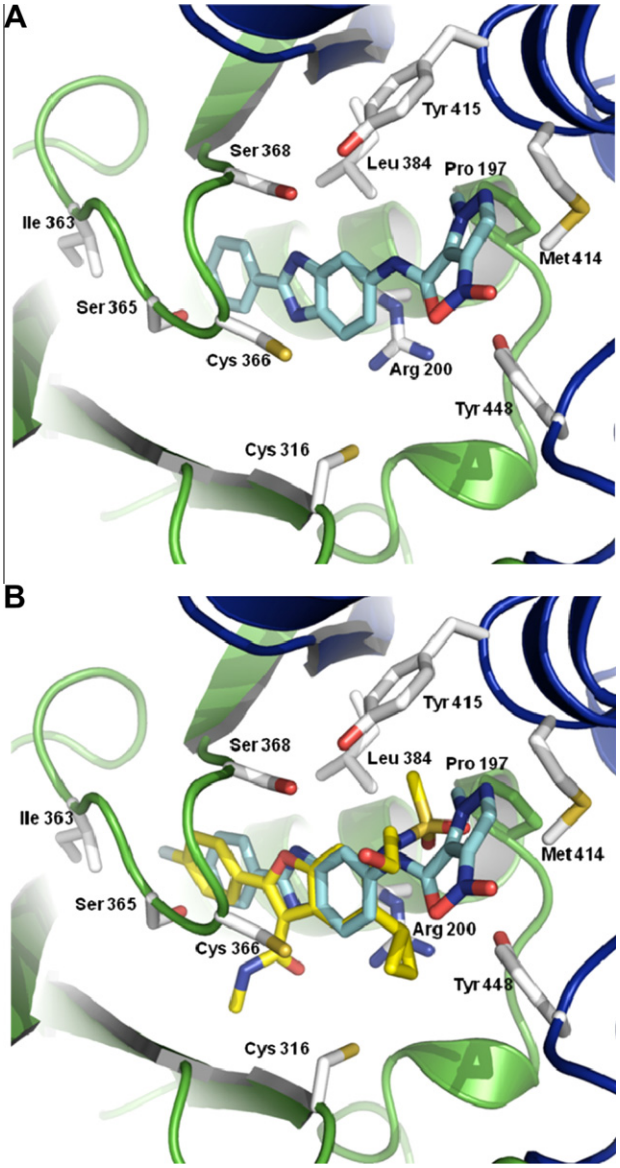


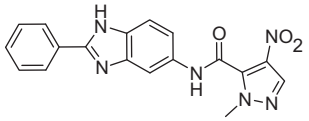
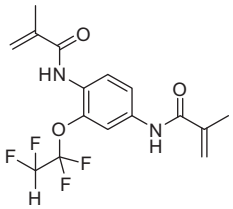
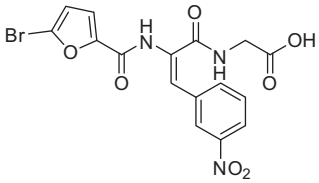
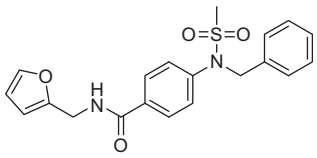
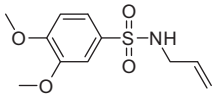
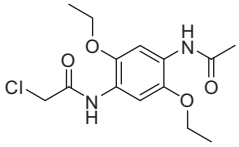
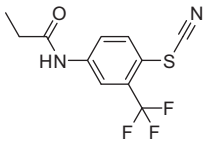
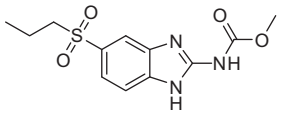
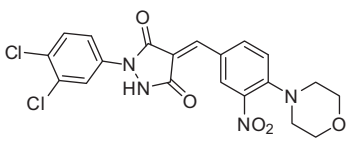
Figure 2. Molecular docking model of compound **1** with HCV NS5B polymerase. (A) The molecular docking model of compound **1** was predicted by Autodock4 simulation. (B) Comparison of the proposed binding modes of compound **1** to HCV NS5B NNI-IV binding site with HCV-796 (yellow) originating from the X-ray structure. Compound **1** is presented in cyan color.

Finally, we obtained 18 compounds for further testing, in vitro enzyme assay.

We investigated whether the virtually screened 18 compounds inhibit RdRp activity in vitro. We initially evaluated the inhibitory effects of candidate compounds at the single concentration of 20 μM. Through the first in vitro screening, two compounds (Compounds **1** and **2**) were shown to inhibit the RdRp activity of genotype 1b, while the other compounds had no significant inhibitory activity. The active compounds of **1** and **2** have each exhibited 54% and 48% inhibitions at 20 μM. Further, we tested two compounds (compounds **1** and **2**) for anti-HCV activity in a HCV replicon-based assay. From these experiments, the EC_{50} value of compound **1** was estimated at 2.16 μM, and the cytotoxic activity was identified. Human hepatoma cell lines, HuH-7, were treated with various concentrations of compound **1** for 72 h. The CC_{50} value of compound **1** was estimated at 34.5 μM (Table 1).

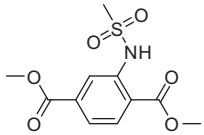
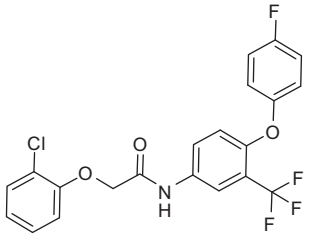
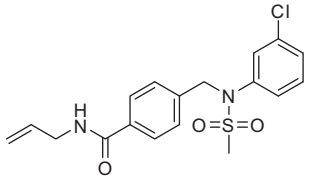
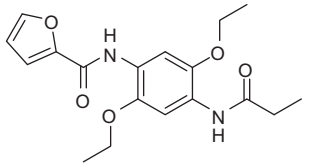
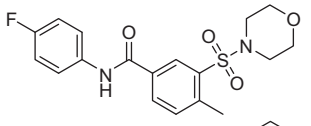
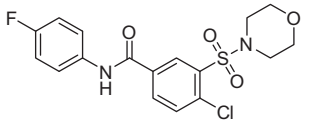
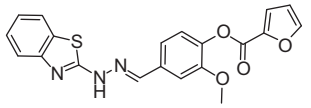
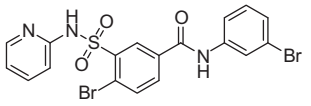
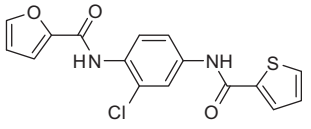
In order to validate the activity of the chemical scaffold of the compound **1**, we obtained commercially available analogs of compound **1**. The results in Table 2 illustrate derivatives of compound **1** for anti-HCV activity, replicon-based assay, and cytotoxicity test,

Table 1
Anti-HCV activity and cytotoxicity for virtually screened 18 compounds

Compound	Structure	RdRp Activity (@20 μM)	Replicon Activity EC_{50} (μM)	Cytotoxicity CC_{50} (μM)
1		54%	2.16	34.5
2		48%	–[a]	29.0
3		–	–	>50.0
4		8%	–	32.7
5		–	–	48.6
6		–	–	>50.0
7		9%	–	45.8
8		–	–	<5
9		13%	–	27

(*continued on next page*)

Table 1 (*continued*)

Compound	Structure	RdRp Activity (@20 μM)	Replicon Activity EC_{50} (μM)	Cytotoxicity CC_{50} (μM)
10		–	–	43.5
11		–	–	14.2
12		–	–	33
13		–	–	36.1
14		–	–	16.5
15		–	–	15.6
16		–	–	30.5
17		–	–	>50.0
18		–	–	18.0

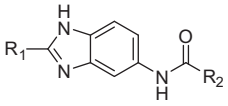
[a] No activity.

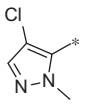
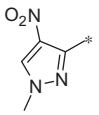
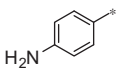
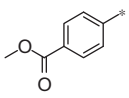
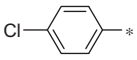
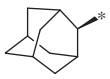
and showed that compounds **24** and **30** had more improved inhibitory effect on HCV polymerase than the compound **1**. However, these compounds displayed weak or no activity of replicon-based assay. We speculate that a poor cell permeability or cytotoxicity could be one of the reasons for the low activity of replicon cell-based assay. Further study is undergoing for the improvement of its cytotoxicity and cell permeability.

To further compare the interactions between newly identified compound **1** and HCV-796 with HCV NS5B polymerase, we performed a molecular docking study. Compound **1** was docked into NNI-IV site of HCV NS5B by the Autodock4 program.[21] As shown in Figure 2A, the benzimidazole group of compound **1** was shown to interact with the side chains of Arg200, Cys366 and Ser368 of HCV NS5B. Besides, a 1-methyl-3-nitro-1*H*-pyrazole group of compound **1** was able to interact with hydrophobic pocket surrounded mainly by Pro197, Met414, Tyr415, and Tyr448 residues. These results show that the binding manner of compound **1** to HCV NS5B polymerase is similar to that of the co-crystal structure of HCV-796 complex to the NNI-IV site (Fig. 2B).

In this study, we successfully employed pharmacophore-based virtual screening in order to identify novel compounds with HCV replicon activity and their ability to inhibit HCV polymerization. Initially, a pharmacophore model was built on the basis of HCV-796 binding site, and the hit compounds selected by the pharmacophore-based virtual screening were assayed for HCV NS5B polymerization inhibition. Among the hit compounds, compound **1**

Table 2
Anti-HCV activity and cytotoxicity of compound **1** derivatives



Compound	R1	R2	RdRp Activity (@20 μM)	Replicon Activity EC_{50} (μM)	Cytotoxicity CC_{50} (μM)
19			14%	—[a]	<5
20			37.7%	8.3	17.3
21			—[a]	—[a]	<5
22			20%	—[a]	20
23			19.7%	—[a]	<5
24			90%	—[a]	<5
25			—[a]	—[a]	<5
26			42%	—[a]	25
27			18.5%	—[a]	20
28			58.6%	—[a]	24.8
29			42.4%	—[a]	20
30			49.9%	—[a]	20

[a] No activity.

showed promising efficacy in both polymerization assay at 20 μM and cell-based HCV replicon assay. Although further biochemical study is required, for instance, on characteristic mutations at the palm and thumb binding sites of NS5B polymerase on the binding affinities of non-nucleoside inhibitors, it would be now possible to provide useful information for the development of new chemical entities targeting HCV.

Acknowledgment

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19. Structure-based pharmacophore models were generated using the structure-based focusing method. (Accelrys Inc., San Diego, CA, USA) The program generates a LUDI interaction map describing all the possible interactions (hydrogen-bond donors and acceptors, hydrophobic) that a ligand can establish with a given active site. A map was generated for all atoms within a radius of 10 Å of the geometric center of the HCV-796 molecule. The pharmacophore features corresponding to the HCV-796 interactions with active site residues of HCV NS5B polymerase. Exclusion volume spheres were placed on each heavy atom of the protein found in this sphere, using a 1.0 Å radius. A 3D compound database was built with commercially available chemical library from Asinex Ltd (http://www.asinex.org) Compounds were generated 3D multiple conformer by catDB command of the Catalyst. The Catalyst-formatted database was screened with the generated pharmacophores as three-dimensional queries using Catalyst's catSearch. Among the pharmacophore-based virtual screening hit compounds, those exhibiting unfavorable interactions with the binding site or unrealistic conformations were filtered out by visual inspection. Finally, we selected 18 compounds for further testing, in vitro enzyme assay.
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