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Fragment-based discovery of hepatitis C virus NS5b RNA polymerase inhibitors

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Abstract—Non-nucleoside inhibitors of HCV NS5b RNA polymerase were discovered by a fragment-based lead discovery approach, beginning with crystallographic fragment screening. The NS5b binding affinity and biochemical activity of fragment hits and inhibitors was determined by surface plasmon resonance (Biacore) and an enzyme inhibition assay, respectively. Crystallographic fragment screening hits with ~1–10 mM binding affinity (K_D) were iteratively optimized to give leads with ~200 nM biochemical activity and low μM cellular activity in a Replicon assay.

Hepatitis C virus is the leading cause of chronic liver disease throughout the world. Patients infected with HCV are at risk of developing cirrhosis of the liver and subsequent hepatocellular carcinoma, and hence HCV is one of the major reasons for liver transplantation. HCV NS5b polymerase has been the focus of many drug discovery efforts,[1] but has proven to be a difficult target as evidenced by the small number of clinical candidates targeting this enzyme to date.[2]

Fragment-based lead discovery has been recently reviewed.[3] Crystallographic fragment-based screening seemed particularly well suited for a challenging target such as HCV polymerase, since previous work has shown that the high screening hit rate for such small, low MW compounds can often identify novel, tractable hits that can be optimized into potent leads with good 'drug-like' properties.[4] In particular, crystallographic fragment screening can identify hits that may be too weak to be identified in biochemical assays. Crystallographic fragment screening also provides unambiguous proof of binding to the target site and reveals the details of the hits binding mode, providing clear direction for how they may be optimized into more potent lead compounds. Cocrystal structures of HCV NS5b RNA dependent RNA polymerase (NS5b) with small molecule inhibitors have been previously reported.[5] We developed experimental protocols for growing NS5b crystals that were suitable for determining inhibitor cocrystal structures by soaking.

Fragments in the screening library were chosen to be consistent with 'lead-like' properties,[6] and also to include two or more substituents to facilitate analog synthesis. The fragment library had a MW range of 100–220D and an average MW of 160.[7] About half of the fragments contain an aromatic bromine, both as a convenient substituent for synthesis and to facilitate determination of bound hits by collecting X-ray data at the bromine anomalous dispersion wavelength. Ninety-six mixtures of 10 fragments (5 mM concentration each) were incubated with the NS5b protein crystals for 24 h. Datasets were obtained for 89 of 96 mixtures at a typical resolution of 1.7 Å (range 1.6–2.1 Å). Twenty individual hits were identified, 17 of which were bound

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at an allosteric site in the thumb domain.[8] In most cases the hit was unambiguously identified directly from the mixture screen. Other hits with less well-defined electron density were identified by soaking the individual compounds from the mixture.

Surface Plasmon Resonance (SPR) technology as implemented with BIAcore instruments was applied initially to detect protein–protein interactions, followed by protein–ligand interactions with high affinity (e.g., sub-μM). Recent hardware and software improvements have enabled the determination of binding affinities of smaller, weaker ligands, such that SPR analysis can be used to detect fragment binding. Using this technique we were able to measure equilibrium binding affinities (K_D) for fragments and elaborated molecules with modest affinity.

The fragments found to bind to the thumb site had a number of common features (Fig. 1), most notably the frequent presence of an aryl or heteroaryl bromide, and a preponderance of carboxylic acids. The hits tolerated a range of substituents meta and para to the bromine. Interestingly, the corresponding chloro substituted analogs were not observed crystallographically.

A number of hits were observed to interact with the carboxylic acid binding site (S476 and Y477) in a manner analogous to the published Shire inhibitor **6**.[9,10] For example, **5** is involved in two hydrogen bonds to S476 and Y477 backbone NHs. These interactions were also observed with other carboxylic acids. In the case of **2**, the carboxylic acid binds to S476 and Y477 while the bromophenyl occupies a hydrophobic pocket in the floor of the binding site. In a biochemical assay, which measures the inhibition of ^{3}H UTP incorporation into RNA using a poly A RNA template,[11] the IC_{50} value of fragments discovered in the crystallographic screen was in the 200–500 μM range, or was too weak to be measured. The binding affinity of **1** and **4** to the NB5b protein was in the millimolar range.[12]

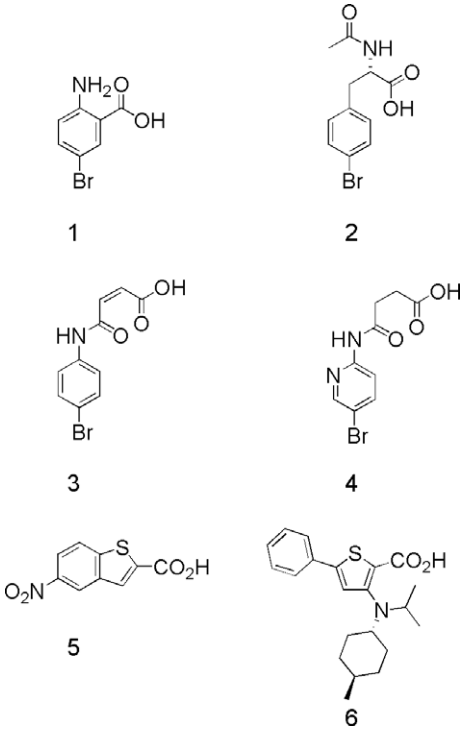

Figure 1. Some of the fragment hits observed at Thumb site in NS5b crystal structures, and Reference inhibitor **6** from reference 2.

In parallel, the biochemical assay was used to identify hits from the same fragment library screened at high concentration. None of the biochemical hits, which had IC_{50} values ranging from 2 to >100 μM, were observed to bind to the NS5b protein crystals in soaking experiments. SPR analysis of the biochemical hits also failed to detect any binding affinity.

The initial optimization efforts were focused on **1** which offered a number of possibilities for elaboration. The goal was to increase the affinity and biochemical activity by creating additional interactions in the binding pocket between R501 and S476 and Y477 (Fig. 2).

A second class of fragments was also discovered in the initial crystallographic fragment screen (**3** and **4** are representative examples). In this case, the bromoaryl moiety binds in the pocket as in **1**, but the amide carbonyl oxygen interacts with the S476 backbone NH in the carboxylic acid binding site. The succinic acid electron density is well defined, with the carboxylate interacting with the S476 hydroxyl on the surface of the protein. Since this is a surface residue, it was unclear how much binding affinity was due to this interaction and whether it was critical for binding. An initial library of anthranilamides **7** was made using a diverse selection of primary, secondary, and aromatic amines (Scheme 1). A second set of amines, distinct from the first, was selected and coupled to 5-bromo anthranilic acid **1**, and then treated with succinic anhydride to give the succinamide products **8** (Scheme 1).

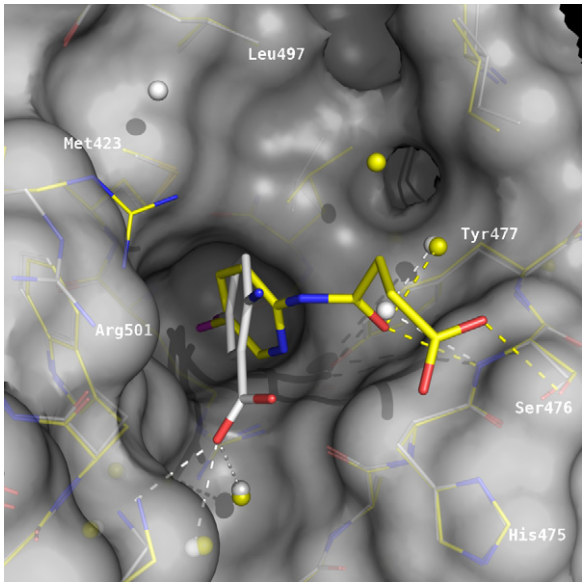

Figure 2. Crystal structure of fragments **1** and **4** bound to NS5B.