Neutron crystallography to inform drug design targeting SARS-CoV-2 main protease

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Source: Radoslavy Zimny/GItty Images
SARS-CoV-2 life cycle

- Proteolysis of pp1a and pp1ab by 3CL M\textsuperscript{pro} and PL\textsuperscript{pro} produces NSPs
- Action by the two enzymes is vital for the viral replication cycle
- Inhibition of the proteases can stop the viral replication

Main protease is the heart of SARS-CoV-2 replication

Stopping the heart of SARS-CoV-2
1) Polyproteins are cleaved into components of the replication machinery
2) Protease inhibitors bind active site, blocking substrate processing
3) Viral replication is prevented
Active site of SARS-CoV-2 $M^{\text{pro}}$
Targetable active site features

- Non-canonical catalytic Cys145-His41 dyad
- Room for ~6-7 peptide/inhibitor groups (P2'-P5)
- Characteristic oxyanion hole

Kneller et al. 2020 IUCR J. 7, 1028-1035
M^pro active site: Where are the hydrogens?

108 combinations of protonation states and tautomers are possible.
Room-temp joint XN structure of M$^{\text{pro}}$ in the native form @ 2.5Å resolution

First cysteine (coronavirus) protease neutron structure: non-canonical catalytic dyad is zwitterionic

Kneller et al. 2020 Nat. Commun. 18, 688-699
Kneller et al. 2020 J. Biol. Chem. 295, 17365-17373
Hemithioketal is protonated with short H-bond to His41, but unfavorable geometry
M<sub>pro</sub> active site is malleable adapting to ligand size
Subsites S2 and S4 are cryptic
Tunability and malleability of M\textsuperscript{pro}

- Active site protonation states are tunable, but overall electrostatic charge is maintained at +1

- Active site conformation dynamically adapts to inhibitor properties

- Cryptic binding subsites and plasticity presents challenges for inhibitor design and \textit{in silico} modeling
Structure-activity relationship (SAR) study for noncovalent inhibitors:
Exploring structural, electrostatic and electronic determinants for binding to subsites S1 and S2
Supercomputer screen identifies noncovalent inhibitor

6.5 Million ligand library

336 Billion conformer dockings

72 tested by *in vitro* assay

1 active compound

\[ K_i = 2.9 \, \mu \text{M} \]

1 room temperature X-ray structure

Direct characterization of protein-ligand hydrogen bond network

Neutrons enable observation of new catalytic water molecule orientation

First neutron structure of Mpro-Non-covalent inhibitor complex
Neutron structure-led VR-assisted SAR

HL-3 series:

X = H, Cl, CF₃
Y = H, Cl, F, Br, I, CN, CF₂, CH₃, CHO, CH₂OH
Z = H, Cl

HL-3-69
HL-3-51 (S), HL-3-53 (R)
SAR study guided by the neutron structure M^{pro}-1 complex (HL-3 series)

- P1 must have H bond acceptor capability and correct geometry to make H bond with protonated His163.
- Substituents on the aromatic P2 group should have both moderate steric size and electronegativity.
- P2 group with one substituent on the aromatic ring is disadvantageous.
- Highly electronegative substituents, such as -F or -CF_{3} are disadvantageous, as are less electronegative but sterically larger ones.
- A third substituent such as -Cl at P2 group is favorable.

IC_{50} = 0.61 \mu M

IC_{50} = 0.68 \mu M

IC_{50} = 1.4 \mu M

IC_{50} = 1.4 \mu M

IC_{50} = 6.2 \mu M

IC_{50} = 8.8 \mu M

IC_{50} = 6.4 \mu M

U.S. Provisional Patent Application serial No. 63/232,331
In vitro assessment of chemical modifications

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition $K_i$ (μM)</th>
<th>Affinity $K_d$ (μM)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$ (cal/mol·K)</th>
<th>$\Delta G$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCULE-5948770040</td>
<td>2.9</td>
<td>1.30</td>
<td>-8.32</td>
<td>-0.7</td>
<td>-8.11</td>
</tr>
<tr>
<td>HL-3-68</td>
<td>0.89</td>
<td>0.69</td>
<td>-7.75</td>
<td>2.4</td>
<td>-8.5</td>
</tr>
<tr>
<td>Mcule-CSR-494190-S1</td>
<td>1.4</td>
<td>1.32</td>
<td>-9.1</td>
<td>-3.16</td>
<td>-8.15</td>
</tr>
</tbody>
</table>

MCULE-5948770040

\[
\text{MCULE-5948770040}
\]

HL-3-68

\[
\text{HL-3-68}
\]

Mcule-CSR-494190-S1

\[
\text{Mcule-CSR-494190-S1}
\]
M\textsuperscript{pro} is structurally and electronically malleable

- One atom difference can significantly alter inhibitor binding potency
- VR allowed true 3D structural analysis and inhibitor building
- VR allowed inhibitor structures to be tailored to the binding site
Design of covalent inhibitors based on hepatitis C virus protease inhibitors
Hepatitis C virus protease inhibitors bind and inhibit M\textsuperscript{pro}

PDB 6XQS

IC50 = 18 µM

PDB 6XQU

IC50 = 3 µM

PDB 6XQT

IC50 = 5 µM

Kneller et al. 2020 Structure 28, 1313-1320
Design of covalent hybrid inhibitors of $M^{\text{pro}}$

- Peptidomimetic inhibitors

Boceprevir

Narlaprevir

GC-376

BBH-1

BBH-2

NBH-2
Unique binding of BBH-1 to M<sup>pro</sup>: A neutron structure perspective
Unique binding of BBH-1 to $\text{M}^{\text{pro}}$: A neutron structure perspective
Covalent inhibitors with nitrile warhead

- Nirmatrelvir (NMV)

BBH-2

NBH-2

PF-07321332
**Isothermal titration calorimetry**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_d$, $\mu$M</th>
<th>Stoichiometry, $N$</th>
<th>$\Delta H$, kcal mol$^{-1}$</th>
<th>$\Delta S$, cal mol$^{-1}$ K$^{-1}$</th>
<th>$\Delta G$, kcal mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBH-2</td>
<td>0.030 ± 0.007</td>
<td>1.000 ± 0.005</td>
<td>−8.74 ± 0.08</td>
<td>5.40</td>
<td>−10.4</td>
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<tr>
<td>NBH-2</td>
<td>0.026 ± 0.016</td>
<td>0.990 ± 0.009</td>
<td>−8.76 ± 0.17</td>
<td>5.63</td>
<td>−10.5</td>
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<tr>
<td>PF-07321332</td>
<td>0.007 ± 0.003</td>
<td>0.990 ± 0.003</td>
<td>−10.75 ± 0.70</td>
<td>1.57</td>
<td>−11.2</td>
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<tr>
<td>GC-376</td>
<td>0.15 ± 0.03$^a$</td>
<td>0.99 ± 0.01</td>
<td>−6.7 ± 0.1</td>
<td>9.1</td>
<td>−9.4</td>
</tr>
</tbody>
</table>
**Antiviral data**

### Compound

<table>
<thead>
<tr>
<th></th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; µM (without CP-100356)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; µM (with CP-100356)</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBH-1</td>
<td>16.1</td>
<td>1.5</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>BBH-2</td>
<td>15.4</td>
<td>0.88</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>NBH-2</td>
<td>13.9</td>
<td>1.82</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>PF-07321332</td>
<td>0.88</td>
<td>0.25</td>
<td>&gt; 10</td>
</tr>
</tbody>
</table>
Hybrid inhibitors – fruitful path to clinical drugs

• Protonation states adapt to a specific inhibitor

• Active site geometry adapts to inhibitor steric and electronic properties

• Hybrid inhibitors are conceptually superior to previous designs