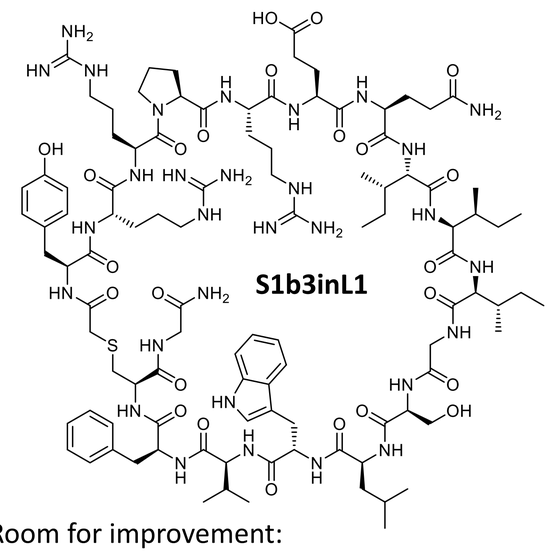
Chemists Don’t Want You to Know This Simple Trick!

A Nanomolar Potency Macrocyclic Peptide Inhibitor of SARS-CoV-2 Spike through Deep Mutational Scanning

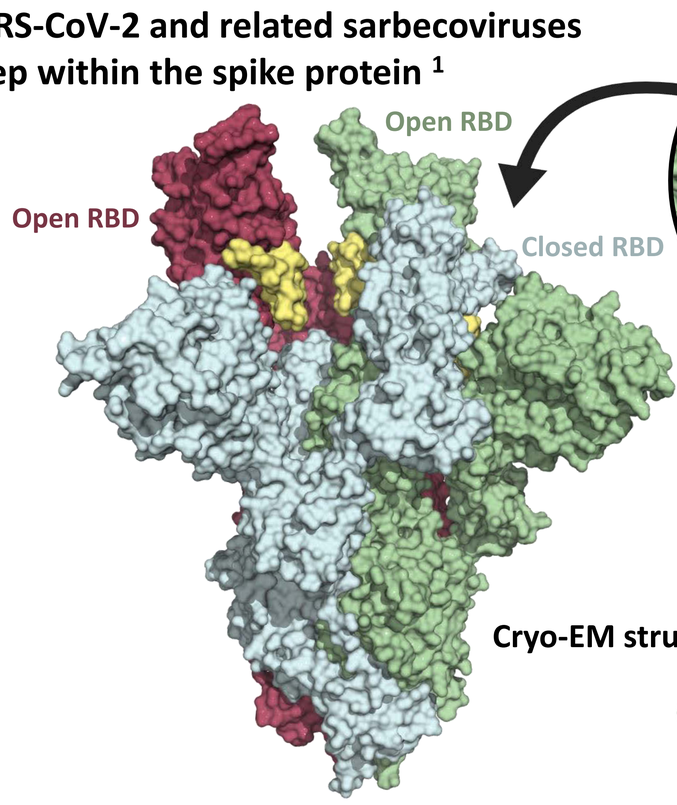
Antonius (Twan) J.P. Hopstaken[1], Oliver J. Debski-Antoniak[2], Preeti Hooda[2], Daniel L. Hurdiss[2], Seino A.K. Jongkees[1]
[1] Vrije Universiteit Amsterdam, Netherlands, [2] Utrecht University, Netherlands
https://doi.org/10.17952/37EPS.2024.P1152

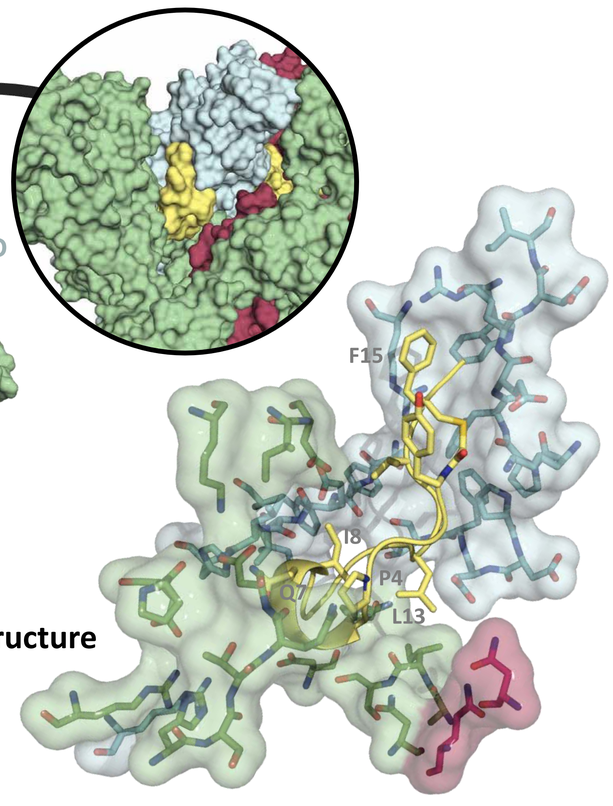
Our previous mRNA display hit inhibits SARS-CoV-2 and related sarbecoviruses through binding of a conserved pocket deep within the spike protein [1]



Room for improvement:

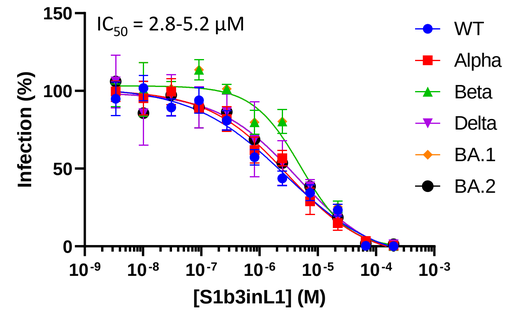
- **Potency** of virus infection inhibition
- **Solubility**
- Little information about tolerated mutations available from the sequencing data



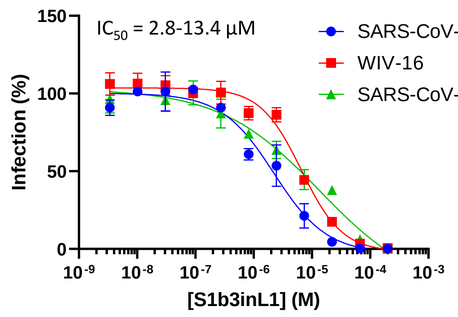


Cryo-EM structure

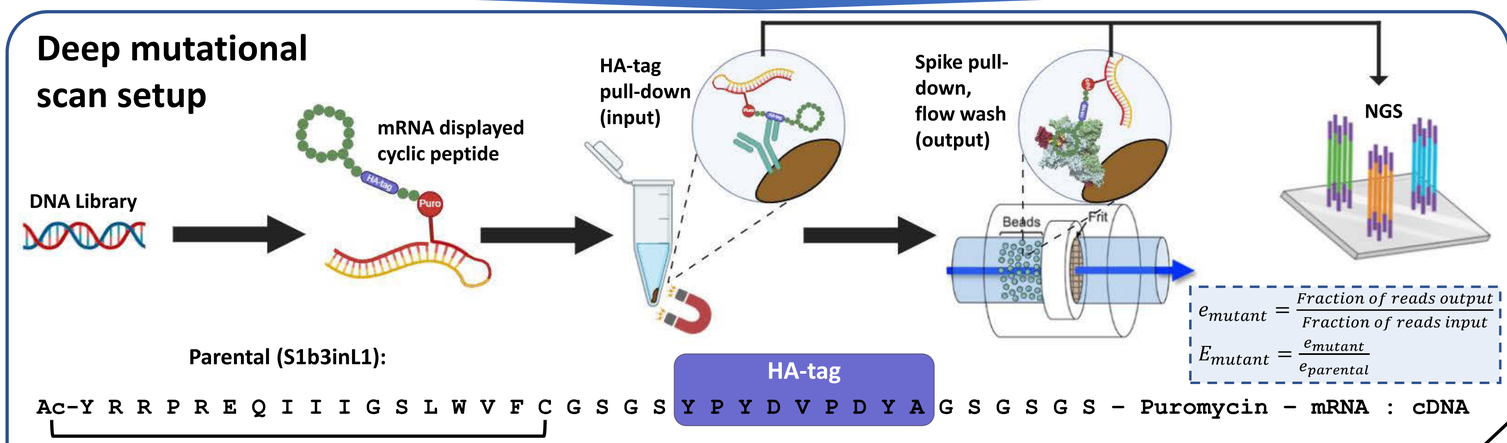
Variants of concern pseudovirus neutralization



Sarbecovirus pseudovirus neutralization



Deep mutational scan setup



$$e_{mutant} = \frac{Fraction\ of\ reads\ output}{Fraction\ of\ reads\ input}$$

$$E_{mutant} = \frac{e_{mutant}}{e_{parental}}$$

Parental (S1b3inL1):

Ac-Y R R P R E Q I I I G S L W V F C G S G S **Y P Y D V P D Y A** G S G S G S – Puromycin – mRNA : cDNA

HA-tag

Scanning site saturation mutagenesis libraries

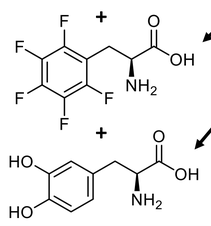
Single mutation per peptide
Library size: 315 peptides

* R P R E Q I I I G S L W V F
R * P R E Q I I I G S L W V F
R R P R E Q I I I G S L W * F
R R P R E Q I I I G S L W V *

Two semi-paired mutations per peptide
Library size: 24696 peptides

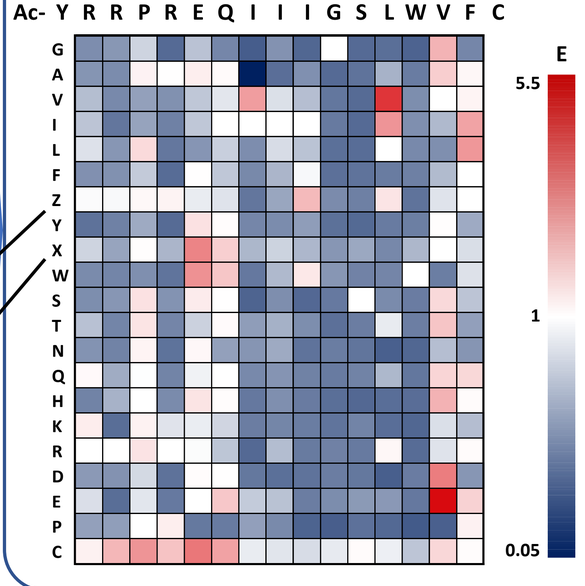
* R P R E Q I I I G S L W V F
R * P R E Q I I I G S L W V F
R R P R E Q I I I G S L W * F
R R P R E Q I I I G S L W V *

AAs: 1x *, random position
* = 19 canonical AAs (-Met)



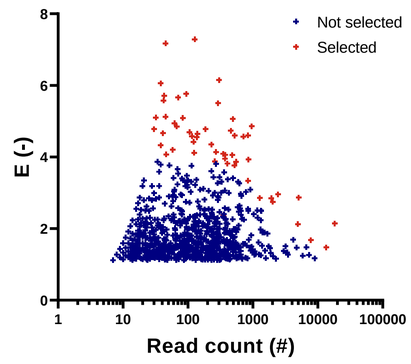
Enrichment scores

Single mutation library



Semi-paired mutation library

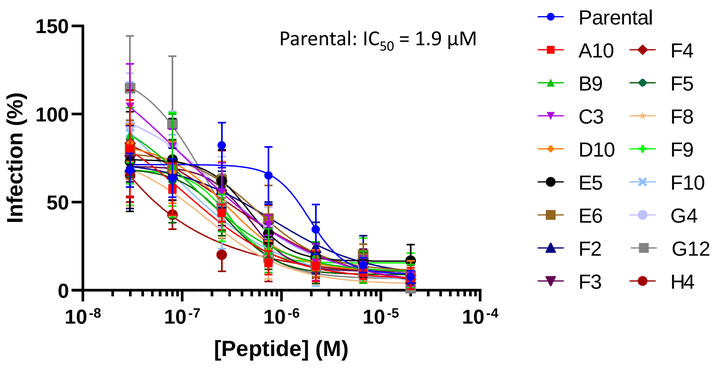
Top 1000 E-scores



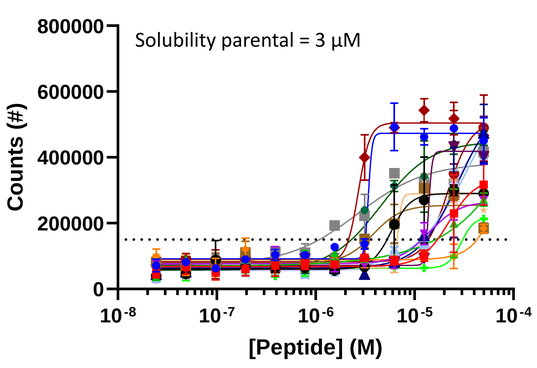
- **Positions 3, 9, 11, 12, 14** completely **conserved**
- High E-scores of cysteine mutations likely an artifact caused by dimerization, since cysteines were not enriched at similar positions in the larger library

Purified peptides all show improved potency and generally better solubility

SARS-CoV-2 pseudovirus neutralization



Nephelometry



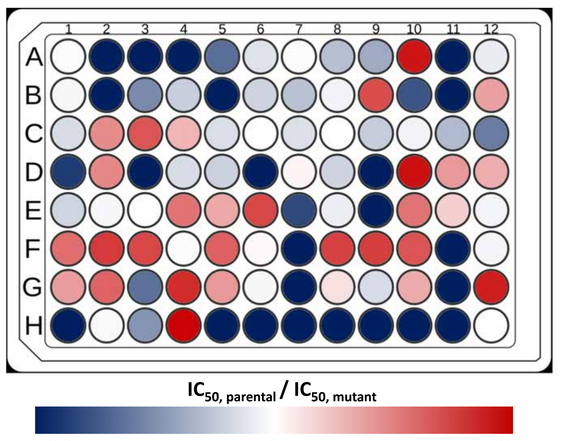
- **H04** is the **most potent hit**, combining 5 mutations that by themselves increase potency (Q7E, I8V, L13V, V15T, F16L) and 1 mutation that is detrimental as single mutation (P4S)
- SPPS of H4-P4S for further testing

- All peptides show **improved solubility** compared to parental **except** those with a mutation introducing another **tryptophan**
- Nephelometry is an extremely sensitive method for the detection of small particles, no turbidity could be observed by eye for any of the peptides even at 50 µM

Effects of single mutations on potency are additive when combined

- **96 peptides** were selected for **SPPS** based on favorable **E-scores** and predicted improved **solubility**
  - **25** from the single mutation libary + **6** shorter cycle peptides
  - **51** double or triple mutants from the larger library
  - **13** peptides with increasing amount of mutations
- **Crude peptides** tested for **potency**

SARS-CoV-2 pseudovirus neutralization

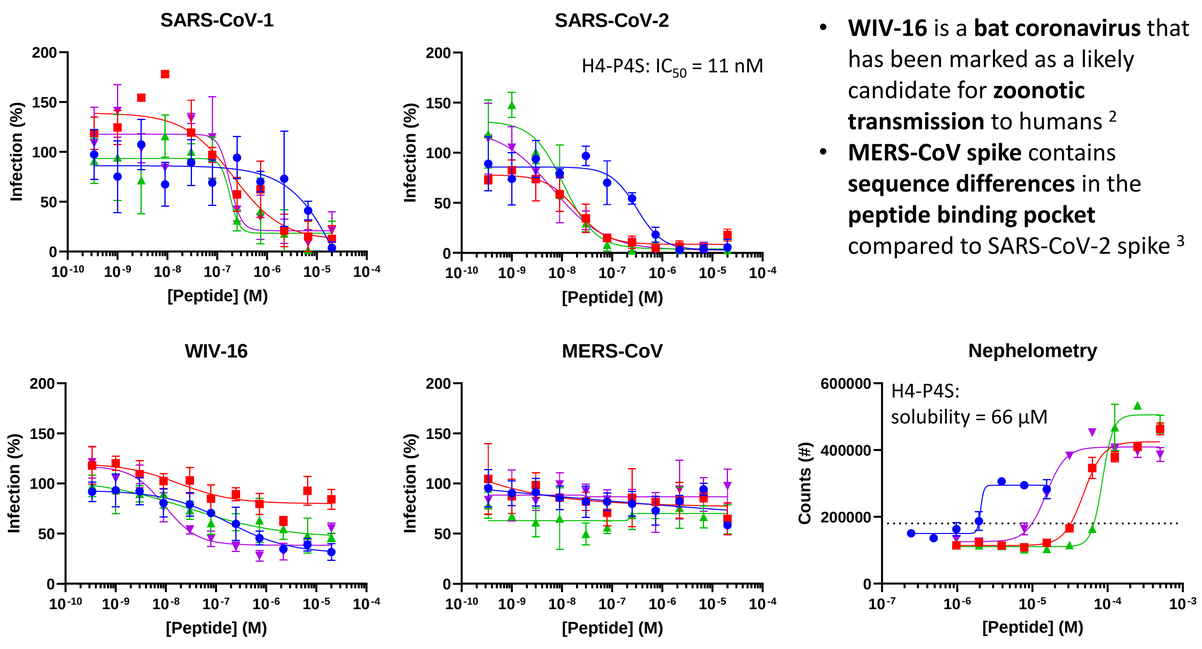


A01	R2K	C02	R2K, L13V	E03	E6X, V15A	G04	P4A, I8V, F16P
A02	P4L	C03	P4A, V15T	E04	E6X, V15T	G05	P4S, I8V, F16P
A03	P4S	C04	P4D, I8V	E05	E6N, I8V	G06	P4S, I8V, V15H
A04	R5Z	C05	P4D, V15T	E06	E6N, V15T	G07	E6X, I10Z, V15D
A05	E6X	C06	P4E, V15T	E07	E6S, V15S	G08	E6W, Q7X, V15E
A06	E6W	C07	P4K, V15T	E08	E6T, V15T	G09	I8V, L13V, V15G
A07	Q7E	C08	P4L, I8V	E09	E6W, I10Z	G10	L13V, V15E, F16L
A08	Q7X	C09	P4L, V15A	E10	E6W, L13V	G11	R5Z, I8V, L13V, V15E
A09	Q7W	C10	P4N, I8V	E11	E6W, V15A	G12	P4L, E6W, I8V, L13V, V15E
A10	I8V	C11	P4N, V15T	E12	E6Y, F16P	H01	P4S, E6X, I8V, I10Z, V15T
A11	I10Z	C12	P4R, V15T	F01	E6Y, V15T	H02	E6X, Q7E, I8V, V15E, F16Q
A12	L13I	D01	P4S, F16P	F02	Q7D, I8V	H03	P4S, Q7E, I8V, L13V, V15D, F16E
B01	L13V	D02	P4S, I8V	F03	Q7L, V15E		
B02	L13Z	D03	P4S, V15Q	F04	Q7W, L13V	H04	P4S, Q7E, I8V, L13V, V15T, F16L
B03	V15D	D04	P4T, V14A	F05	Q7W, V15E		
B04	V15E	D05	**Parental**	F06	Q7W, V15Q	H05	P4L, E6X, Q7W, I8V, I10Z, L13V, V15E, F16L
B05	V15G	D06	P4T, V15Q	F07	I8V, I10Z		
B06	V15H	D07	P4T, V15T	F08	I8V, L13V	H06	R2K, P4L, R5P, E6X, Q7W, I8V, I10Z, L13V, V15E, F16L
B07	V15Q	D08	P4V, V15T	F09	I8V, V15E		
B08	V15S	D09	P4Z, V15T	F10	I8V, V15Q	H07	V15C, del16-17
B09	V15T	D10	R5P, V15T	F11	I10Z, F16L	H08	Q7C, C17A
B10	F16E	D11	R5V, V15T	F12	L13I, F16L	H09	E6C, C17A
B11	F16I	D12	E6H, V15T	G01	L13V, F16L	H10	R5C, C17A
B12	F16L	E01	E6X, F16P	G02	L13V, V15E	H11	P4C, C17A
C01	F16Q	E02	E6X, L13V	G03	V15T, F16E	H12	R3C, C17A

- **I8V**, **V15T** and **F16L** result in the **highest increase** in **potency** (with I8V > V15T > F16L)
- **No paired effects** observed for double or triple mutants, effect of single mutations additive when combined

Mutant peptides maintain activity across related sarbecoviruses

Pseudovirus neutralization

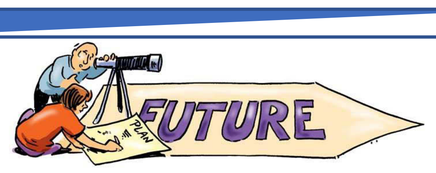


- **WIV-16** is a **bat coronavirus** that has been marked as a likely candidate for **zoonotic transmission** to humans [2]
- **MERS-CoV spike** contains **sequence differences** in the **peptide binding pocket** compared to SARS-CoV-2 spike [3]

Summary

- We found multiple **point mutations** that **improved** the **potency** with which our peptide **inhibits SARS-CoV-2** pseudovirus **infection**, most notably **I8V** and **V15T**
- When mutations were combined the **effect** on **potency** was **additive**
- Increase in potency went hand in hand with **increase** in **solubility**, likely because of the focus on mutations to soluble resiudes in the selection of the first set of 96 peptides
- A small set of our most promising hits showed **broad activity**, targeting **closely related coronaviruses** as well

Outlook



- **Potency** tests on **actual viruses**
- Further improve **solubility** of peptides
- For future deep mutational scans: see if additive nature of point mutations translates across all peptides / targets
- If so, a logical workflow would be: synthesize all peptides with point mutations that are tolereated in the deep mutational scan, then test for a metric and combine the best mutations

References

1. Thijssen, V. et al. A broad-spectrum macrocyclic peptide inhibitor of the SARS-CoV-2 spike protein. Proceedings of the National Academy of Sciences 120, e2303292120 (2023).
2. Yang, X.-L. et al. Isolation and Characterization of a Novel Bat Coronavirus Closely Related to the Direct Progenitor of Severe Acute Respiratory Syndrome Coronavirus. Journal of Virology 90, 3253–3256 (2016).
3. Hatmal, M. M. et al. Comprehensive Structural and Molecular Comparison of Spike Proteins of SARS-CoV-2, SARS-CoV and MERS-CoV, and Their Interactions with ACE2. Cells 9, 2638 (2020).