Corso di laurea in Scienze Biologiche
Corso di laurea magistrale in Scienze Biomolecolari e dell'Evoluzione

Materiale didattico di supporto

Tutto il materiale fornito a supporto delle lezioni e reperibile nel minisito dell'insegnamento o sulla piattaforma online UniFE deve essere inteso come traccia degli argomenti svolti e non sostituisce il libro di testo.

Sommario Macromolecole Covid 19 (in pillole) 2

Le proteasi

- sia quelle delle cellule (polmonari) umane bersaglio capaci di "maturare" ed attivare lo spike e la fusione virus –cellula
- che quelle codificate dal genoma virale capaci di produrre singole proteine dalle grandi ORF (poliproteine)

sono state identificate, caratterizzate e sono stati identificati inibitori molto efficaci

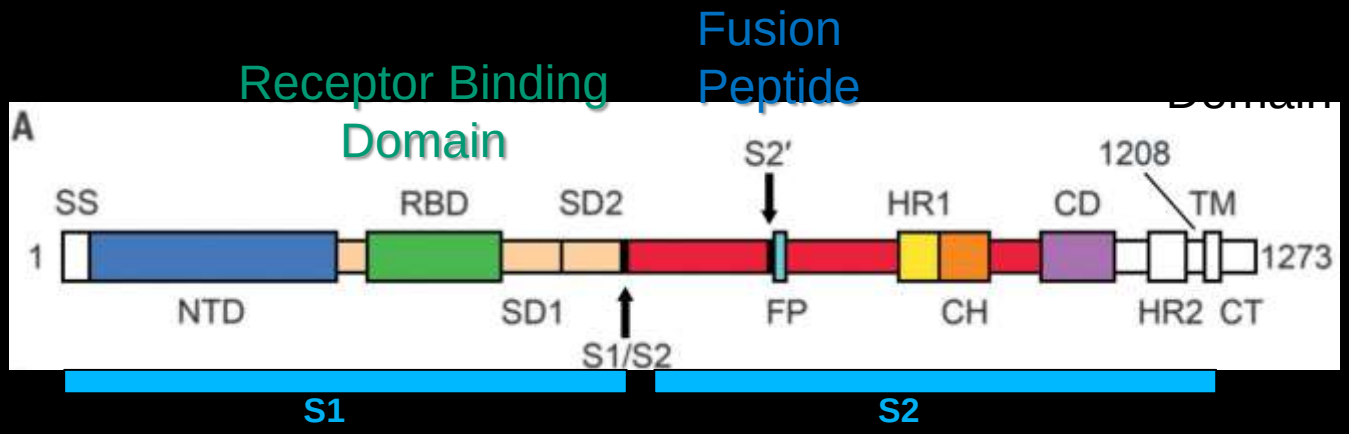
Queste informazioni permettono di sviluppare molecole

(inibitori delle proteasi)

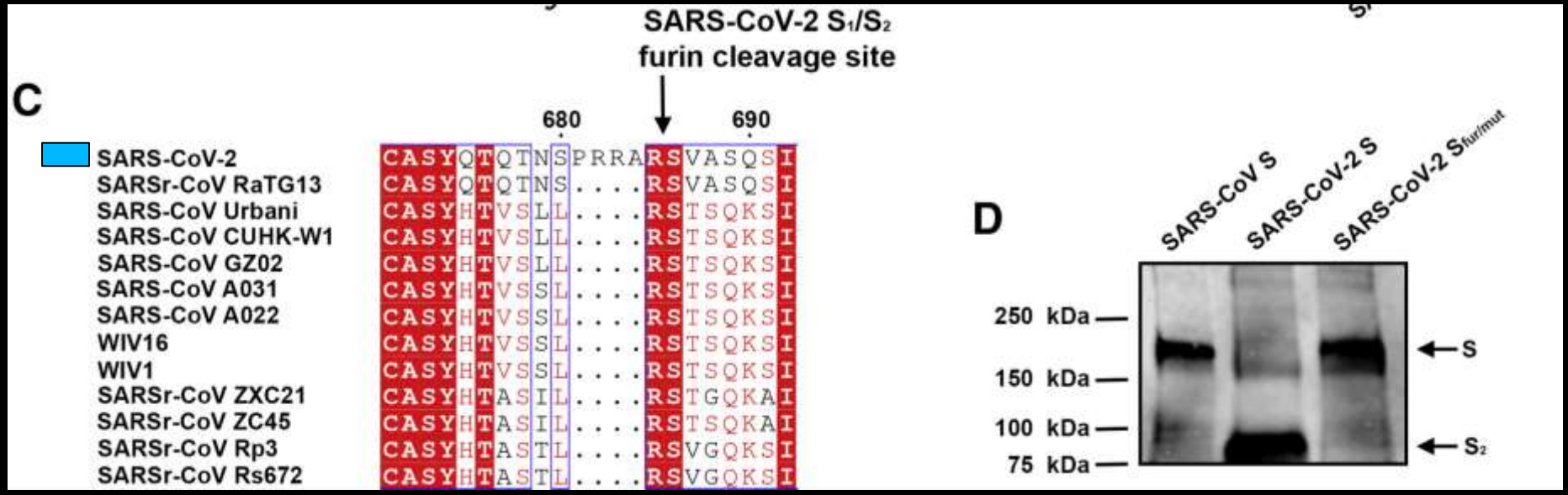
per inibire l'attacco o ridurre lo sviluppo virale dopo l'attacco

Lo «Spike»- struttura e …maturazione proteolitica

S is cleaved at the boundary between the S1 and S2 subunits, which remain non-covalently bound in the prefusion conformation



an S1/S2 furin cleavage site in this novel COV-2 coronavirus



SARS-CoV-2 S glycoprotein harbors a furin cleavage site at the boundary between the S1/S2 subunits, which is processed during biogenesis
SARS-CoV-2 S differs from SARS-CoV and SARS-related CoVs

Cell DOI: (10.1016/j.cell.2020.02.058)

IPOTESI

A polybasic cleavage site (RRAR) in the fusion glycoprotein of SARS-CoV-2 could putatively expand its tropism and/or enhance its transmissibility, compared with SARS-CoV

Sostenuta da

due to the near-ubiquitous distribution of furin-like proteases and their reported effects on other viruses

SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor

Markus Hoffmann, Hannah Kleine-Weber, Simon Schroeder, Nadine Krüger, Tanja Herrler, Sandra Erichsen, Tobias S. Schiergens, Georg Herrler, Nai-Huei Wu, Andreas Nitsche, Marcel A. Müller, Christian Drosten, Stefan Pöhlmann

Cell

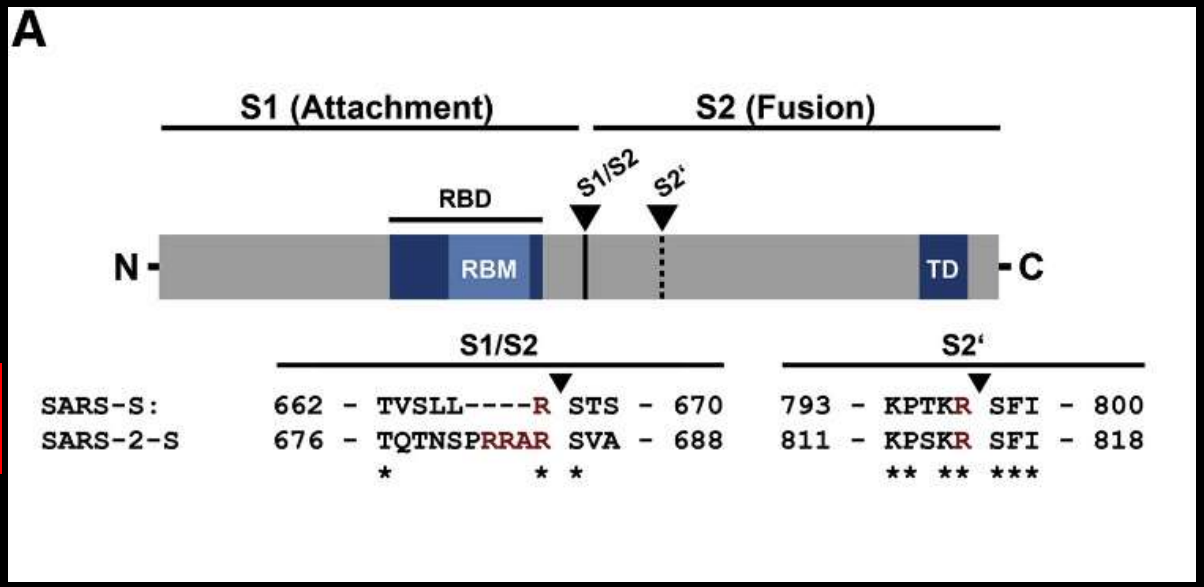
DOI: 10.1016/j.cell.2020.02.052

The spike (S) protein of coronaviruses facilitates viral entry into target cells.

Entry depends on binding of the surface unit, S1, of the S protein to a cellular receptor (ACE2), which facilitates viral attachment to the surface of target cells.

In addition, entry requires S protein priming by cellular proteases, which cleave S protein at the S1/S2 and the S2' site and allows fusion of viral and cellular membranes, a process driven by the S2 subunit

Figure 1



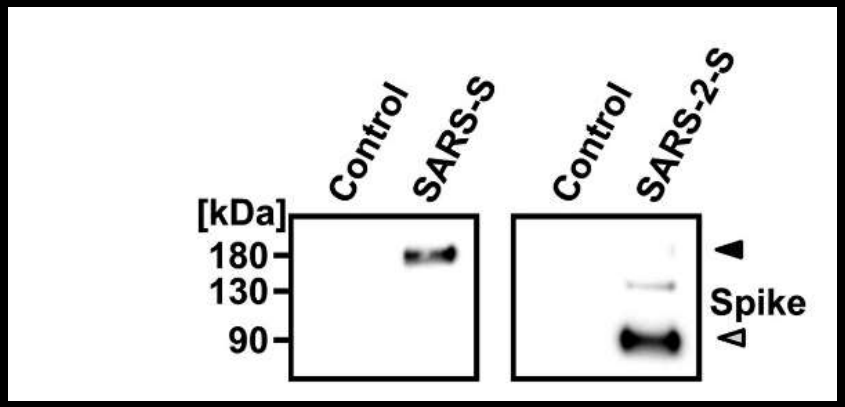
SARS-CoV
SARS-CoV-2

presence of several arginine residues at the S1/S2 cleavage site of SARS-2-S but not SARS-S

In contrast, the S2′ cleavage site of SARS-2-S was similar to that of SARS-S.

Cell DOI: (10.1016/j.cell.2020.02.052)

Figure 1



efficient proteolytic processing of SARS-2-S in human cells, in keeping with the presence of several arginine residues at the S1/S2 cleavage site of SARS-2-S but not SARS-S

Cell DOI: (10.1016/j.cell.2020.02.052)

INFORMAZIONI DA STUDI PRECEDENTI

SARS-CoV can use the endosomal cysteine proteases cathepsin B and L (CatB/L) and the serine protease TMPRSS2 for S protein priming in cell

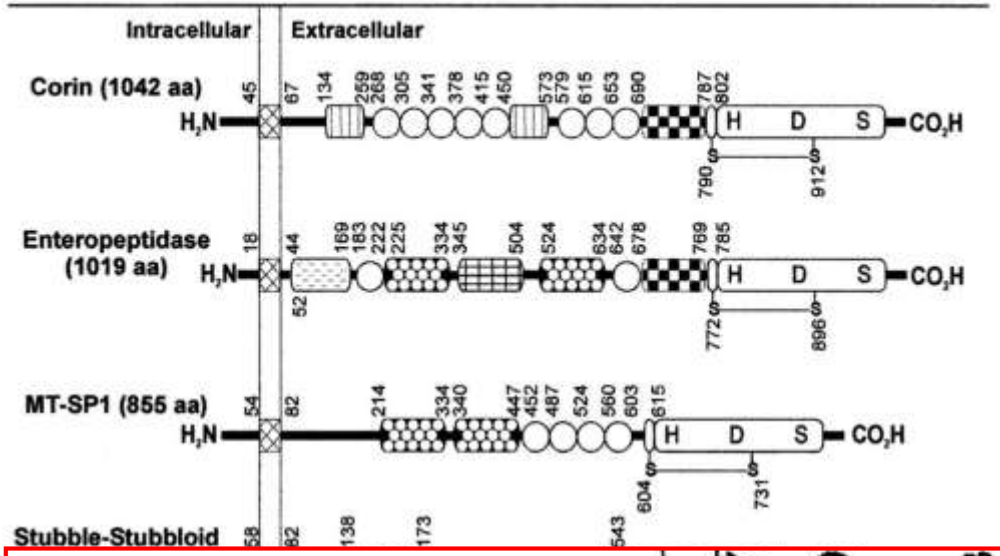
Inhibition of both proteases is required for robust blockade of viral entry

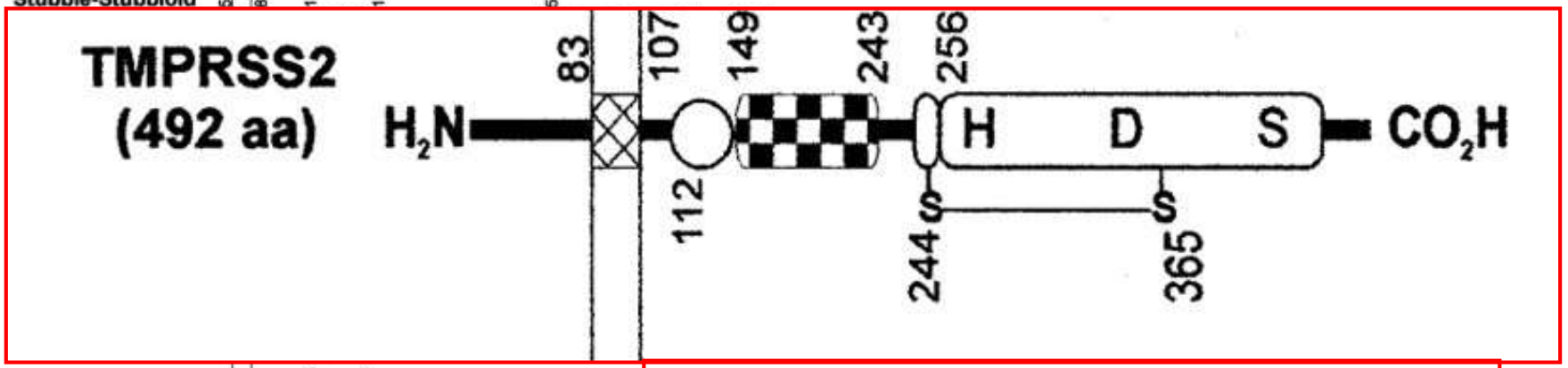
Only TMPRSS2 activity is essential for viral spread and pathogenesis in the infected host whereas CatB/L activity is dispensable

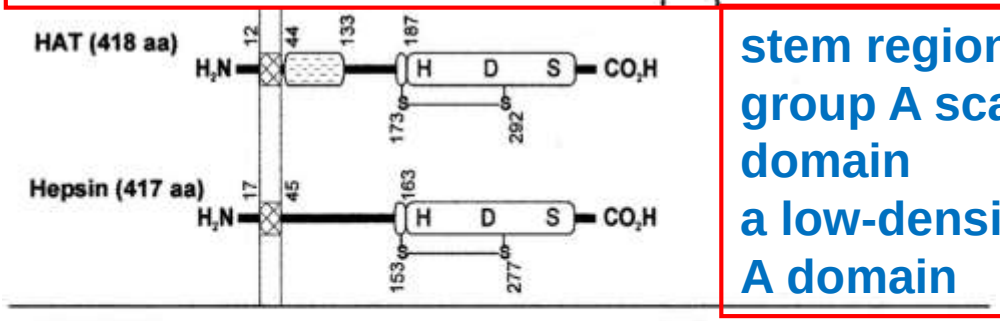
Quesito sperimentale

Does SARS-2-S employ TMPRSS2 for S Protein priming in Human Lung Cells?

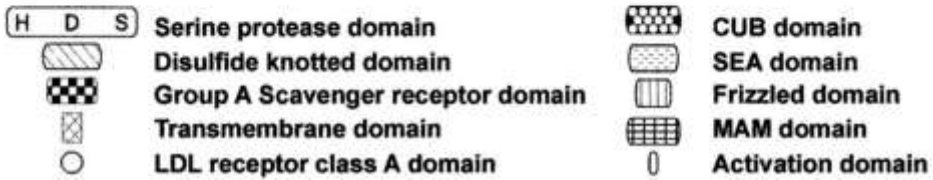
Type II transmembrane trypsin-like serine proteases (TTSPs)

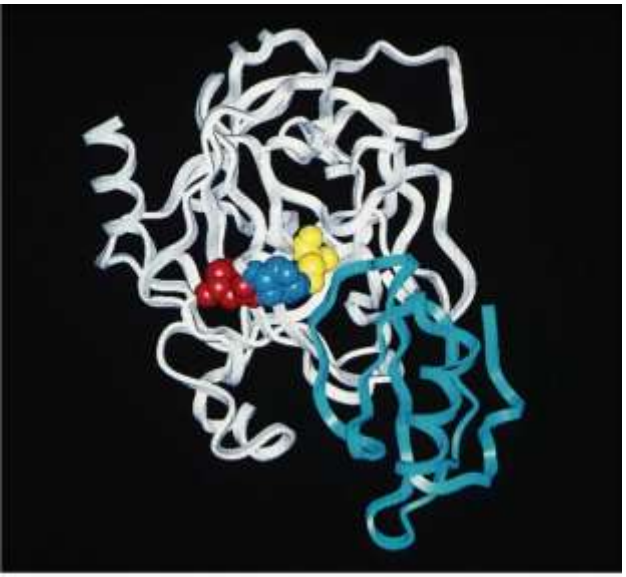


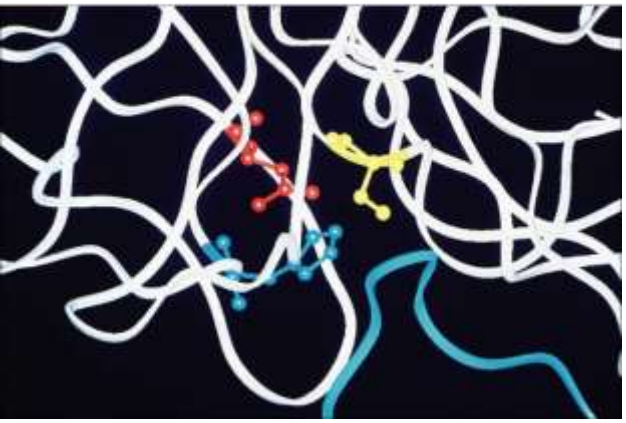


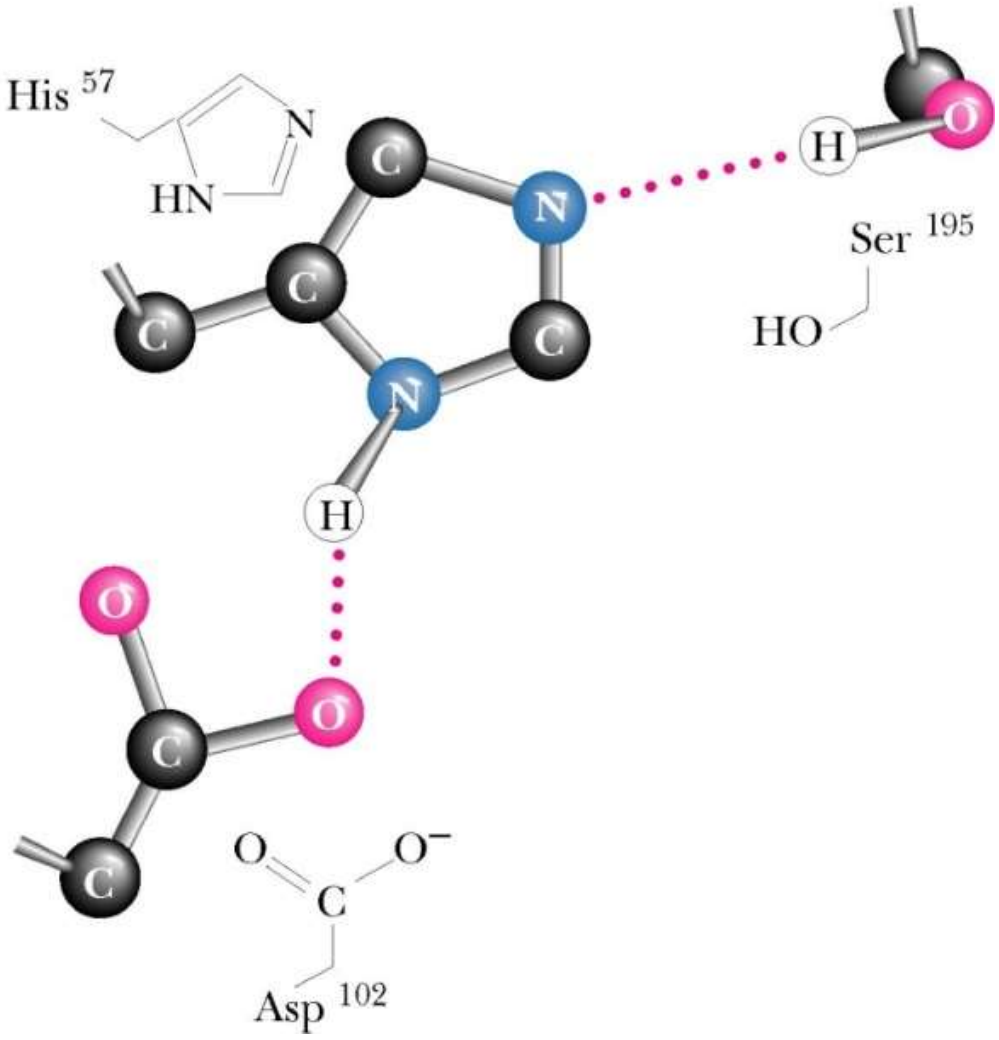


stem region
group A scavenger receptor cys-rich domain
a low-density lipoprotein receptor class A domain









Tre residui polari (His [57], Asp[102], Ser[195]) formano la cosiddetta *triade catalitica* in corrispondenza del sito attivo dell'enzima

TransMembranePRotease - TMPRSS2

Cell surface proteolysis has emerged as an important mechanism for the generation of biologically active proteins that mediate a diverse range of cellular functions.

These enzymes are ideally positioned to interact with other proteins on the cell surface as well as soluble proteins, matrix components, and proteins on adjacent cells.

These membrane-spanning proteases have cytoplasmic N-terminal domains, suggesting possible functions in intracellular signal transduction.

They contain six conserved cysteine residues within their catalytic domain, which form three intradomain disulfide bonds.

They have high affinity towards substrates containing an **Arg residue in the P1 position compared to Lys**

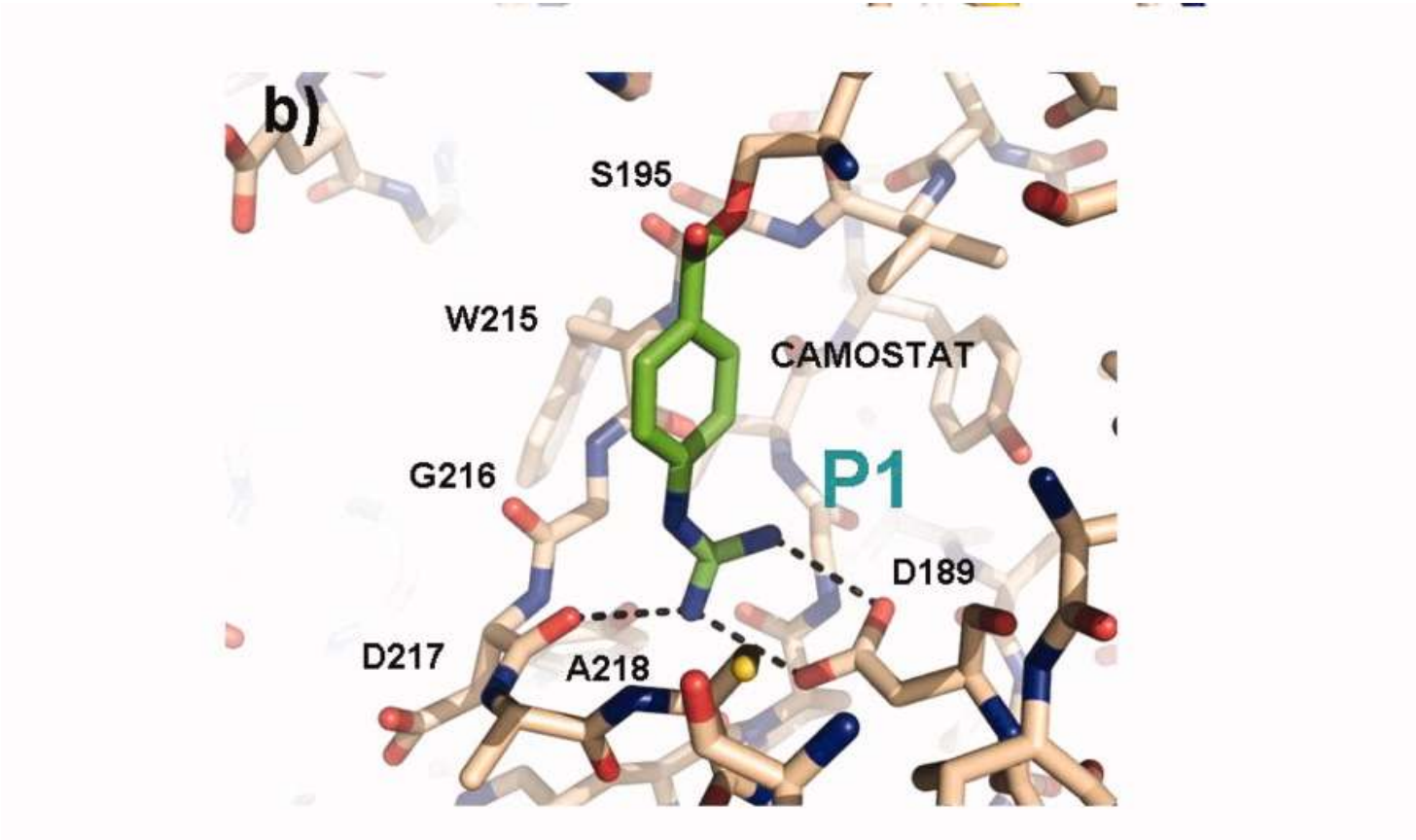
SARS-2-S Employs TMPRSS2 for S Protein Priming in Human Lung Cells

Human Lung cells were pre-incubated with the indicated concentrations of camostat mesylate and subsequently inoculated with pseudovirus

camostat mesylate blocks the activity of the protease TMPRSS2

Cell DOI: (10.1016/j.cell.2020.02.052)

Serine Active site inhibited by camostat



Protein Science, Volume: 18, Issue: 5, Pages: 1081-1094, First published: 16 March 2009, DOI: (10.1002/pro.118)

SARS-2-S Employs TMPRSS2 for S Protein Priming in Human Lung Cells

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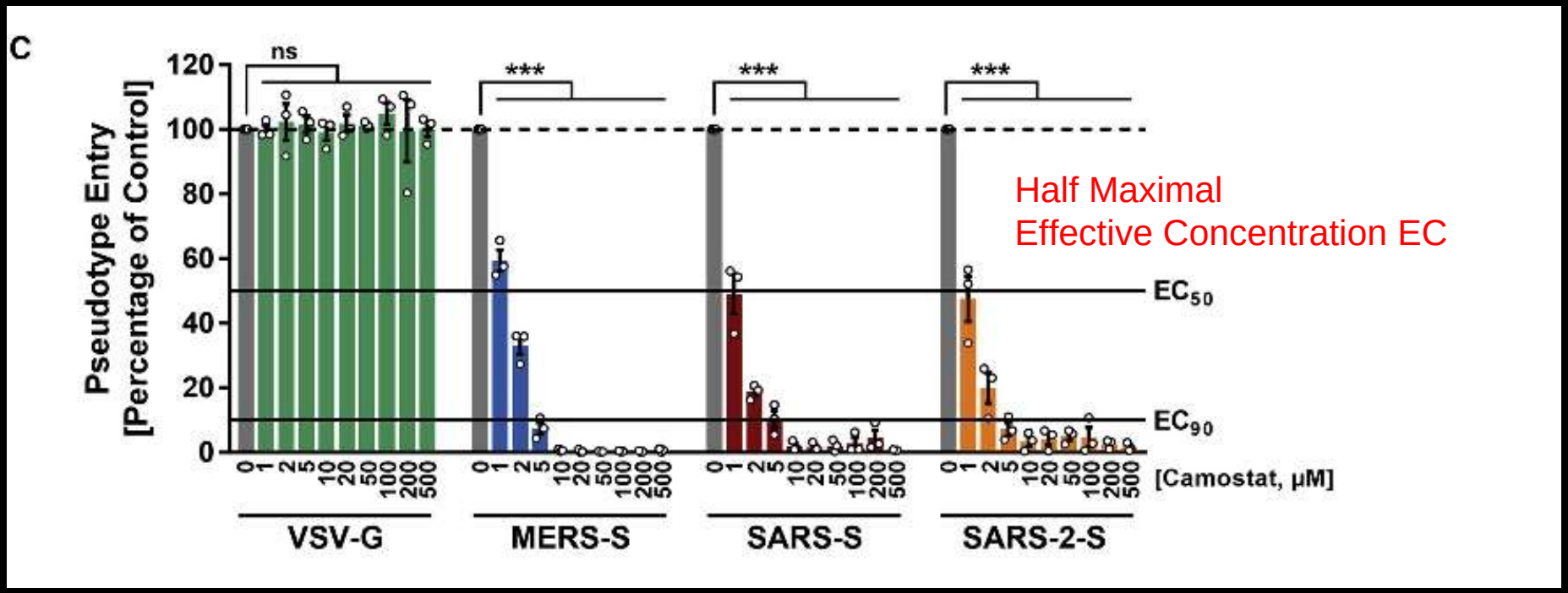
camostat mesylate blocks the activity of the protease TMPRSS2

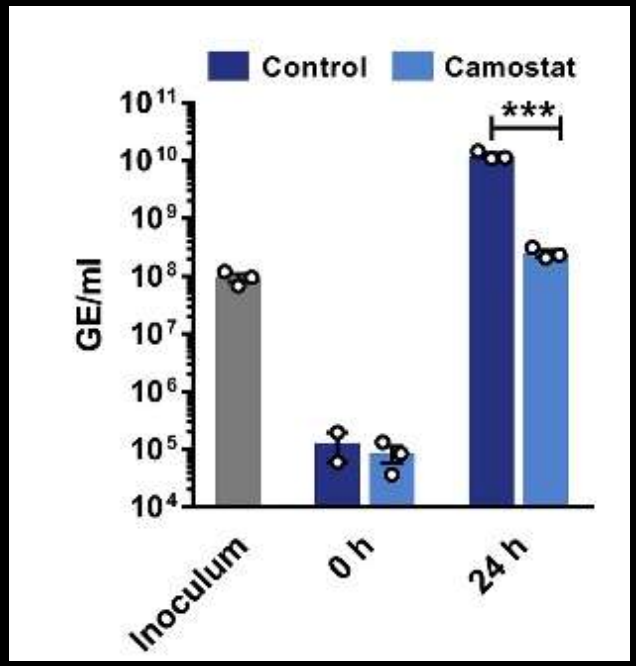


camostat mesylate significantly reduced MERS-S-, SARS-S-, and SARS-2-S

Cell DOI: (10.1016/j.cell.2020.02.052)

Figure 4

SARS-2-S Employs TMPRSS2 for S Protein Priming in Human Lung Cells



Human Lung cells were pre-incubated with camostat mesylate and infected with SARS-CoV-2.

Genome equivalents (GE) in culture supernatants were determined by quantitative RT-PCR.

camostat mesylate blocks the activity of the protease TMPRSS2

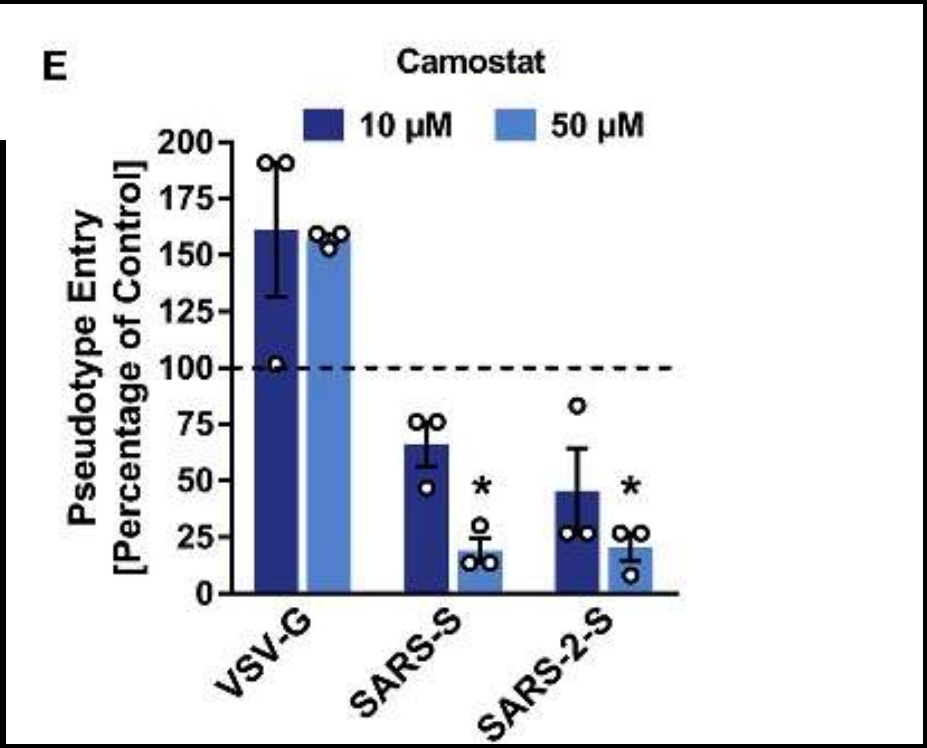
camostat mesylate treatment significantly reduced Lung Cell Calu-3 infection with authentic SARS-CoV-2 !!

Cell DOI: (10.1016/j.cell.2020.02.052)

Figure 4

SARS-2-S Employs TMPRSS2 for S Protein Priming in Human Lung Cells

camostat mesylate blocks the activity of the protease TMPRSS2



In order to investigate whether serine protease activity is required for SARS-2-S-driven entry into human lung cells, **primary human airway epithelial cells** were incubated with camostat mesylate prior to transduction.

camostat mesylate treatment inhibited SARS-S- and SARS-2-S- entry into primary human lung cells

Cell DOI: (10.1016/j.cell.2020.02.052)

We analyzed whether TMPRSS2 usage is required for SARS-CoV-2 infection of lung cells.

SARS-CoV-2 can use TMPRSS2 for S protein priming and camostat mesylate, an inhibitor of TMPRSS2, blocks SARS-CoV-2 infection of lung cells and exerted no unwanted cytotoxic effects

APPROCCIO TERAPEUTICO Premesse

- TMPRSS2 is dispensable for development and homeostasis (Kim et al., 2006) and thus constitutes an attractive drug target.

Mol Cell Biol. 2006 26(3) Phenotypic analysis of mice lacking the Tmprss2-encoded protease. Kim TS

We generated Tmprss2-/- mice by disrupting the serine protease domain through homologous recombination. Compared to wild-type, Tmprss2-/- mice developed normally, survived to adulthood with no discernible abnormalities in organ histology or function

- The serine protease inhibitor camostat mesylate, which blocks TMPRSS2 activity has been approved in Japan for human use.

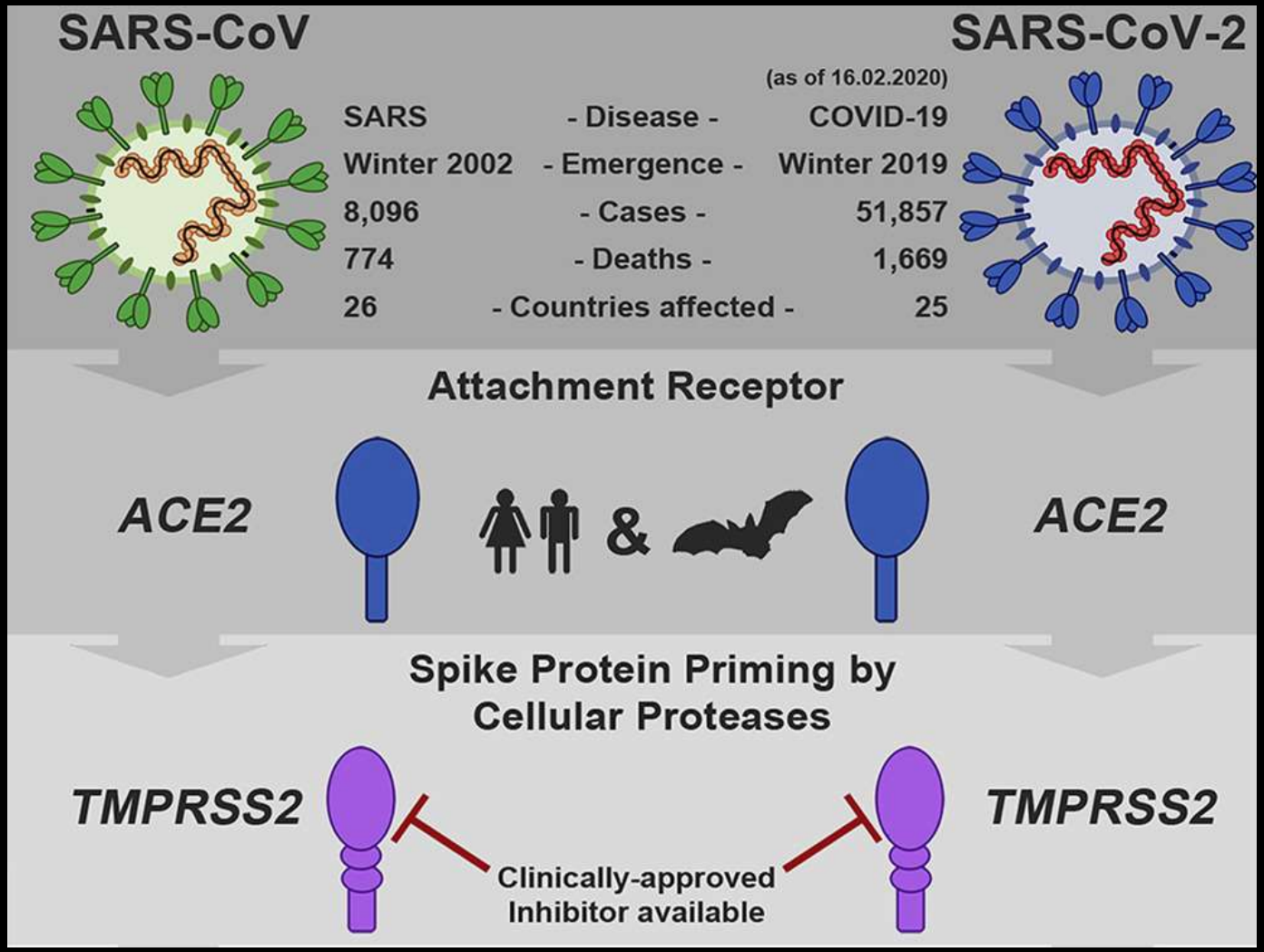
Digestion. 2019;99(4).Camostat Mesilate Therapy Improves Epigastric Pain in Early Chronic Pancreatitis Yamawaki H

APPROCCIO TERAPEUTICO

TMPRSS2 is a host cell factor that is critical for spread of several clinically relevant viruses, including influenza A viruses and coronaviruses

The present study provides evidence that host cell entry of SARS-CoV-2 can be blocked by a clinically proven inhibitor of the cellular serine protease TMPRSS2

These results reveal a target for therapeutic intervention Camostat Mesilate or related compounds with potentially increased antiviral activity could thus be considered for treatment of SARS-CoV-2-infected patients.



Cell DOI: (10.1016/j.cell.2020.02.052)

Sommario Macromolecole Covid 19 (in pillole) 2

Le proteasi

- codificate dal genoma virale e capaci di produrre singole proteine dalle grandi ORF (poliproteine)

sono state identificate, caratterizzate e sono stati identificati inibitori molto efficaci

Queste informazioni permettono di sviluppare molecole

(inibitori delle proteasi)

per inibire l'attacco o ridurre lo sviluppo virale dopo l'attacco

SARS-CoV polyprotein proteolytic maturation

Polyprotein proteolytic maturation is primarily achieved by the 33.1-kD HCoV main proteinase (**Mpro**)

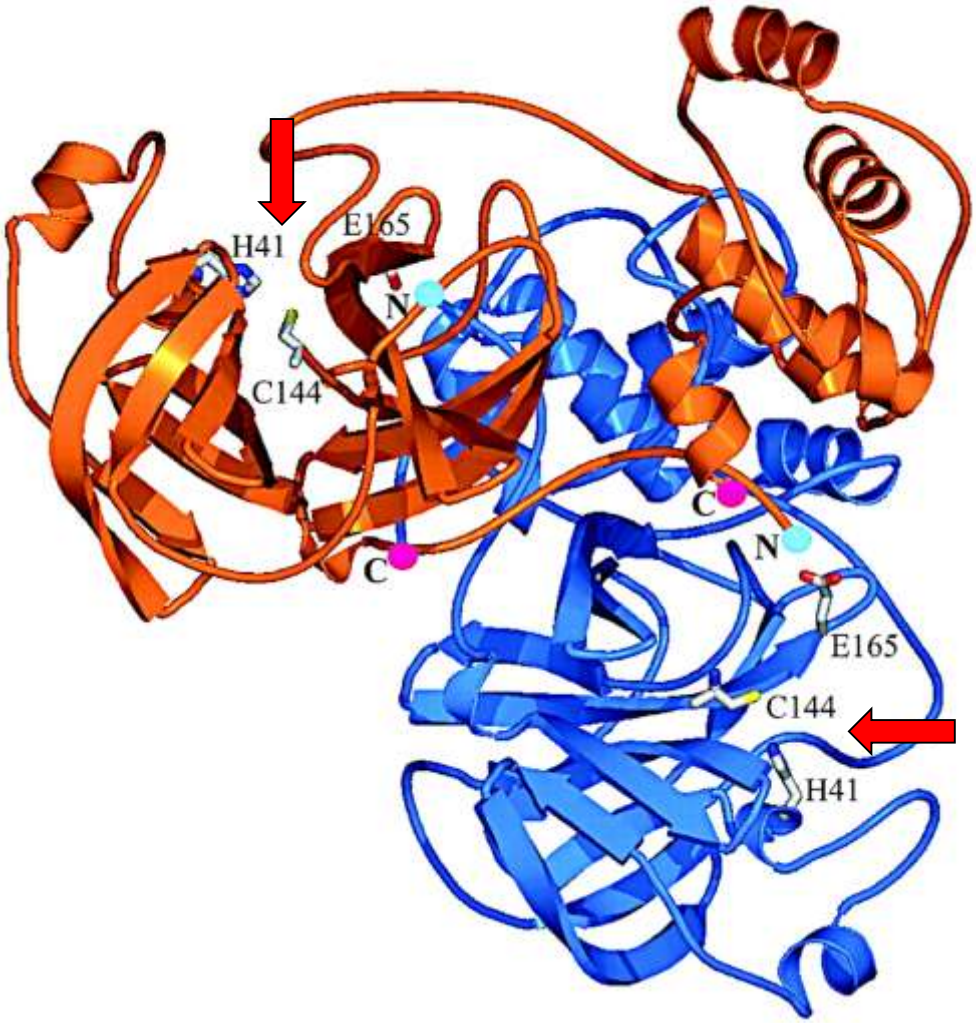
The Mpro (3CLpro) cleaves the polyprotein at no less than **11 conserved sites** involving Leu Gln / (Ser,Ala,Gly) sequences

Cleavage pattern appears to be conserved in the Mpro from SARS coronavirus (SARS-CoV)

SARS-CoV polyprotein proteolytic maturation

The functional importance of Mpro in the viral life cycle makes this proteinase an attractive target for the development of drugs directed against coronavirus infections.

Fig. 2. Dimer of HCoV Mpro.

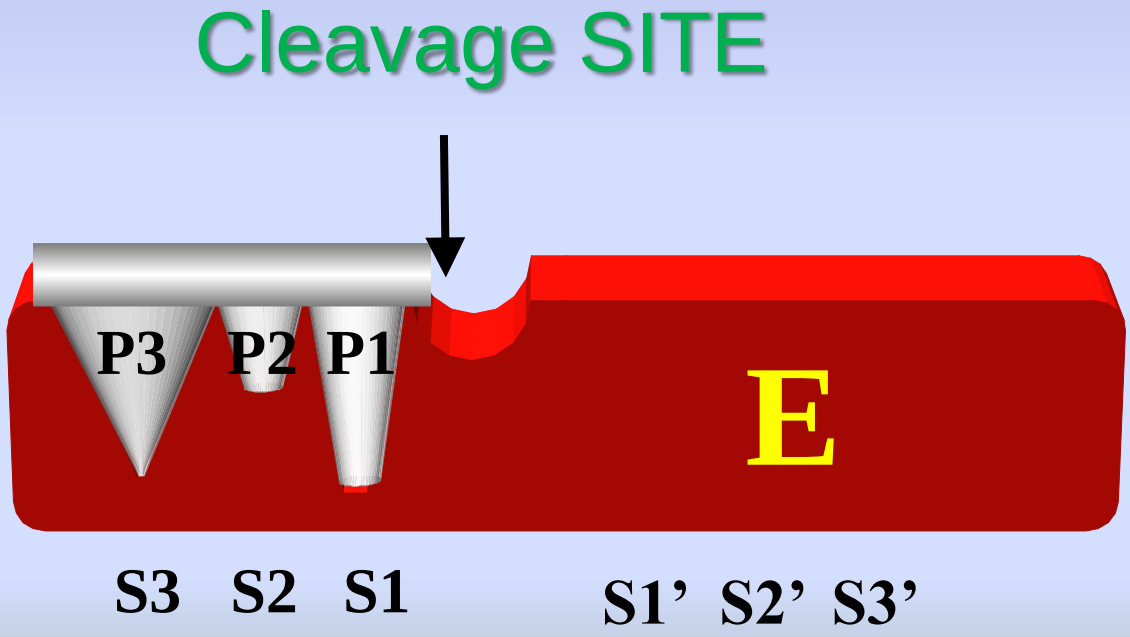


American Association for the Advancement of Science

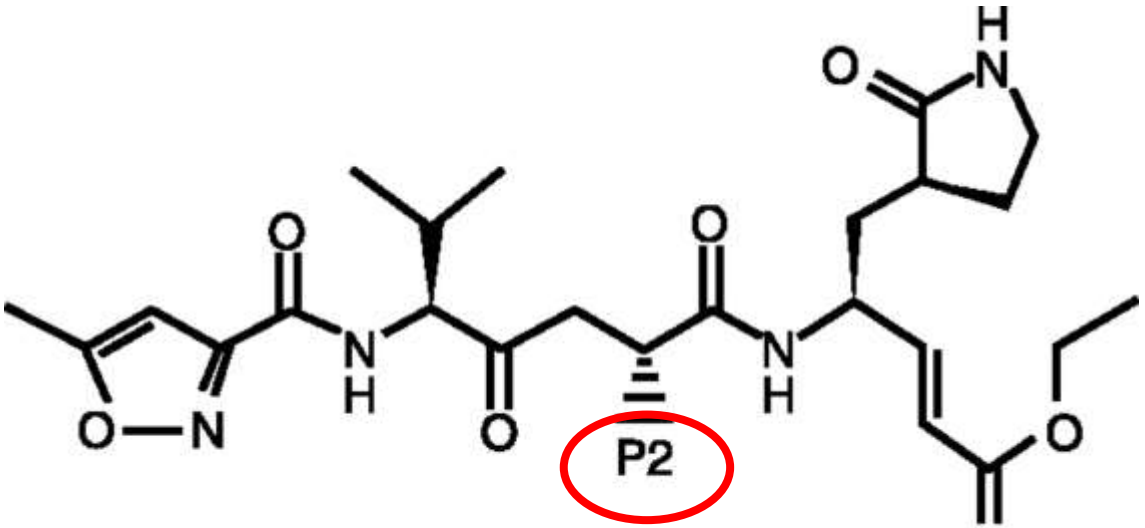
Kanchan Anand et al. Science 2003;300:1763-1767

Science
AAAS

SARS-CoV polyproteins have three noncanonical Mpro cleavage sites with Phe, Met, or Val in the P2 position



Mpro inhibitor



P2 = *p*-fluoro-benzyl: AG7088

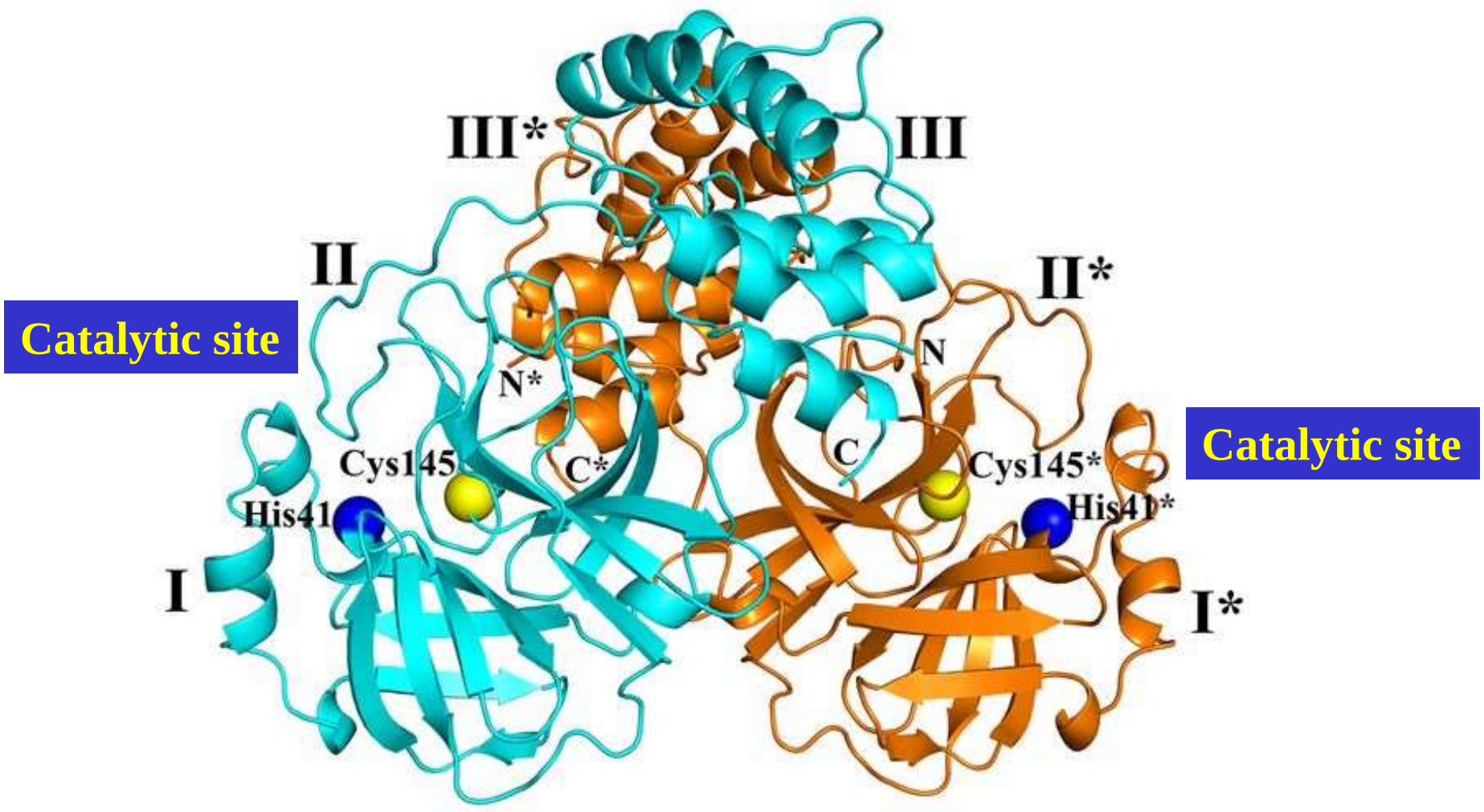
Kanchan Anand et al. Science 2003;300:1763-1767

Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α-ketoamide inhibitors

by Linlin Zhang, Daizong Lin, Xinyuanyuan Sun, Ute Curth, Christian Drosten, Lucie Sauerhering, Stephan Becker, Katharina Rox, and Rolf Hilgenfeld

Science
Volume ():eabb3405
March 23, 2020

Fig. 2 Three-dimensional structure of SARS-CoV-2 Mpro dimer



Linlin Zhang et al. Science 2020;science.abb3405

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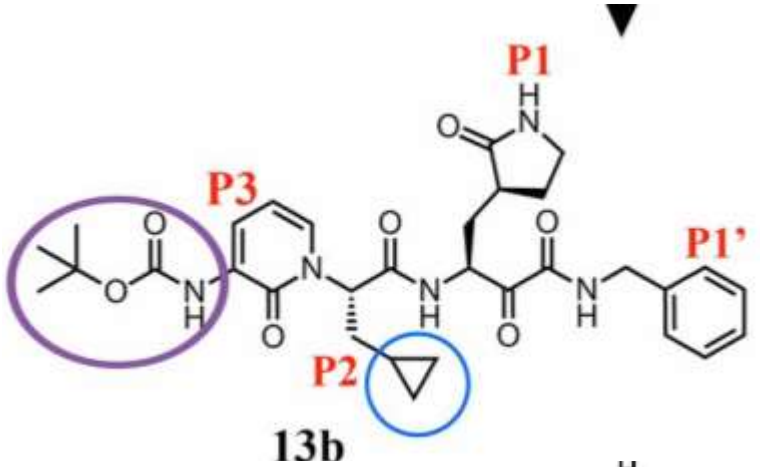
Catalytic efficiency of the protease

SARS-CoV-2 M^{pro} (k_{cat}/K_m = 3426.1 ± 416.9 $s^{-1}M^{-1}$)

SARS-CoV M^{pro} (k_{cat}/K_m = 3011.3 ± 294.6 $s^{-1}M^{-1}$)

Fig. 1 Chemical structures of α-ketoamide inhibitor 13b



P2 = cyclopropyl

Linlin Zhang et al. Science 2020;science.abb3405

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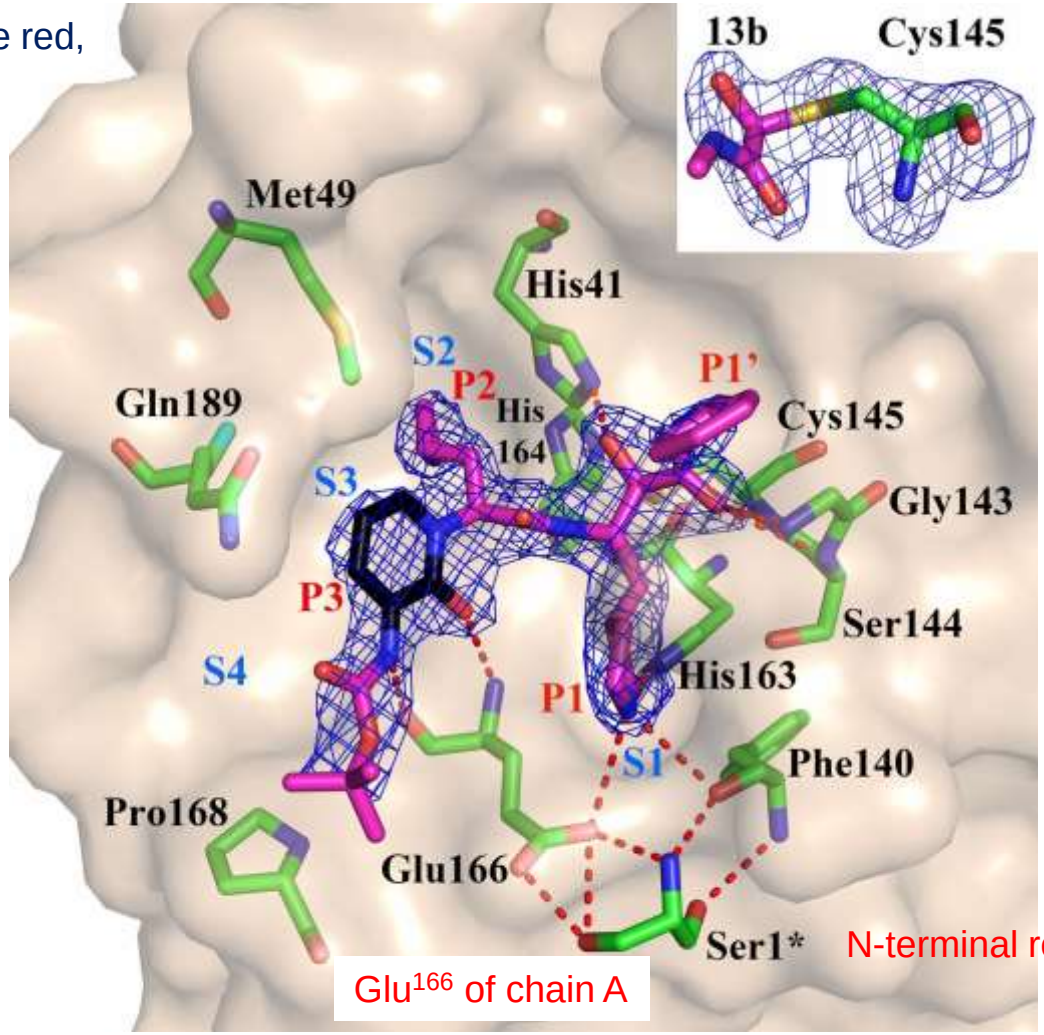
AAAS

Fig. 3 Compound 13b in the substrate-binding cleft located between domains I and II of the Mpro,

oxygen atoms are red,
nitrogens blue,
and sulfur yellow



nucleophilic attack of the
catalytic cysteine onto
the α-carbon of the inhibitor

N-terminal residue of chain B, Ser1*,

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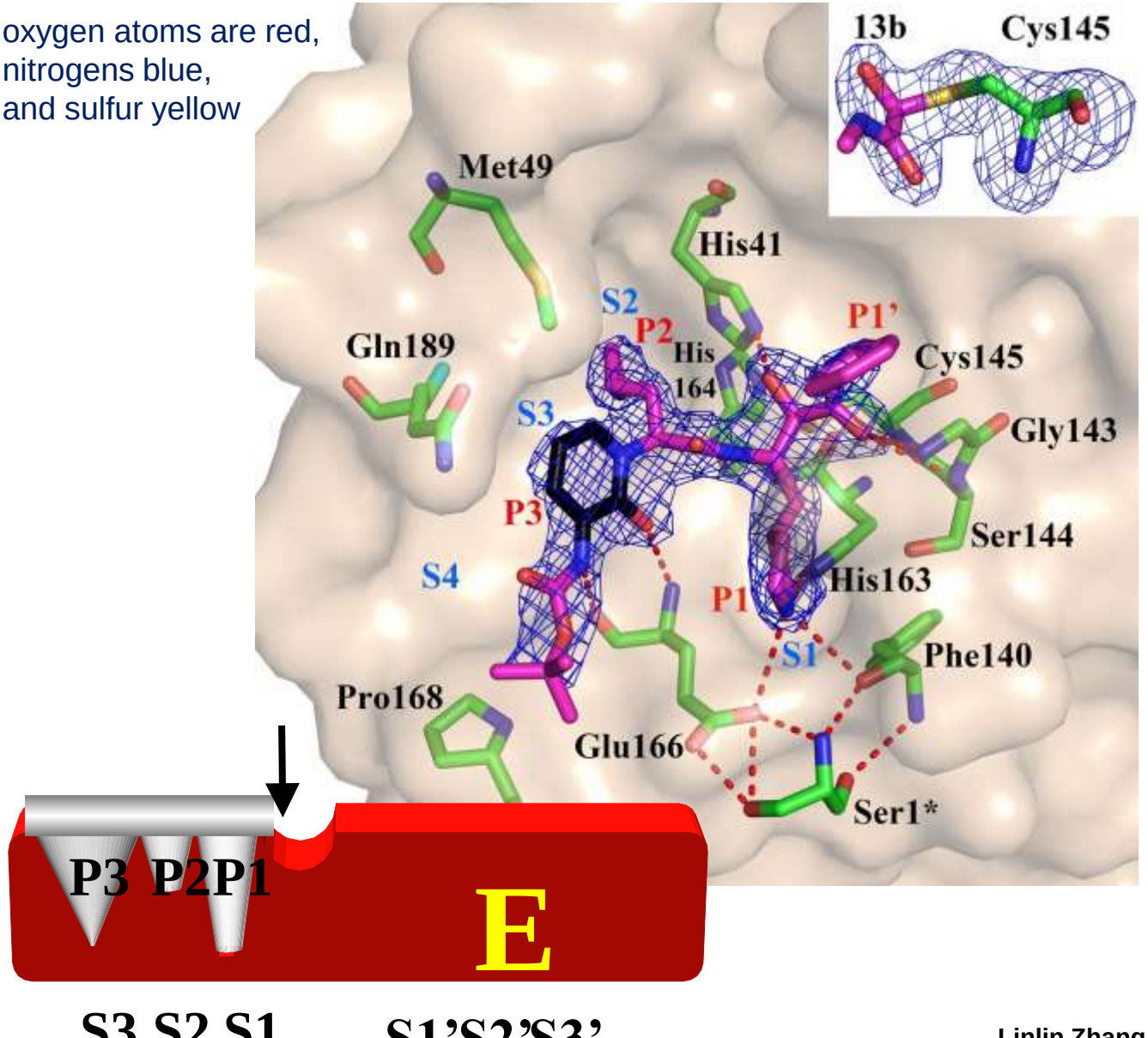
Linlin Zhang et al. Science 2020;science.abb3405

Fig. 3 Compound 13b in the substrate-binding cleft located between domains I and II of the Mpro,

oxygen atoms are red, nitrogens blue, and sulfur yellow



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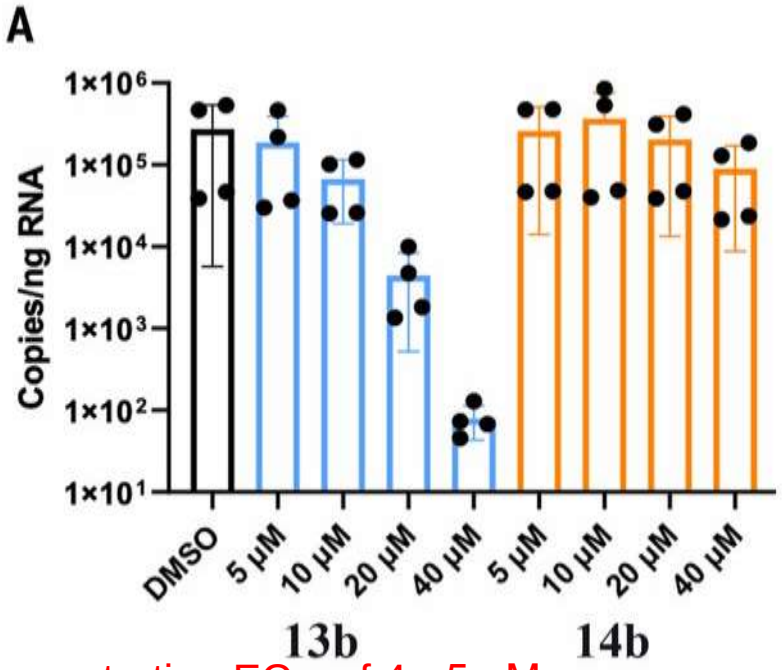
Linlin Zhang et al. Science 2020;science.abb3405

Fig. 4 Compound 13b inhibits SARS-CoV-2 replication in lung cells.

13b inhibits the purified recombinant SARS-CoV-2 M^{pro} with half maximal inhibitory concentration IC_{50} = 0.67 ± 0.18 μM

SARS-CoV-2 RNA 24Hrs



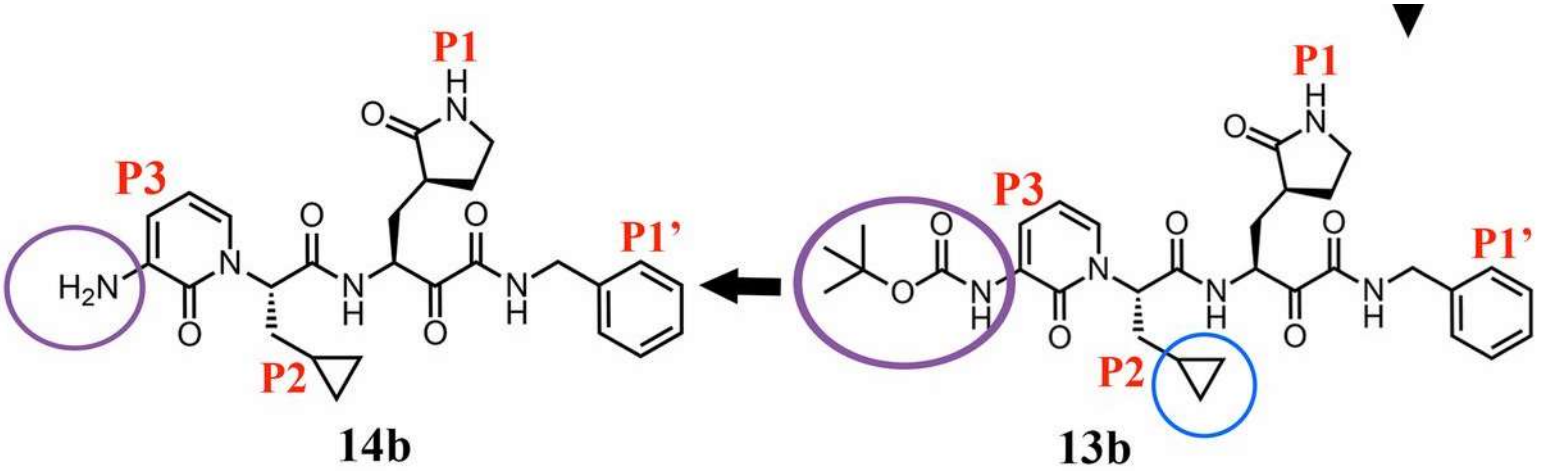
Half Maximal Effective concentration EC_{50} of 4 - 5 μM

Human Calu-3 cells infected with SARS-CoV-2 using an MOI of 0.05

Linlin Zhang et al. Science 2020;science.abb3405

Science AAAS

Fig. 1 Chemical structures of α-ketoamide inhibitors 13b and 14b.



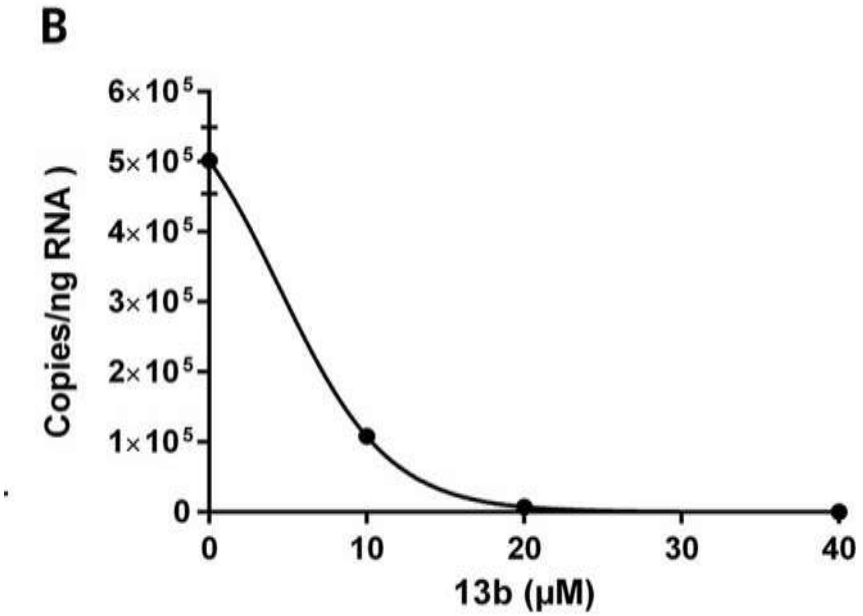
Linlin Zhang et al. Science 2020;science.abb3405

Science
AAAS

Fig. 4 Compound 13b inhibits SARS-CoV-2 replication in human Calu3 lung cells.

dose-response curve

SARS-CoV-2 RNA 24Hrs



RNA replication is inhibited with half maximal effective concentration (EC50)= 1.75 ± 0.25 μM

Linlin Zhang et al. Science 2020;science.abb3405

Science AAAS

Table S3. Pharmacokinetic parameters of 13b after subcutaneous administration 3 mg/kg

		13b 3 mg/kg sc
$t_{1/2}$ [h]		1.8 ± 0.5
T_{max} [h]		0.7 ± 0.3
C_{max} [ng/mL]		126.2 ± 31.0
AUC_{0-t} [ng/mL*h]		306.6 ± 80.4
MRT [h]		2.7 ± 0.8
V_z/F [L/kg]		20.4 ± 5.4
Cl/F [mL/kg/min]		131.6 ± 26.0

$t_{1/2}$: half-life, T_{max}: time point at which maximal concentration is reached, C_{max}: maximal concentration, AUC_{0-t}: area under the cure from time point 0 until t, MRT: mean residence time, V_z: volume of distribution, Cl: clearance, F: fraction/bioavailability

Table S3. Pharmacokinetic parameters of 13a and 13b after subcutaneous administration (20 mg/kg and 3 mg/kg, resp.)

	13a 20 mg/kg sc	13b 3 mg/kg sc
$t_{1/2}$ [h]	1.0 ± 0.1	1.8 ± 0.5
T_{max} [h]	0.4 ± 0.1	0.7 ± 0.3
C_{max} [ng/mL]	334.5 ± 109.2	126.2 ± 31.0
AUC_{0-t} [ng/mL*h]	551.2 ± 67.7	306.6 ± 80.4
MRT [h]	1.6 ± 0.2	2.7 ± 0.8
V_z/F [L/kg]	48.6 ± 7.7	20.4 ± 5.4
Cl/F [mL/kg/min]	565.6 ± 61.0	131.6 ± 26.0

$t_{1/2}$: half-life, T_{max}: time point at which maximal concentration is reached, C_{max}: maximal concentration, AUC_{0-t}: area under the cure from time point 0 until t, MRT: mean residence time, V_z: volume of distribution, Cl: clearance, F: fraction/bioavailability

Lung tropism and administration through inhalation!

During the pharmacokinetic study with 13b, we monitored its **lung tissue** levels. After 4 hours, around 13 ng/g 13b were still found in lung tissue.

This lung tropism is beneficial given that COVID-19 affects the lungs.

In addition to subcutaneous administration, 13b was **nebulized** using an inhalation device at 3 mg/kg.

After 24 hours, 33 ng/g were found in lung tissue. Inhalation was tolerated well and mice did not show any adverse effects, suggesting that direct administration of the compound to the lungs would be possible.

Given these favorable pharmacokinetic results, our study provides a useful framework for development of anticoronaviral inhibitors

Sommario Inibizione Proteasi Covid 19 (in pillole)

Sono state identificati e caratterizzati

- Inibitori (camostat mesylate) della protease TMPRSS2 capace di "maturare" ed attivare lo spike e la fusione virus –cellula in cellule polmonari umane bersaglio. L'inibitore è gia stato impiegato nell'uomo
- Inibitori (13b) della protease **Mpro** codificata dal genoma virale e capace di maturare le proteine virali. 13 b è gia stato saggiato nel topo

Molecole candidate ad inibire l'attacco o ridurre lo sviluppo virale nell'uomo