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CORONA ACCELERATED R&D IN EUROPE

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CARE – Corona Accelerated R&D in Europe

WP1 – Anti-coronavirus drug discovery in phenotypic virus-cell-based assays

D1.3 Reverse-engineered drug-resistant viruses

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Introduction

Reverse genetic (RG) Engineering is a powerful tool in virology that enables the generation of synthetic viruses starting from a virus's genetic sequence rather than the virus itself. This technique allows researchers to precisely manipulate viral genomes, introducing targeted mutations or entirely redesigning genetic structures to study specific gene function, pathogenicity, and replication processes. By recreating viruses from scratch, reverse genetics provides critical insights to allow the study of emerging pathogens such as monitoring viral evolution, dissection of host-virus interactions, creation of attenuated viruses for use as vaccines (including both inactivated and live-attenuated vaccine strains) and also offers a controlled platform to design therapeutics. These techniques have proven valuable in understanding and combating infectious diseases such as influenza and coronaviruses.

There is a great need for therapeutics as part of interventional strategies to control newly emerged diseases, including bridging potential gaps until vaccines are developed and in the treatment of patients. However, antiviral treatments can also be associated with driving the development of drug-resistant viruses, as has been extensively described in the treatment of (chronic/persistent or acute) viral infections caused by HIV, hepatitis B virus, hepatitis C virus, herpesviruses, and influenza [1, 2]. It is therefore important to understand the risk of development of resistance and to characterise therapeutic associated changes in viral genes and proteins. In the context of the CARE project, reverse genetics has been used to generate specific SARS-CoV-2 genomes, enabling the study of how genetic mutations contribute to drug resistance. This is particularly useful for understanding antiviral mechanisms and to help in developing better combinational treatments and to predict how viruses might naturally evolve resistance, allowing for proactive drug design and treatment strategies.

Coronaviruses are positive strand RNA viruses with some of the largest RNA virus genomes ranging from 26.4 to 31.7 kb. They belong to the *Coronaviridae* virus family in the order of *Nidovirales* [3]. They generally cause respiratory tract infections in humans and include common cold coronaviruses, MERS, SARS-CoV and SARS-CoV-2. Genomes of large RNA viruses such as those from coronaviruses are cumbersome to clone and manipulate in bacteria owing to the size and occasional instability of the genome [4-6]. Several strategies have been developed to overcome these issues and allow the generation of reporter and mutant RNA viruses.

The aim of this deliverable is therefore to illustrate the use of reverse-engineered viruses in the process of developing antiviral drugs by highlighting some of the work done within the remit of the CARE project.

Methods

In the context of the CARE project, four strategies for Reverse-Engineering viruses were used and these are outlined below:

Transformation-associated recombination (TAR) cloning [7]

The principle of TAR cloning is based on the ability of yeast to recombine overlapping DNA fragments *in vivo* [8]. A broad range of starting material can be used to generate infectious RNA to rescue viable virus including viral isolates, cloned viral DNA, clinical samples or synthetic DNA. In-yeast genome reconstruction requires one-step delivery of overlapping DNA fragments that cover the viral genome and a TAR vector (Fig. 1). In the case of SARS-CoV-2, the genome was divided into 12 overlapping fragments. These transformed DNA fragments are assembled by homologous recombination in yeast to generate a Yeast Artificial Chromosome (YAC) that contains the full-length viral cDNA

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sequence. In vitro production of infectious-capped viral RNA starts with the isolation of the YAC, followed by plasmid linearization to provide a DNA template for run-off T7 RNA polymerase-based transcription. Virus rescue is then initiated by electroporation of BHK-SARS N cells after which virus production and amplification is carried out by culturing the virus with susceptible Vero-E6 cells [7].

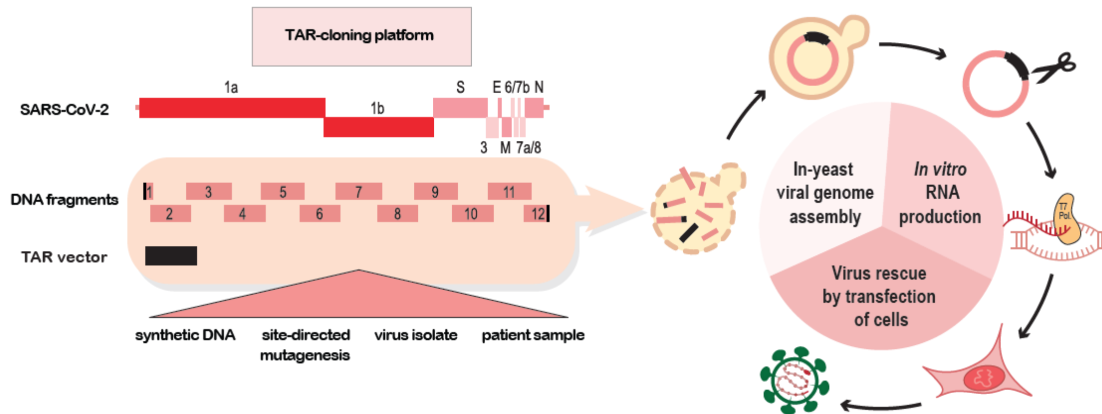

Figure 1. Generation of SARS-CoV-2 by TAR cloning and overview of viral rescue.

Mutations were identified in an ALG-097161 screen [9] that were implicated in development of resistance to nirmaltrelvir. They were incorporated into the SARS-CoV-2 genome by adapting fragment 5 which includes the whole of the 3CLpro sequence. Briefly, newly generated and overlapping PCR products were constructed to introduce the amino acid changes L50F, E166A L157F, or L50F E166A L167E. This was done by taking RNA purified from viral RNA of the virus stock obtained during repeated selection with ALG-097161 and generating cDNA using LunaScript RT SuperMix (NEB). PCRs specifically targeting the 3CLpro gene were then performed using Q5 high-fidelity DNA polymerase (NEB), and the resulting PCR products mixed and matched to produce the desired mutation combinations which were used for TAR cloning and viral production as described above.

Circular polymerase extension reaction (CPER) cloning [10]

Briefly, to generate SARS-CoV-2 by CPER, viral RNA can be used as a template to generate first-strand cDNA (Fig. 2). The cDNA is then used to PCR amplify six fragments that collectively encompass the SARS CoV-2 genome using a high-fidelity DNA polymerase. Each fragment contains overlapping sequences of only 20 nucleotides, selected for high GC content to allow the use of more optimal annealing temperatures. To facilitate DNA circularisation and viral RNA transcription in eukaryotic cells, the linker fragment contains the last 20 nucleotides of SARS CoV-2 3'UTR (that overlap with fragment F6), a polyA tail containing 30 adenines and the hepatitis delta virus ribozyme (HDVr) to generate the authentic 3' end of viral RNA, and SV40 polyA signal for efficient transcription termination, a spacer sequence to separate the functional elements, a CMV promoter for in vivo transcription of viral RNA by the cell RNA polymerase II, and the first 37 nucleotides of SARS-CoV-2 5'UTR (that overlap with fragment F1).

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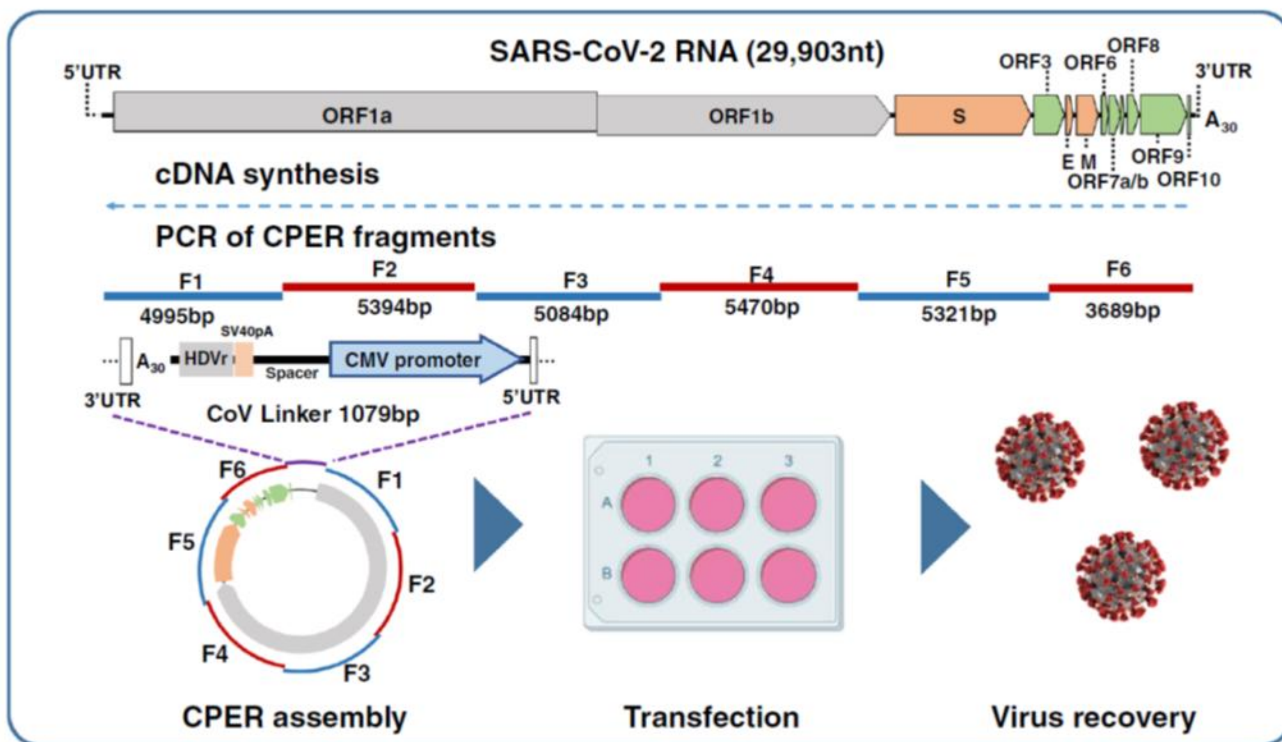

Figure 2. Generation of SARS-CoV-2 by CPER. Schematic of SARS-CoV-2 genome and overlapping SARS-CoV-2 fragments amplified from SARS-CoV-2 cDNA and circularised with a linker fragment containing the last 20 nucleotides of SARS-CoV-2 3'UTR, 30As, hepatitis delta virus ribozyme (HDVr), SV40 pA signal for transcription termination, space sequence, CMV promoter, and first 37 nucleotides of SARS-CoV-2 5'UTR. The resultant SARS-CoV-2 CPER products can then be directly transfected into HEK293T cells, and cocultured with Vero E6 cells for virus recovery [10].

Two-step en passant recombination using BAC vectors [11]

Bacterial artificial chromosomes (BACs) have been widely used to clone large DNA sequences, and this technology has also been applied to coronaviruses [12]. Firstly, a BAC vector is constructed that contains the complete genome of SARS-CoV-2. Next, to facilitate introduction of specific mutations, a donor fragment encompassing the desired modification is designed. On transformation of the BAC into a bacterial host, homologous recombination occurs between the BAC and donor resulting in a modified BAC carrying the new genetic information within the context of the viral genome. In the second step, the modified BAC can undergo further recombination with another BAC or with a donor construct that includes additional modifications (e.g. promotor regions, reporter genes, or mutations). The two-step process allows for the sequential addition of multiple genetic elements without the need for extensive purification or separate cloning procedures. When designing the donor fragments including the required mutations, additional translationally silent marker mutations are introduced near the site of mutagenesis to rule out possible reversions or potential contamination with parental virus. The resulting DNA was used for *in vitro* transcription and virus launching, achieved by transfecting full-length RNA transcripts into BHK-21 cells which were then mixed with susceptible Vero E6 cells. Cell culture supernatants are collected when full cytopathic effects are observed and used for downstream experimentation.

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In vitro Ligation Approach "Seven plasmid" system [13]

This approach is similar to that used to construct infectious clones of SARS-CoV and MERS-CoV [14, 15] which directionally assembles full-length cDNA of the SARS-CoV-2 genome (Fig. 3). Briefly viral RNA is used to produce seven contiguous cDNA fragments which cover the whole viral genome. Each fragment is then individually cloned into a plasmid vector. To facilitate directional assembly, each cDNA fragment is flanked by a class IIS restriction endonuclease site (BsaI or Esp3I) which cleave outside their recognition sequence and generate unique cohesive overhangs. After digestion with BsaI or Esp3I, the seven fragments can be directionally ligated to assemble the genome-length cDNA. The unique cohesive ends of each fragment ensure one directional, seamless assembly of the seven fragments with the concomitant loss of the restriction enzyme sites. A T7 promoter and polyA tail were engineered upstream of fragment 1 (F1) and downstream of F7 respectively so that *in vitro* transcription of the ligated F1-F7 DNA would produce a 5′ capped and 3′ polyadenylated genome-length RNA. To recover recombinant SARS-CoV-2, *in vitro*-transcribed RNA was electroporated in Vero E6 cells.

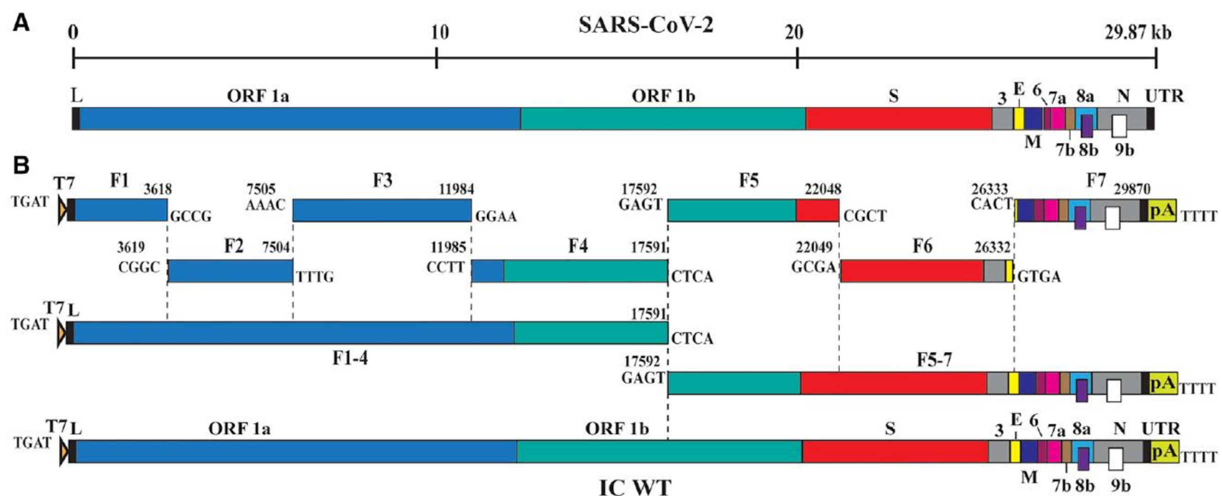

Figure 3. Assembly of a Full-Length SARS-CoV-2 Infection cDNA clone. A) Genome structure of SARS-CoV-2 with the open reading frames from the full genome are indicated. B) A strategy for *in vitro* assembly of an infectious cDNA clone of SARS-CoV-2. The nucleotide sequences and genome locations of the cohesive overhangs are indicated. The WT full-length (FL) cDNA of SARS-CoV-2 (IC WT) was directionally assembled using *in vitro* ligation.

Results

Understanding development pathways of drug resistance through the use of reverse-genetic technology [9] (Partners 21-EDI-IVI and 11-KUL)

The SARS-CoV-2 main protease (3CLpro) has an indispensable role in the viral life cycle and is a therapeutic target for the treatment of COVID-19. 3CLpro cleaves the two polyproteins (pp1a and pp1ab) of SARS-CoV-2 resulting in various nonstructural proteins

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which are key for viral replication [16] and therefore presents itself as a highly desirable drug target [17]. The potential for these inhibitors to select for drug-resistant variants needs to be established. To accomplish this, SARS-CoV-2 was passaged *in vitro* in the presence of increasing concentrations of ALG-097161, a compound designed in a 3CLpro drug discovery programme and inhibits 3CLpro. A combination of amino acid substitutions in 3CLpro were identified (L50F, E166A, L167F) after whole-genome sequencing which correlated with an increased EC_{50} for ALG-097161, and cross-resistance to nirmatrelvir and PF-00835231.

To dissect whether the observed amino acid substitutions in 3CLpro are sufficient to produce the resistance phenotype, virus stocks with individual, or combinations of identified mutations were engineered from an infectious clone [7] by Partner 21-EDI-IVI. Three viruses were constructed (i) L50F, (ii) E166A L167F and (iii) L50F E166A L167E. It was not possible to rescue E166A or L167F. All viruses were sequenced to confirm the presence of the mutations and rule out the presence of any other nonsynonymous mutations in the genome.

To assess if these engineered viruses also exhibited the expected resistance, they were tested with antivirals (Table 1). Interestingly the results demonstrated that L50F on its own is not associated with resistance, while E166A L167F and L50F E166A L167F cause a 4.6x and 10.3x increase respectively, in EC_{50} for ALG-098161. Cross resistance was also found with other known 3CLpro inhibitor, nirmatrelvir and ensitrelvir. Additionally, and importantly, no resistance to the polymerase inhibitor remdesivir was observed, indicating a 3CLpro specific activity as all the viruses remained fully sensitive.

Table 1. Phenotypic resistance associated with the L50F, E166A L167F, and L50F E166A L167F mutation profiles as determined using reverse-engineered SARS-CoV-2 viruses in the USA-WA1 background on VeroE6 cells[a].

Compound	SARS-CoV-2 WT-USA-WA1 EC_{50} (μM) (range) (n = 4)	EC_{50} (μM) [FC][d] (range) L50F (n = 4)	E166A L167V	L50F E166A L167V
ALG-97161	0.60[b] (0.51–0.71)[c]	0.98 [1.6] (0.83–1.1)	2.9 [4.6] (1.5–4.5) (n = 4)	6.2 [10.3] (4.8–7.3) (n = 4)
Nirmatrelvir	0.11 (0.09–0.12)	0.16 [1.5] (0.12–0.2)	1.1 [10.0] (0.67–1.8) (n = 4)	3.2 [29] (2.3–3.5) (n = 6)
Ensitrelvir	0.21 (0.18–0.27)	0.24 [1.1] (0.18–0.31)	8.0 [38] (2.8–12.7) (n = 4)	9.3 [44] (3.5–15) (n = 6)
Remdesivir (GS-441524)[e]	0.86 (0.53–1.1)	1.1 [1.3] (0.8–1.1)	1.5 [1.7] (1.1–1.9) (n = 4)	1.2 [1.4] (0.91–1.7) (n = 6)

[a] EC_{50}, 50% effective concentration. Note: all assays on VeroE6 cells were performed in the presence of 0.5 μM CP-100356.
[b] Median value.
[c] 25th to 75th percentile; EC_{50}, 50% effective concentration.
[d] FC, fold change of EC_{50}.
[e] GS-441524 is the parent nucleoside of remdesivir; it is intracellularly converted to the same active metabolite.

A second experiment was undertaken to confirm the resistance profile of the engineered viruses in the context of a cell-based report assay of SARS-CoV-2 3CLpro enzymatic function [18]. Briefly, in this gain-of-signal assay, inhibition of 3CLpro results in an increased enhanced green fluorescent protein signal. This data confirmed the previous observed loss of potency for ALG-097161 and nirmatrelvir when all three amino acid changes L50F E166A and L167F were introduced into the construct (Table 2).

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Table 2. Phenotypic resistance associated with the L50F E166A L167F mutation profile as determined in a cell-based 3CLpro reporter assay.

Compound	WT EC_{50} (μM) (range)	L50F E166A L167F EC_{50} (μM) [FC][c] (range)
ALG-097161	1.7[a] (1.2–3.8)[b] ($n = 6$)	39 [23] (10–39) ($n = 3$)
Nirmatrelvir	0.96 (0.65–1.2) ($n = 5$)	27 [28] (17–36) ($n = 4$)
PF-00835231	1.6 (1.2–6.0) ($n = 6$)	11 [6.9] (7.6–15) ($n = 3$)

[a]Median value.
[b]25th to 75th percentile.
[c]FC, fold change of EC_{50}.

<u>Investigating the molecular target of the cluster 613 series through the use of reverse-engineering technology (Partner 2-Janssen)</u>

Reverse genetic engineering was used to study the effect of the mutations observed during in vitro resistance selection (IVRS; P10S, T123I and P133S) on the antiviral activity of COVS19954. Individual and double Nsp8 mutations were introduced into the SARS-CoV-2 viral genome using a CPER-based reverse engineering system. The mutant viruses were able to produce viral progeny and had similar growth kinetics compared to the recombinant wild type viruses. In a standard High Content Imaging (HCI)-based antiviral potency shift assay in A549-hACE2 cells, phenotypic resistance was observed for viruses carrying P10S, T123I and P133S mutations, and respectively resulted in EC_{50} shifts of 3-fold, 5-fold and 8-fold. The double mutant T123I P133S resulted in 167-fold resistance to Cluster 613 (Table 3).

Janssen generated further Nsp8 site-directed mutants in the helical region of Nsp8 (E48G, E48V) where cluster 613 compounds may bind. Additionally, Nsp8:T123C was generated based on the observations that another mutation in residue T123 was found through IVRS and that MERS-CoV (which is resistant to cluster 613) possesses a Cysteine at this position. Nsp8:T123C, Nsp8:E48G and Nsp8:E48V did not show potency shifts in the standard HCI assay (Table 3).

To further investigate whether mutations in other viral proteins (e.g., Nsp13, Nsp14) might also contribute to resistance against cluster 613 compounds, additional site-directed mutants were generated (Nsp13:L280F and Nsp14:K304E). These Nsp13 and Nsp14 mutants also did not show reduced potency of cluster 613 compounds (Table 3).

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Table 3. Phenotypic resistance associated with mutations in Nsp8, Nsp13 and Nsp14 reverse-engineered in the SARS-CoV-2/GHB-03021/2020 background (GenBank No. MW368439.1) in A549-hACE2 cells.

Site-directed mutation	EC_{50} shift against COVS19954 (fold change ± SD)	EC_{50} shift against remdesivir (fold change ± SD)
Nsp8: P10S	3.21 ± 0.71	1.46 ± 0.08
Nsp8:T123I	5.18 ± 0.69	1.28 ± 0.23
Nsp8:P133S	8.69 ±0.98	0.98 ± 0.10
Nsp8:P133S+T123I	166 ± 36	1.44 ± 0.11
Nsp8:T123C	0.60 ± 0.09	1.12 ± 0.04
Nsp8:E48G	1.82 ± 0.38	0.83 ± 0.24
Nsp8:E48V	1.60 ± 0.44	0.96 ± 0.16
Nsp13:L280F	1.99 ± 0.26	1.04 ± 0.17
Nsp14:K304E	1.05 ± 0.17	1.11 ± 0.12

Investigating the molecular target of compound CIM-834 through the use of reverse-engineering technology (Partners 11-KUL and 12-LUMC) (Accepted for publication in Nature).

CIM-834 is a small molecule inhibitor that has been demonstrated by Partner 11-KUL to inhibit SARS-CoV-2. CIM-834 was obtained through high-throughput phenotypic antiviral screening followed by medicinal chemistry efforts and target elucidation. CIM-834 inhibits the replication of SARS-CoV-2 (including a broad panel of variants) and SARS-CoV. In SCID mice and Syrian hamsters, intranasally infected with SARS-CoV-2, oral treatment reduces lung viral titres to nearly undetectable levels, even (as shown in mice) when treatment is delayed until 24hr before endpoint. Treatment of infected hamsters prevents transmission to untreated sentinels. Transmission electron microscopy studies demonstrate that virion assembly is completely absent in CIM-834 treated cells. Single-particle cryo-electron microscopy reveals that CIM-834 binds and stabilizes the M protein in its short form thereby preventing the conformational switch to the long form, required for successful particle assembly.

To elaborate on the molecular target of CIM-834, drug-resistant viruses were selected through serial passage of SARS-CoV-2 in the presence of gradually increasing concentration of CIM-834 in an *in vitro* selection assay to identify if any mutations emerge. The treated viruses were then sequenced using whole genome sequencing and identified a number of mutations localised to the M protein. To assess the impact of some of the mutations in terms of viral replication and fitness, reverse genetic engineering was used to construct viruses with individual M protein mutations (M91K, S99A, N117K and P132S). Results are shown in Figure 4. Reverse-engineered SARS-CoV-2 Wuhan with a P132S substitution in the membrane protein gene was constructed by mutating nucleotide positions 26916-26918 from CCG to AGC in a bacterial artificial chromosome (BAC) vector containing a full-length cDNA copy of the genome of SARS-CoV-2 strain SARS-CoV-2/human/NLD/Leiden-0008/2020. This work was performed at partner 12-LUMC. Changes were also reverse-engineered in the SARS-CoV-2 BF.7 (Omicron) backbone, starting from the seven-plasmid reverse genetics system [13] for strain SARS-CoV-2/USA_WA1/2020 [a kind gift from Pei Yong Shi via the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA)] and an RNA extract from a clinical isolate of SARS-CoV-2 Omicron subvariant BF.7 (generously donated by Prof. Piet

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Maes, Rega Institute). This was performed at partner 11-KUL.

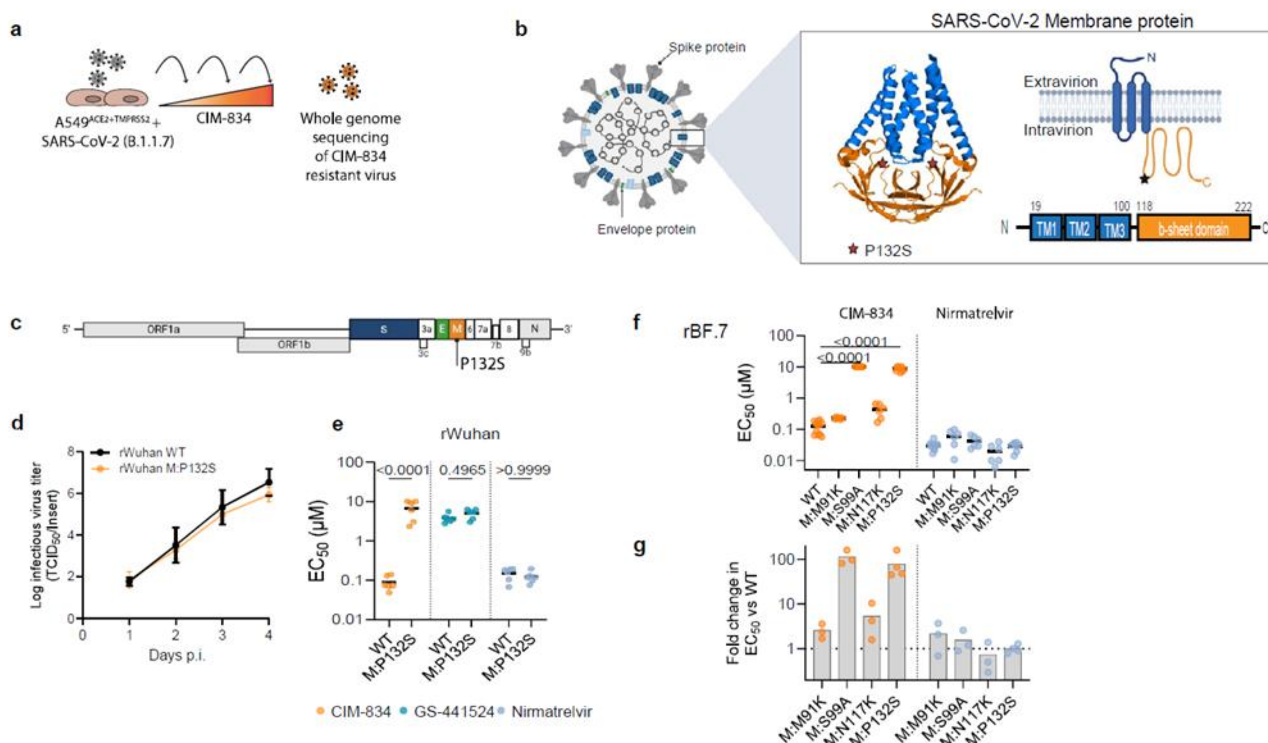

Figure 4. Amino substitutions in the M protein are selected by CIM-834 and are associated with antiviral resistance. a, In vitro resistance selection was performed by passaging SARS-CoV-2 B.1.1.7 in presence of increasing concentrations of CIM-834 in A549ACE2+TMPRSS2 cells. b, The P132S substitution is located in the C-terminal intra-virion domain of the M protein. c, Introduction of M:P132S in the background of SARS-CoV-2 (Wuhan and Omicron BF.7) through reverse genetics. d, Replication kinetics of rWuhan-WT and the M:P132S mutant in human nasal epithelial airway cultures grown at the air-liquid interface. Infectious virus titers from apical washes collected at different time points were determined (mean ± s.e.m., n=6 biologically independent experiments). e, Susceptibility of rWuhan WT and M:P132S for CIM-834 and reference inhibitors GS-441524 and nirmatrelvir, in A549ACE2+TMPRSS2 cells (individual and median values, n=6 biologically independent experiments). Two-way ANOVA with Sidak's multiple comparisons test was used to compare EC50's. f, Level of compound resistance associated with other M substitutions elected in other experiments. Mutations were reverse- engineered in the omicron BF.7 background (individual and median values, n=3 or 4 (P132S) biologically independent experiments with each two technical repeats). Two-way ANOVA with Dunnett's multiple comparisons test was used to compare EC50's. g, Fold changes in EC50 values of M mutants vs WT virus (same data as f, individual and average values, n = 3 or 4 (P132S) biologically independent experiments).

Discussion

Antivirals play a crucial role in alleviating the burden of disease on both patients and healthcare systems, particularly in the early phases following the emergence of a new pathogen. Repurposing existing, approved drugs to target newly identified viruses can significantly shorten the time and cost associated with drug discovery and regulatory approval, enabling rapid emergency use authorisation (EUA). This approach is especially valuable before novel drugs and vaccines become widely available, helping to mitigate the virus's impact.

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During the COVID-19 pandemic, remdesivir received EUA on the 1st May 2020 from the FDA for treating hospitalised patients with severe COVID-19, months before the first vaccine was authorised on the 11th December 2020. As the pandemic progressed, antivirals continued to play a vital role in managing infections, even among vaccinated individuals. Their importance in prophylaxis and post-exposure settings should not be underestimated, as they proved an important tool in controlling outbreaks.

As the COVID-19 pandemic transitions into an endemic phase, the emergence of drug-resistant SARS-CoV-2 variants has offered critical insights into viral evolution and this in turn has influenced treatment strategies. Ongoing viral evolution underscores the urgent need for potent, safe and effective antiviral therapeutics against pathogenic coronaviruses. Notably, SARS-CoV-2 has shown the ability to develop resistance to key antiviral treatments such as remdesivir, molnupiravir, and nirmatrelvir (the active ingredient of Paxlovid) and reports suggest that as many as 45% of patients [19] have a risk of treatment resistance [20], which could lead to rebound with monotherapy [19].

The work outlined here highlights the vital role of reverse genetic engineering in antiviral drugs development. This technology not only helps validate mechanisms of action but also enhances our understanding of how drug resistance emerges. An important first step to monitor clinical drug resistance is to identify the mutations involved. By passaging SARS-CoV-2 in the presence of increasing concentrations of an antiviral drug, potential mutations facilitating resistance can be identified. After sequencing, strategies for reverse-genetic engineering can be used to produce viruses with precise sequence-verified mutations for use in downstream studies. Within the CARE project, 16 distinct drug-resistant viruses were successfully rescued providing invaluable data for future research.

Initiatives like the CARE project have significantly expanded our understanding of antiviral resistance, equipping us to respond more effectively to future pandemics and outbreaks. The rapid emergence of drug-resistant variants further emphasises the necessity for combinatorial therapies, as well as second- and third-generation antivirals. On-going research should prioritise the development of broad-spectrum antivirals that target multiple coronaviruses and other RNA virus families, ensuring a more robust defense against emerging threats.

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