Remdesivir, Molnupiravir and Nirmatrelvir remain active against SARS-CoV-2 Omicron and other variants of concern.

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Abstract

The *in vitro* effect of GS-441524, remdesivir, EIDD-1931, molnupiravir and nirmatrelvir against the various SARS-CoV-2 VOCs, including Omicron, was determined. VeroE6-GFP cells were pre-treated overnight with serial dilutions of the compounds before infection. The number of fluorescent pixels of GFP signal, determined by high-content imaging on day 4 post-infection, was used as read-out, and the EC_{50} of each compound on a viral isolate of each VOC was calculated. These experiments were performed in the presence of the Pgp-inhibitor CP-100356 in order to limit compound efflux. A SARS-CoV-2 strain grown from the first Belgian patient sample was used as ancestral strain. All the other isolates were obtained from patients in Belgium as well.

Our results indicate that GS-441524, remdesivir, EIDD-1931, molnupiravir and nirmatrelvir retain their activity against the VOCs Alpha, Beta, Gamma, Delta and Omicron. This is in accordance with the observation that the target proteins of these antivirals are highly conserved.

Main Text

One and a half year after the start of the global COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), multiple variants have emerged. As coronaviruses are constantly evolving, variants were to be expected. These can be harmless or slightly beneficial for the virus, causing for example increased transmission, virulence, or immune escape [1-3]. SARS-CoV-2 genetic diversification was initially considered slow when the virus was spreading in early 2020. The first official *variant*, a single spike D614G mutation found in early European lineages, was linked to more efficient transmission [4] and rapidly spread to become the dominant viral strain worldwide. Late 2020, multiple variants emerged that spiked regional epidemics. Lineages characterized by recurring mutations that carry an increased risk to public health are referred to as 'variants of interest' or 'variants of concern' (VOC). At the moment, five such concern lineages have been identified (Alpha, Beta, Gamma, Delta and Omicron). All have characteristic mutations (www.ecdc.europa.eu/en/covid-19/variants-concern). The spike (S) glycoprotein appears especially prone to accumulate mutations, more particular in the amino (N)-terminal domain (NTD) and in the receptor binding domain (RBD)[5].

As the RBD is essential for the primary contact with the ACE2 receptor on the host cell, mutations in this RBD can alter affinity and thus greatly impact spread of the variant [6]. Such increased transmissibility was very apparent in the Delta variant, which rapidly spread to become the dominant strain. However, according to the first data, Omicron is expected to soon conquer this questionable position in many countries it is spreading extremely fast [7].

Besides being responsible for ACE-2 receptor interaction, the spike protein is also the dominant target of neutralizing antibodies against SARS-CoV-2. Variations can thus alter the antigenicity and promote immune escape, which in turn promotes spread of the variant as well [8]. For example, the previously mentioned E484K mutation has been reported to impact neutralization of post-vaccination sera or antibodies in general. In fact, all of the circulating VOCs have some mutations that favor evasion from the host immune response [9].

Starting in 2020, numerous spike-protein based vaccines were developed and vaccination programs are running at full speed. However, studies of sera and emerging real-world evidence indicate that Omicron escapes the immunity whether from previous infection or vaccination [10].

Several direct-acting antivirals against SARS-CoV-2 have been approved or are advancing in clinical development. They can be divided in two classes, monoclonal antibodies (mAbs) directed against the Spike protein and small molecules interfering with the viral replication machinery. mAbs are administered intravenously but studies are underway to explore intramuscular or subcutaneous administration which would overcome the requirement of hospital settings. Presently, mAbs can be produced at large scale but a single-dose regimen continues to be expensive, particularly for low- and middle-income countries [11]. Recent cell culture data indicates that the SARS-CoV-2 variant of concern (VOC) Omicron is not susceptible to most of these mAbs making it unlikely that their clinical efficacy will be maintained [12, 13].

The direct-acting small-molecule SARS-CoV-2 antivirals that have received approval or emergency use authorization do not target the variable spike-protein but target either the conserved viral RNA-dependent RNA polymerase (RdRp) or the conserved viral main protease (Mpro or 3CL protease). Remdesivir, a monophosphoramidate prodrug of the nucleoside GS-441524, originally developed to treat Ebola virus infections, inhibits the RdRp of SARS-CoV-2. It was the first antiviral approved or authorized for emergency use to treat COVID-19 in several countries. Remdesivir improves clinical outcomes in patients hospitalized with moderate-to-severe disease and it prevents disease progression in outpatients [14, 15]. While remdesivir requires intravenous administration, an oral

prodrug of GS-441524 is being developed [16]. Molnupiravir (MK-4482 or EIDD-2801), a prodrug of the nucleoside analogue EIDD-1931 (β-D-N4-hydroxycytidine), is another inhibitor of the viral RdRp and was originally developed against different RNA viruses such as influenza [17]. A phase 2a clinical trial of molnupiravir in patients with COVID-19 shows accelerated SARS-CoV-2 RNA clearance and elimination of infectious virus [18]. This orally bioavailable drug was recently authorized in the UK for use in people who have mild to moderate COVID-19 and who have at least one risk factor for developing severe illness. Also, the U.S. FDA issued an emergency use authorization (EUA) in infected adults who are at high risk for progression to severe COVID-19, and for whom alternative COVID-19 treatment options are not accessible or clinically appropriate.

Another target for antiviral drugs is the viral main protease Mpro (or 3CL protease), a cysteine protease which cleaves the two polyproteins (pp1a and pp1ab) of SARS-CoV-2 at multiple locations, resulting in the various non-structural proteins, which are key for viral replication. Nirmatrelvir (PF-07321332), is an irreversible inhibitor of SARS-CoV-2 Mpro that is co-formulated with ritonavir allowing an oral route of administration (known as Paxlovid). When treatment is initiated during the first days after symptom onset, it results in significant protection against severe COVID-19 and hospitalization [19]. Even though the Mpro-gene can be slightly affected by evolutionary mutations, the antiviral potency does not seem to be compromised [20].

In this paper, we assess the in vitro effect of GS-441524, remdesivir, EIDD-1931, molnupiravir and nirmatrelvir against the various SARS-CoV-2 VOCs, including Omicron.

To this end VeroE6-GFP cells (kindly provided by Janssen Pharmaceutica, Beerse, Belgium), were used as described previously [21, 22]. Since VeroE6 cells show a high efflux of some chemotypes the antiviral assays were performed in the presence of the P-glycoprotein (Pgp) efflux inhibitor CP-100356 (0.5 µM). A SARS-CoV-2 strain grown from the first Belgian patient sample after excessive passaging on VeroE6 cells (GHB-03021/2020), was used as ancestral strain as it is closely related to the prototypic Wuhan-Hu-1 2019-nCoV (GenBank accession number MN908947.3) [23]. All the other isolates were obtained from patients in Belgium and more information can be found in GISAID (Alpha = EPI_ISL_791333; Beta = EPI_ISL_896474; Gamma = EPI_ISL_1091366; Delta = EPI_ISL_2425097; Omicron = EPI_ISL_6794907). The multiplicity of infection (MOI) was kept constant for the different VOC to allow comparison of the potency.

Our results indicate that GS-441524, remdesivir, EIDD-1931, molnupiravir and nirmatrelvir retain their activity against all current VOCs including Omicron (Figure 1, Table 1). This is in accordance with the observation that the target proteins of these antivirals are highly conserved. For the RdRp there are two amino acid changes (P313L in all VOCs and G661S in Delta) when compared with the ancestral lineage. As these are distant from the active site, a different susceptibility towards remdesivir or molnupiravir is not to be expected. For the Mpro there are three amino acid changes (K90R in Alpha, P132H in Omicron and V296I in Delta). Similar as with the RdRp, these are not part of the Mpro active site, and hence no difference in susceptibility for nirmatrelvir is expected.

These results indicate that when more VOCs arise, due to antigenic drift, there is a high probability that they will remain sensitive towards current antivirals. It is therefore important to continue both vaccine development as well as antiviral research, as they are both essential armor and complement each other in the strategy to control the current pandemic [24]. Though the current focus lies on SARS-CoV-2, we wish to emphasize the need for broad-spectrum antivirals for future viral outbreaks.

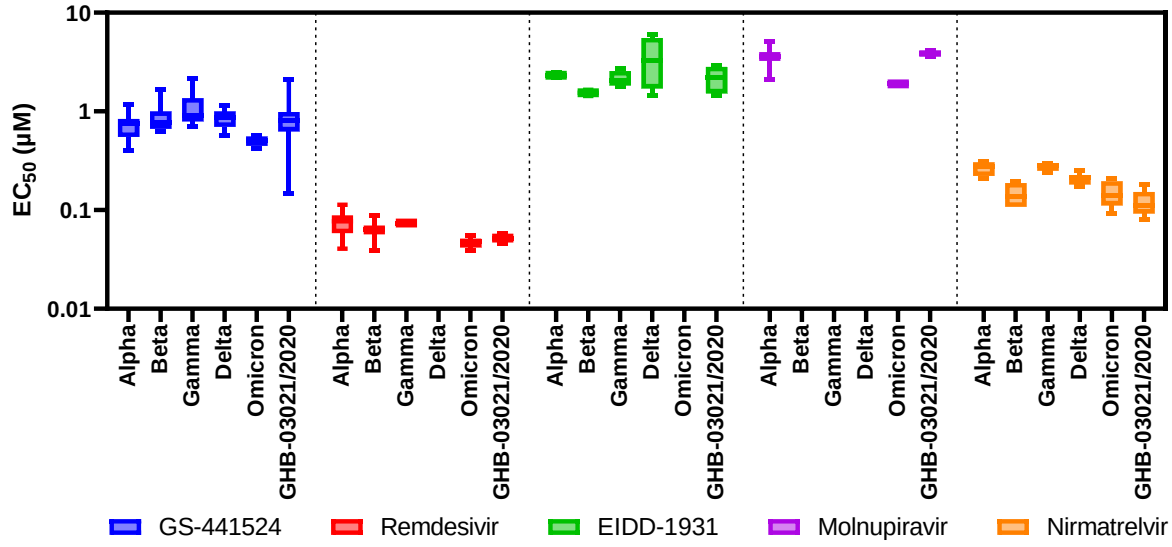

Figure 1: Activity of antivirals upon infection of VeroE6-GFP cells with different VOC in the presence of a Pgp-inhibitor. VeroE6-GFP cells were pre-treated overnight with serial dilutions of the compounds. The next day, cells were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.001 tissue culture infectious dose (TCID50) per cell. The number of fluorescent pixels of GFP signal, determined by high-content imaging on day 4 post-infection, was used as read-out. Percentage of inhibition was calculated by subtracting background (number of fluorescent pixels in the untreated infected control wells) and normalizing to the untreated-uninfected control wells (also background subtracted). The 50% effective concentration (EC_{50}, the concentration of compound required for fifty percent recovery of cell-induced fluorescence) was determined using logarithmic interpolation. These experiments were performed in the presence of the Pgp-inhibitor CP-100356 (0.5 µM) in order to limit compound efflux. This graph was created using Graphpad Prism 9.2.0. The boxes extend from the 25th to 75th percentiles while the whiskers indicate the minimal and maximal values.

Table 1: Descriptive statistics of the data depicted in Figure1

EC_{50} (µM)	GS-441524					
	Alpha	Beta	Gamma	Delta	Omicron	GHB-03021 /2020
n	14	12	10	12	6	206
25% Percentile	0.55	0.67	0.80	0.70	0.45	0.63
Median	0.76	0.77	0.90	0.87	0.50	0.81
75% Percentile	0.84	1.00	1.36	1.00	0.55	0.99

EC_{50} (µM)	Remdesivir					
	Alpha	Beta	Gamma	Delta	Omicron	GHB-03021 /2020
n	6	2	2	Not	6	2
25% Percentile	0.058	0.039	0.072	Determ.	0.042	0.046
Median	0.077	0.063	0.074		0.048	0.052
75% Percentile	0.089	0.087	0.076		0.052	0.058

EC_{50} (µM)	EIDD-1931					
	Alpha	Beta	Gamma	Delta	Omicron	GHB-03021 /2020
n	2	2	4	4	Not	4
25% Percentile	2.2	1.4	1.8	1.7	Determ.	1.5
Median	2.3	1.5	2.0	3.3		2.2
75% Percentile	2.5	1.7	2.6	5.5		2.8

EC_{50} (µM)	Molnupiravir					
	Alpha	Beta	Gamma	Delta	Omicron	GHB-03021 /2020
n	2	Not	Not	Not	2	4
25% Percentile	2.1	Determ.	Determ.	Determ.	1.9	3.6
Median	3.6				1.9	3.9
75% Percentile	5.1				2.0	4.1

EC_{50} (µM)	Nirmatrelvir					
	Alpha	Beta	Gamma	Delta	Omicron	GHB-03021 /2020
n	4	4	4	6	22	14
25% Percentile	0.22	0.11	0.25	0.18	0.11	0.09
Median	0.28	0.14	0.28	0.21	0.14	0.11
75% Percentile	0.30	0.19	0.29	0.23	0.20	0.15

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References

1. Davies, N.G., et al., *Estimated transmissibility and impact of SARS-CoV-2 lineage B.1.1.7 in England.* Science, 2021. **372**(6538).
2. Sabino, E.C., et al., *Resurgence of COVID-19 in Manaus, Brazil, despite high seroprevalence.* Lancet, 2021. **397**(10273): p. 452-455.
3. Mahase, E., *Covid-19: Novavax vaccine efficacy is 86% against UK variant and 60% against South African variant.* BMJ, 2021. **372**: p. n296.
4. Volz, E., et al., *Evaluating the Effects of SARS-CoV-2 Spike Mutation D614G on Transmissibility and Pathogenicity.* Cell, 2021. **184**(1): p. 64-75 e11.
5. Saputri, D.S., et al., *Flexible, Functional, and Familiar: Characteristics of SARS-CoV-2 Spike Protein Evolution.* Front Microbiol, 2020. **11**: p. 2112.
6. Barton, M.I., et al., *Effects of common mutations in the SARS-CoV-2 Spike RBD and its ligand, the human ACE2 receptor on binding affinity and kinetics.* Elife, 2021. **10**.
7. del Rio, C., S.B. Omer, and P.N. Malani, *Winter of Omicron—The Evolving COVID-19 Pandemic.* JAMA, 2021.
8. Planas, D., et al., *Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization.* Nature, 2021. **596**(7871): p. 276-280.
9. Khateeb, J., Y. Li, and H. Zhang, *Emerging SARS-CoV-2 variants of concern and potential intervention approaches.* Crit Care, 2021. **25**(1): p. 244.
10. Cohen, J., *Omicron sparks a vaccine strategy debate.* Science, 2021. **374**(6575): p. 1544-1545.
11. Kumar, S., A. Chandele, and A. Sharma, *Current status of therapeutic monoclonal antibodies against SARS-CoV-2.* PLoS Pathog, 2021. **17**(9): p. e1009885.
12. Alexander Wilhelm, M.W., Katharina Grikscheit, Tuna Toptan, Barbara Schenk, Christiane Pallas, Melinda Metzler, Niko Kohmer, Sebastian Hoehl, Fabian A. Helfritz, Timo Wolf, Udo Goetsch, Sandra Ciesek, *Reduced Neutralization of SARS-CoV-2 Omicron Variant by Vaccine Sera and monoclonal antibodies* medrxiv, 2021.
13. *ASPR pauses allocation of bamlanivimab and etesevimab together, etesevimab alone, and REGEN-COV - https://www.phe.gov/emergency/events/COVID19/therapeutics/update-23Dec2021/Pages/default.aspx.*

14. Beigel, J.H., et al., *Remdesivir for the Treatment of Covid-19 - Final Report.* N Engl J Med, 2020. **383**(19): p. 1813-1826.
15. Gottlieb, R.L., et al., *Early Remdesivir to Prevent Progression to Severe Covid-19 in Outpatients.* N Engl J Med, 2021.
16. Cox, R.M., et al., *Oral prodrug of remdesivir parent GS-441524 is efficacious against SARS-CoV-2 in ferrets.* Nat Commun, 2021. **12**(1): p. 6415.
17. Painter, W.P., et al., *Human Safety, Tolerability, and Pharmacokinetics of Molnupiravir, a Novel Broad-Spectrum Oral Antiviral Agent with Activity Against SARS-CoV-2.* Antimicrob Agents Chemother, 2021.
18. Fischer, W.A., 2nd, et al., *A Phase 2a clinical trial of Molnupiravir in patients with COVID-19 shows accelerated SARS-CoV-2 RNA clearance and elimination of infectious virus.* Sci Transl Med, 2021: p. eabl7430.
19. Owen, D.R., et al., *An oral SARS-CoV-2 M(pro) inhibitor clinical candidate for the treatment of COVID-19.* Science, 2021: p. eabl4784.
20. Sven Ullrich, K.B.E., Gottfried Otting, Christoph Nitsche, *Main protease mutants of SARS-CoV-2 variants remain susceptible to PF-07321332.* bioRxiv, 2021.
21. Do, T.N.D., et al., *A robust SARS-CoV-2 replication model in primary human epithelial cells at the air liquid interface to assess antiviral agents.* Antiviral Res, 2021. **192**: p. 105122.
22. Rana Abdelnabi, C.S.F., Dirk Jochmans, Laura Vangeel, Steven De Jonghe, Patrick Augustijns, Raf Mols, Birgit Weynand, Thanaporn Wattanakul, Richard M. Hoglund, Joel Tarning, Charles E. Mowbray, Peter Sjö, Fanny Escudié, Ivan Scandale, Eric Chatelain, Johan Neyts, *The oral protease inhibitor (PF-07321332) protects Syrian hamsters against infection with SARS-CoV-2 variants of concern* bioRxiv, 2021.
23. Boudewijns, R., et al., *STAT2 signaling restricts viral dissemination but drives severe pneumonia in SARS-CoV-2 infected hamsters.* Nat Commun, 2020. **11**(1): p. 5838.
24. Torneri, A., et al., *A prospect on the use of antiviral drugs to control local outbreaks of COVID-19.* BMC Med, 2020. **18**(1): p. 191.