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Very low levels of remdesivir resistance in SARS-COV-2 genomes after 18 months of massive usage during the COVID19 pandemic: A GISAID exploratory analysis

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ABSTRACT

Massive usage of antiviral compounds during a pandemic creates an ideal ground for emergence of resistant strains. Remdesivir, a broad-spectrum inhibitor of the viral RNA-dependent RNA polymerase (RdRp), was extensively prescribed under emergency use authorization during the first 18 months of the COVID19 pandemic, before randomized controlled trials showed poor efficacy in hospitalized patients. RdRp mutations conferring resistance to remdesivir are well known from *in vitro* studies, and the huge SARS-CoV-2 sequencing effort during the ongoing COVID19 pandemic represents an unprecedented opportunity to assess emergence and fitness of antiviral resistance *in vivo*. We mined the GISAID database to extrapolate the frequency of remdesivir escape mutations. Our analysis reveals very low levels of remdesivir resistance worldwide despite massive usage.

1. Introduction

Antiviral drugs are hard to develop and drug resistance is a serious concern, especially when these drugs are used in massive quantities, as it invariably happens during pandemic settings. In pandemics with new infectious agents there are usually no specific drugs to the microbe and the first impulse is to test available agents for antimicrobial efficacy, in so called repurposing drug efforts. Consequently, when the COVID-19 pandemic began physicians initially turned to known drugs to find some that were active against SARS-CoV-2, including antiviral agents developed against other viruses. The most successful example of repurposing a drug for COVID-19 was remdesivir, a drug originally designed as a therapeutic for Ebolavirus. Given the absence of other small molecule antivirals remdesivir was widely used in massive quantities during the first year of the pandemic, at the beginning within clinical trials as well as off-label and emergency authorized prescriptions, and later for fully authorized prescriptions.

The largest Coronaviridae polyprotein PP1ab is encoded by ORF1ab, which is cleaved by the proteases PLpro and 3CLpro into the non-structural proteins (Nsp1-16) to form the replicase machinery (Romano et al., 2020). Among them, the RNA-dependent RNA polymerase (RdRp), which consists of Nsp12-Nsp7-Nsp8, is targeted by numerous nucleoside analogs. Some polymerase inhibitors (Gordon et al., 2020; Shen et al., 2020) are currently being tested in clinical studies to target SARS-CoV-2 RdRp, including favipiravir (T-705) (Furuta et al., 2013), remdesivir (GS-5734, Veklury®) (Agostini et al., 2018), ribavirin (Morgenstern et al., 2005), penciclovir (Wang et al., 2020a), galidesivir (BCX-4430) (Lim et al., 2017) and ponatinib (Imnovid®) (Chan et al., 2021; Najjar et al., 2015). Additionally, molecular docking studies predict potential efficacy for other drugs such as simeprevir, filibuvir and tegobuvir (Ruan et al., 2021) on the basis of anticipated binding to RdRp. Recently, the orally available molnupiravir (MK-4482, EIDD-2801, Lagevrio®, Molxvir®) showed efficacy in phase 3 randomized controlled trials (RCT) in COVID-19 patients. Among these drugs remdesivir and molnupiravir were the most promising candidates, being able to evade the proofreading exonuclease (Agostini et al., 2018) and inducing a phenomenon variably known as “error catastrophe” (Eigen and Schuster, 1977), “lethal mutagenesis” (Loeb et al., 1999) or

Abbreviations: RdRp, RNA-dependent RNA polymerase; Nsp, non-structural protein.

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"mutational meltdown" (Lynch and Gabriel, 1990) during viral RNA replication. More in details, it consists of G-to-A and C-to-U transitions via 2-step mutagenesis, where increased mutations hinder viral fitness (Kabinger et al., 2021; Malone and Campbell, 2021). However, by targeting mitochondrial RNA polymerase (Sheahan et al., 2020; Sticher et al., 2020), they are also intrinsically mutagenic for human RNA and DNA (Zhou et al., 2021). Accordingly, remdesivir has been postulated to increase the rate of immune escape mutations (Colson et al., 2021). Furthermore, remdesivir has poor penetration in lungs (Wang and Chen, 2020), and could facilitate emergence of compartmentalized resistance (Boshier et al., 2021).

The characterization of a remdesivir-resistant signature has relevant implications for clinical management of individual patients and for pandemic surveillance (Szemiel et al., 2021), and is an important goal of clinical laboratory test development (Kumar et al., 2021a). Hence, we moved from *in vitro* evidence to investigate the global spread of such mutations of concern.

2. Methods

On November 24, 2021, we searched PubMed (which also indexes the medRxiv and bioRxiv preprint servers) for *in vitro* studies showing remdesivir resistance and identified mutations of concern in RdRp and other SARS-CoV-2 genes involved in the RNA replication machinery. The following queries were used: "SARS-CoV-2 AND mutation AND remdesivir" and "SARS-CoV-2 AND polymerase AND remdesivir". Results were manually filtered for relevance.).

The Outbreak. info web portal was then used to assess prevalence of such mutations among the 5,212,176 SARS-CoV-2 sequences deposited in GISAID as of November 24, 2021. The function "Variants"> "Lineage|Mutations tracker" > "Select my own lineage and/or mutations(s)" was used selecting individual mutations within ORF1B. Frequencies of mutations were reported in Table 1 together with the main PANGOLIN lineages where each one had been found.

Mutations within the RNA-dependent RNA polymerase (RdRp, Nsp12) associated with remdesivir resistance were mapped onto the cryo-EM model of the pre-translocation complex (PDB 7C2K) using the PyMOL Molecular Graphics System (Schrödinger, version 2.4.1).

3. Results

Table 1 summarizes mutations in different proteins of the replication machinery that have been associated with remdesivir resistance *in vitro*. Overall, few articles reported remdesivir resistance *in vitro*, and a single case report detailed emergence of resistance *in vivo*. GISAID frequencies for those mutations were exceedingly low. Fig. 1 reports the 3D location of mutations within the RdRp associated with remdesivir resistance, mapped onto the cryo-EM model of the pre-translocation complex.

Fig. 2 shows the temporal trends in prevalence of those mutations among the SARS-CoV-2 sequences deposited in GISAID, showing extremely low and stable frequencies in the last 2 years.

4. Discussion

The ligand binding strategy of the SARS-CoV-2 RdRp (nsp12) features entry and exit channels for the primer-template complex, NTPs and nascent RNA strand which interact strongly with anionic nucleic acids due to a positive electric potential along these grooves. NTP entry, RNA template entry, and primer strand entry channels are defined by K545/R553/R555, motifs F/G, and motif E/thumb subdomains, respectively. These all proceed toward to active site composed of motifs A and C, where residues D760, D761 and D618 form important coordinating interactions with divalent cations. Additionally, these residues combine with R555 to stabilize incoming nucleotides or inhibitors. In the complex structure, N691 stabilizes with the RDV ribose group as it would with a natural substrate, while the hydrophobic V557 likely stabilizes the base pairing interaction between the RDV adenosine analog and the +1 template strand uridine base (Wang et al., 2020b). Of these critical residues, V557 and R555 have been identified in viral sequencing efforts and their substitutions would likely have deleterious effects on ligand binding and polymerase efficiency (Fig. 1).

The pre-insertion form of remdesivir forms hydrogen bonds with the viral RNA template and binds ~100-fold stronger to S549, R555 and D618 in RdRp than the natural substrate ATP (Zhu et al., 2020). The commonest RdRp mutations (e.g., P323L (Pachetti et al., 2020)) do not destabilize RdRp, while mutations conferring resistance to remdesivir are rare in circulating lineages. The latter is likely due to poor fitness of mutants (Agostini et al., 2018).

While treatment with molnupiravir has failed to induce viral-resistance mutations *in vitro* so far (Agostini et al., 2018; Yoon et al., 2018), remdesivir-resistant viral populations have been selected *in vitro* by serially passaging SARS-CoV-2 in the presence of the drug (Szemiel et al., 2021). *In vivo* emergence of RdRp E802D (a.k.a. E793D), a mutation that causes ~6-fold increase in remdesivir $IC_{50,}$ was reported in single nonresponding immunocompromised patient, but the mutation was unfit when remdesivir was removed, and was cleared after REGN-COV-2 (Gandhi et al., 2021).

A former analysis moved from the commonest RdRp mutations to investigating their effect on remdesivir binding. Two potential escape motifs have been identified within RdRp, Nsp12:R467-V493 and Nsp12:L544-Q570. Mutations within these motifs are predicted to affect proofreading ability and binding affinity for the RNA template, respectively (Mari et al., 2021). In the A97V (a.k.a. A88V) RdRp structure remdesivir forms 5 hydrogen bonds with residues K545, S549, K551, T556, and S682 and this was associated with a 20-fold increased resistance to remdesivir; the RdRp P323L (a.k.a. P314L) structure, remdesivir forms hydrogen bonds with T556, S759, T680, S682, and N691, but there is no change in affinity (Mohammad et al., 2021). P323L mutations reduces accessibility to the 321–327 epitope and facilitates immune escape (Mallick Gupta et al., 2021).

Our prevalence results are in line with analyses run during the first pandemic wave (Mari et al., 2021), suggesting stable and rare occurrence of such escape mutations, as confirmed in Fig. 2. Low sequence variation in the RNA replication complex was also observed among human and mink SARS-CoV-2 isolates (Martin et al., 2021).

The paucity of remdesivir resistance mutations despite its widespread use contrasts with the rapid emergence of drug resistance to antiviral monotherapy in other RNA viruses such as HIV. This difference could stem from different causes.

First, it might reflect the pathogenesis of SARS-CoV-2 viral infection and the logistics of antiviral use in these conditions. In most cases, COVID-19 tends to be a disease where individuals improve or succumb to infection, while HIV infection is chronic. Hence, remdesivir is used in individuals of whom the majority will either improve and clear any emerging resistant virus with their immune response or die thus greatly reducing the likelihood of establishment and spread of resistant viral strains.

Second, remdesivir is an intravenous drug that is used only in hospital settings where patients are under infectious disease precautions. Hospital use also reduces compliance issues, which are associated with emergence of resistance in HIV.

Third, remdesivir has been typically administered in-hospital (at a median of 5–7 days since onset of symptoms) after the peak viral loads have been achieved (usually around day 3), so reducing the chances for interfering with RdRp. During the early trials, it was mainly focused in the moderate to severe group of patients and there wasn't a fixed schedule. In the ACTT-1/2 placebo-controlled randomized trials, the duration was 10 days (loading dose of 200 mg at day 1, then 100 mg daily up to day 10) (Beigel et al., 2020; Kalil et al., 2020), but the GS-US-540-5773 showed noninferiority of an alternative schedule of just 5 days (Goldman et al., 2020). A single RCT (NCT04501952) has investigated remdesivir usage in outpatients based on a 3-day schedule

Table 1
Mutations within the RNA replication complex of SARS-CoV-2 investigated for potential remdesivir resistance: bold characters identify the ones leading to confirmed *in vitro* resistance. MHV: murine hepatitis virus. The amino acid residue numbering is provided according to both the universal positioning derived by NCBI Reference Sequence: YP_009725307.1 and the −9 frameshift used by outbreak.info.

gene	polyprotein	target	function	mutation		*in vitro* evidences			*in vivo* evidences of emergence in COVID19 patients	frequency among the 5,212,176 SARS-CoV-2 sequences deposited in GISAID as of November 24, 2021 (former data)
				outbreak.info amino acid numbering	GISAID amino acid numbering	tested virus	impact on IC_{50}	ref		
ORF1a	PP1a	Nsp7	cofactor of Nsp12	-	-	-	-	-	-	-
		Nsp8	cofactor of Nsp12	-	-	-	-	-	-	-
		Nsp9	RNA binding protein	-	-	-	-	-	-	-
		Nsp10	cofactor of Nsp16 and Nsp14	-	-	-	-	-	-	-
ORF1b	PP1ab	Nsp12	RNA-directed RNA polymerase (also with exonuclease activity (Wang et al., 2021))	**A88V**	**A97V**	-	20-fold increase	Mohammad et al. (2021)	n.a.	0.17% = 8792 sequences worldwide (100% of several B.6 sublineages, B.1.431, B.1.104)
				P314L	P323L	-	40-fold decrease	Mohammad et al. (2021)	n.a.	98% = 5,106,626 sequences worldwide (vs. 0.5% in first 90,000 sequences (Martin et al., 2021))
				V464F	V473F	-	-	-	n.a.	0.05% = 2849 sequences worldwide (peaking at 13% within B.1.370) ((Mari et al., 2021))
				F471 L/S/C	**F480 L**/S/C (F476L in MHV numbering)	MHV and mouse-adapted SARS-CoV	5.6-fold increase	Agostini et al. (2018)	n.a.	F471L: 14 sequences F471S: 2 sequences F471C: 8 sequences
				N482S	N491S	-	-	-	-	363 sequences worldwide, mostly B.1.108 ((Mari et al., 2021))
				R546P	R555P	-	-	-	n.a.	4 sequences worldwide ((Mari et al., 2021))
				V548L	**V557L** (V553L in MHV numbering)	MHV and Mouse-adapted SARS-CoV	5.6-fold increase	Agostini et al. (2018)	n.a.	21 sequences worldwide
				E793D/A	**E802D**/A	-	6-fold increase	Szemiel et al. (2021) Gandhi et al. (2021)	Gandhi et al. (2021)	E802D: 0.002% = 129 sequences worldwide (no sublineage with prevalences higher than 0.5%) E802A: 17 sequences worldwide
		Nsp13	helicase, 5′ triphosphatase	-	-	-	-	-	-	-
		Nsp14	3′–5′ exoribonuclease, ExoN; Guanine-N7 methyltransferase, N7 MTase	A504V	-	-	-	-	Gandhi et al. (2021)	mutation has not been detected in available sequence data at outbreak.info
		Nsp15	NendoU, Uridylate-specific endoribonuclease	I115L	-	-	-	-		mutation has not been detected in available sequence data at outbreak.info
		Nsp16	2′-*O*-ribose methyltransferase	-	-	-	-	-	-	-

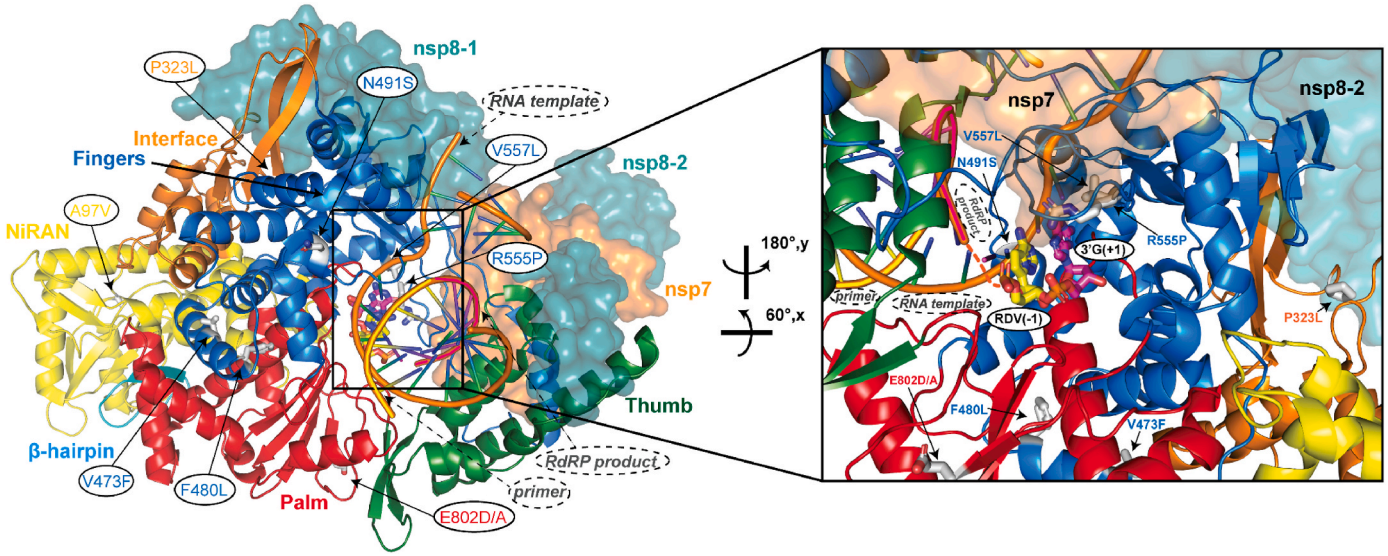

Fig. 1. Mutations within the RNA-dependent RNA polymerase (RdRp, Nsp12) associated with remdesivir resistance are mapped onto the cryo-EM model of the pre-translocation complex (PDB 7C2K) (Wang et al., 2020b). Nsp12 is comprised of a nidovirus RdRp-associated nucleotidyltransferase (NiRAN, residues 60–249) N-terminal domain fused to a right-hand RdRp domain (residues 366–920) through an interface region (residues 250–365) (Gao et al., 2020). The RdRp polymerase domain is highly conserved within the viral polymerase family, consisting of three subdomains: the palm (residues 582–620; 680–815, colored red), fingers (residues 366–581; 621–679, colored dark blue) and thumb (residues 816–920, colored dark green). The NiRAN domain (yellow) contains a unique beta-hairpin (cyan) which distinguishes this protein fold from the SARS-CoV nsp12 homolog and forms stabilizing interactions with the palm subdomain of RdRp domain. The RNA polymerase holoenzyme is composed of nsp12 (RdRP) in complex with two nsp8 cofactors and one copy of nsp7, which are displayed in surface representation and colored teal and orange, respectively. The complex is visualized in a pre-translocation state with template nucleic acid (T33-7) and primer (P10) stalled due to incorporation of remdesivir (RDV) into the RNA polymerization machinery. Template RNA, primer and elongation product are depicted in cartoon representation with an orange backbone and green-blue, yellow-blue, and magenta-blue sticks spanning the base plane, respectively. RDV and 3′-guanosine are depicted as in full stick representation to represent the entire molecules, and colored yellow and magenta, respectively. The complex was rotated and zoomed in to visualize the active site of the polymerase, with RDV and the 3′ guanosine are in the −1 and +1 subsites, respectively. Escape mutation positions on nsp12 associated with remdesivir resistance are visualized as white side chain sticks and labeled with coloration according to their subdomain localization.

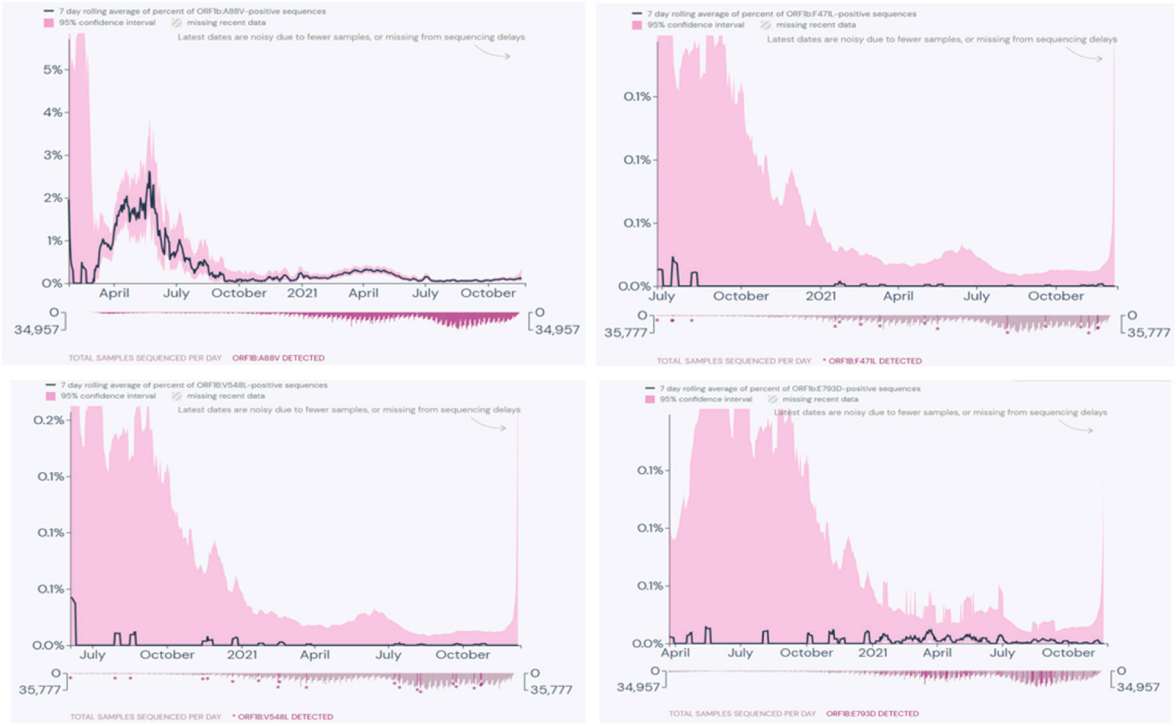

Fig. 2. Variations in time of the prevalence of Nsp12 mutations associated with confirmed remdesivir resistance, i.e. A88V (A97V), F471L (F480L), V548L (V557L) and E793D (E802D) among the SARS-CoV-2 genomes deposited in GISAID as of November 24, 2021 (reproduced from http://www.Outbreak.info webportal).

(200 mg day 1, 100 mg days 2–3) (Gottlieb et al., 2021).

Fourth, the vast majority of immunocompetent individuals can clear the resistant strain, leaving room for emergence of antiviral resistance in immunosuppressed patients only (Shiraki et al., 2021).

Fifth, sampling could have been performed at a time remdesivir has been discontinued, when the resistant sublineage has already succumbed because it was less fit than the susceptible ones. Such phenomenon has been previously reported after treatment failure with sofosbuvir, which similarly targets the NS5B RdRp of HCV (Hayes et al., 2021). Finally, SARS-CoV-2 sequencing is performed at different frequencies in different regions, and isolates selected for genome sequencing from individuals with COVID-19 could be biased towards representing cases with more severe clinical phenotype. As a result, our analysis provides just an estimate of the real frequency of mutations. Furthermore, we note that many SARS-CoV-2 sequences in the database were probably deposited from viruses isolated during initial diagnosis rather that obtained from hospitalized patients. Hence, the database analysis is almost certainly skewed towards viral isolates that were not under remdesivir selection. Consequently, the available genome database may not be fully representative of the SARS-CoV-2 viral sequences in human populations. Lastly, we note that our analysis focused on known remdesivir resistance mutations, while it is possible that other mutations exist that have not been associated with resistance since clinical isolates are not routinely tested for efficacy against SARS-CoV-2.

5. Conclusions

The usage of remdesivir in hospitalized COVID19 patients has been suddenly discontinued in many countries (e.g., Italy) but not others (e.g., USA) since September 2021, when the WHO NOR-Solidarity reported lack of clinical efficacy (2021). Accordingly, the latest revision of WHO guidelines (update 2 published December 8, 2021) issued a conditional recommendation against use of remdesivir in hospitalized patients regardless of disease severity (Agarwal et al., 2020). Nevertheless, earlier (outpatient) usage could restore clinical benefit, and research with oral remdesivir prodrugs continues (e.g. GS-441524 (Cox et al., 2021)).

Nevertheless, it has been massively used worldwide for more than a year and a half, representing an ideal ground for emergence of viral resistance. Despite such deployment, our study shows that in the real-world remdesivir resistance remains exceedingly rare and stable over time, being associated with poor viral fitness. Attention should nevertheless be maintained at high levels for “sublethal mutagenesis” (Sadler et al., 2010), especially when suboptimal oral doses are used (Nelson and Otto, 2021). In case resistance occurs, it can be overcome with increased, nontoxic concentrations of remdesivir (Agostini et al., 2018), RdRp compounds with increased affinity (e.g., SCHEMBL20144212) (Kumar et al., 2021b), or alternative targets such as the 3C-like-protease/M^{pro}/Nsp5 inhibitors (e.g. nirmatrelvir/PF-07321332 plus ritonavir (Paxlovid™)).

Author contributions

D.F. conceived the manuscript; F.M. analyzed the literature; S.M. provided the figure and revised the final version; A.C. revised the final version. All authors approved the final version.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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