Molnupiravir Increases Random SARS-CoV-2 RNA Errors, Without Selection for Resistance

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Introduction

- Molnupiravir (MOV) is an orally administered, small-molecule ribonucleoside prodrug of N-hydroxycytidine (NHC, EIDD-1931), which inhibits viral replication by the mechanism of viral error induction
- The viral error induction mechanism of NHC/MOV has previously been demonstrated both in vitro and in animal infection models of SARS-CoV-2[1,2]
- Treatment with MOV/NHC results in the accumulation of multiple low-frequency errors across the viral genome, associated with a loss of viral infectivity
- In preclinical studies, MOV treatment results in predominantly transition errors as predicted by a 2-step mutagenesis model[3]

Objectives

- Next-generation sequencing (NGS) was performed on clinical samples from the MOVe-OUT (P002) Phase 2/3 trial to characterize the rate, distribution, and type of viral mutations observed after MOV treatment in SARS-CoV-2–infected participants

Methods

Next-Generation Sequencing

- Full genome sequences of SARS-CoV-2 from study participants in P002 (Phase 2, and partial data from Phase 3 available at interim analysis 4, ((IA4)) were amplified from nasopharyngeal (NP) swabs by PCR, and NGS performed on samples with viral RNA titers of ≥22,000 c/mL (Ph2) or ≥1200 c/mL (Ph3/IA4) with Ion Torrent sequencing. Sequences were aligned to the original Wuhan-1 isolate and sequence changes with an allele frequency of 2% or greater, relative to the reference sequence, were reported

Minor variant analysis

- Low-frequency nucleotide errors in the viral quasispecies, were identified from bam data files using the VarScan 2.4 mutation caller with a 0.4% minimum variant allele frequency cut-off

Treatment-emergent (TE) amino acid substitutions

- To identify potential resistance-associated variants, protein sequences from the viral nsp12 (RNA Polymerase), nsp13 (Helicase) and nsp14 (Exonuclease) and Spike from baseline and Day 3 and/or Day 5 samples were compared to determine the number and location of non-synonymous amino acid substitutions. TE variants were defined as variants occurring in 2 or more participants at an allele frequency of ≥5%

Phenotypic assessment of amino acid substitutions

- AA substitutions in nsp12, nsp13 or nsp14 were introduced into an established SARS-CoV-2 replicon model[4] and evaluated for impact on NHC antiviral activity in 293T cells

Results

- At both Day 3 and Day 5, there was a linear dose–response relationship with increasing MOV dose in the geometric mean number of minor variants (NMV) (Table 1, Figure 1A)
- At Day 3 and Day 5, an approximate 5- and 11-fold increase, respectively, in the geometric mean number of mutations was observed for the 800 mg MOV group compared with placebo (Table 1, Figure 1A)

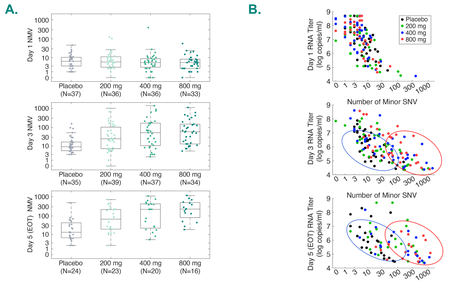
Table 1. Number of minor variants in SARS-CoV-2 RNA genome sequence by visit and dose: (NP samples, Ph2)

Visit	Placebo		MOV 200 mg		MOV 400 mg		MOV 800 mg		
	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	N	GM (95% CI)	P-value for linear trend
Day 1	37	6.6 (4.4, 9.9)	36	6.7 (4.4, 10.2)	36	6.0 (4.0, 9.1)	33	6.1 (3.9, 9.4)	0.7048
Day 3	35	12.5 (8.2, 19.1)	39	30.6 (20.5, 45.7)	37	47.4 (31.3, 71.6)	35	61.5 (40.3, 94.2)	<.0001
Day 5 (EOT)	24	20.6 (12.5, 34.0)	24	83.4 (50.5, 137.4)	20	129.1 (74.8, 223.4)	16	223.4 (121.6, 410.2)	<.0001

Day 3 includes post-baseline records up to Day 4 relative to randomization. Day 5 (EOT) includes post-baseline records from Day 5 (relative to randomization) up to Day 7. EOT visits occurring earlier than Day 5 are included in the Day 3 visit. CI, confidence interval; EOT, end of treatment; GM, geometric mean; MOV, molnupiravir; N, number of participants with available data.

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Figure 1. A) Number of nucleotide minor variants increases with MOV dose and B) controlling for viral RNA titer, number of nucleotide minor variants increases with MOV dose (Ph2)



NMV, number of minor variants; SNV, SARS-CoV-2 nucleotide variants

- Controlling for viral RNA titer, a linear dose–response relationship in mean NMV was observed in Day 3 and Day 5 samples in MOV after controlling for sample RNA titer (Figure 1B)
- There was a dose dependent increase in transition but not transversion errors at Day 3 and Day 5, consistent with the mechanism of action of MOV (Figure 2)

Figure 2. Majority of minor variants were transition errors

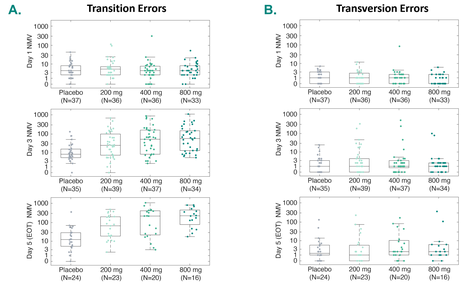


Figure 3. Dose-dependent increase in number of synonymous and nonsynonymous minor variants

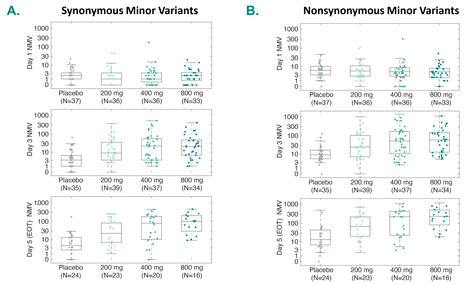


Table 2. Treatment-emergent AA substitutions in replicase proteins were infrequently detected in MOV participants (Ph2/Ph3 IA4)

Replicase gene	AA variant	MOV (pooled) (n=155)	Placebo (n=89)	Comment
Polymerase (nsp12)	N/A	0	0	No treatment-emergent changes
Helicase (nsp13)	A446T E341D	2 2	0 0	1 in 200 mg / 1 in 800 mg group (Ph2) 2 in 800 mg (Ph3/IA4)
Exonuclease (nsp14)	N129D A220S/V V466I	1 2 (1 S, 1V) 2	1 0 0	1 in 800 mg/ 1 placebo 1 in 200 gm / 1 in 400 mg 1 in 400 gm/ 1 in 800 mg

- Among minor variants, increasing MOV dose led to a dose-dependent increase in the number of synonymous and nonsynonymous errors, with a DN/DS ratio of ~2:1 in the MOV group, as predicted by the mechanism of action (Figure 3)

- No treatment-emergent (TE) AA changes in the viral polymerase were detected in post-baseline sequences (compared with baseline) in 2 or more participants in the combined MOV arms (Phase 2) and available Phase 3 (IA4 data) or placebo (Table 2)

Table 3. Treatment-emergent AA changes detected at ≥5% frequency in Spike sequences

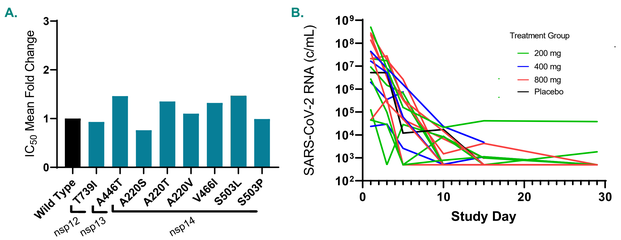
AA substitution	MOV (pooled)	Placebo	Error type	Infectious virus recovered (MOV, Day 5)	Previously reported Spike substitution
	n	n			
Phase 2	**113**	**39**			
≥2 participants					
ΔP139-Y145	1*	0	Deletion	No	Yes
P139S	1*	0	Transition	No	Yes
ΔL141-Y144	1*	0	Deletion	No	Yes
ΔL141-Y144, Fins	1*	0	Deletion	No	Yes
ΔY145	1*	0	Deletion	No	Yes
G261I/V	2	0	Transversion/Other	No	Yes
S267L	1	1	Transition	No	No
T383I	2	0	Transition	No	Yes
E484K	2	0	Transition	No	Yes
P681H	2	0	Transversion	No	Yes
S884F	1	1	Transition	No	Yes
A1022T	2	0	Transition	No	Yes
Phase 3 (IA3/4)	**42**	**50**			
≥2 participants					
D138Y	2†	0	Transversion	No	Yes
G142D	2†	0	Transition	No	Yes
Y144T*	0	1	Deletion/Insertion	No	Yes
ΔY144_T145, insTSN*	1†	0	Deletion/Insertion	No	Yes
K417T	2†	0	Transversion	No	Yes
N501Y	3	0	Transversion	No	Yes
H655Y	2†	0	Transition	No	Yes
P681H/R	2†	1	Transversion	No	Yes
D950N	2†	1	Transition	No	Yes
G1124V	1	1	Transition	No	Yes
V1176F	2†	0	Transition	No	Yes

*Treatment-emergent in only 1 subject but noted as an important spike position.
†Substitution observed in 1 or more MOV participants with different viral genotype (Clade) at baseline (20J or 21H) and Day 5 (20H or 20C), suggestive of a sample error or coinfection.
AA, amino acid.

- Several TE changes were observed in the viral helicase and exonuclease in a small number of participants (Table 2)
- Phenotyping of TE substitutions showed NHC was similarly active (IC_{50} values <1.6-fold) against replicons single AA substitutions in replicase genes (Figure 4A)
- Among participants with nsp13 or nsp14 substitutions, no infectious virus was recovered from samples by Day 5 and no hospitalizations or deaths recorded by Day 29

- TE spike changes were observed in both MOV and/or placebo participants by Day 5, but were more common in the MOV group (Table 3)
- In Phase 2, 14 AA changes in spike were observed in 15/113 MOV participants (4 in 800 mg, 6 in 400 mg, 5 in 200 mg) and 2/39 placebo participants (Table 3)
- In Phase 3 (data available at IA3/4), 11 spike substitutions were observed in 6/42 MOV participants, however, 8 of these changes were attributable to 3 participants; each presenting with 3 or more substitutions. Two participants had AA changes associated with a different viral clade at Day 5 compared with baseline, suggesting a possible sample error, contamination or coinfection (Table 3)
- All spike substitutions (except S267L) have been previously reported in circulating SARS-CoV-2 isolates
- No MOV participant with TE substitutions had infectious virus recovered at Day 5 (Table 3) and had no or only low viral RNA shedding by Day 29 (Figure 4B, Phase 2)

Figure 4. A) NHC activity against SARS-CoV-2 replicons with amino acid substitutions in nsp12, nsp13, and nsp14 and B) viral RNA profiles of participants with post-baseline replicase or Spike amino acid changes



IC_{50}, half-maximal inhibitory concentration.
The nsp T731I and S503L/P substitutions were observed at low frequency (<5%) in several participants but did not meet TE defined criteria.

Conclusions

- MOV treatment resulted in a dose-linear increase in low frequency (0.4–10%) nucleotide transition changes across the viral genome, consistent with the viral error induction mechanism of action
- By Day 5 an 11-fold increase in minor viral nucleotide errors was observed in MOV-treated participants (800 mg) compared with placebo
- TE changes in viral replicase genes were uncommon in MOV treated participants and not associated with high level resistance to MOV (IC_{50} fold change <1.6-fold)
- No participant with TE changes had infectious virus detected by study Day 5 or had high level prolonged viral RNA shedding

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