Poster #153

Molnupiravir exhibits a high barrier to the development of SARS-CoV-2 resistance in vitro

Julie M. Strizki[1]; Nicholas J. Murgolo[1]; John Gaspar[1]; John Howe[1]; Beth Hutchins[1]; Hiroshi Mohri[2]; David D. Ho[2]; Daria J. Hazuda[1]; Jay A. Grobler[1]

[1]Merck & Co, Inc., Rahway, NJ, USA; [2]Aaron Diamond AIDS Research Center, Columbia University Medical Center, New York, NY, USA

Background

- Molnupiravir is authorized for treatment of mild to moderate corona virus disease 2019 (COVID-19) in adults at high risk of progression to severe disease
- Molnupiravir is a small-molecule ribonucleoside prodrug of N-hydroxycytidine (NHC) that has broad preclinical activity against RNA viruses, including severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) and its variants[1]
- Incorporation of NHC into viral RNA causes an accumulation of deleterious errors throughout the viral genome, reducing viral infectivity and replication[1]
- In previous studies with other RNA viruses (including mouse hepatitis virus,[2] Middle Eastern respiratory syndrome coronavirus,[2] respiratory syncytial virus,[3] and influenza virus[3]), a high barrier to the development of resistance was seen for NHC, with only modest shifts (ie, 2-fold) in susceptibility after prolonged exposure to NHC in vitro

Objectives

- To assess the potential for SARS-CoV-2 to develop resistance to NHC or a 3C-like protease inhibitor (MRK-A) by continuous virus passage in cell cultures
- To determine progressive viral sequence changes in cultures and identify any amino acid changes associated with loss of susceptibility to NHC or MRK-A

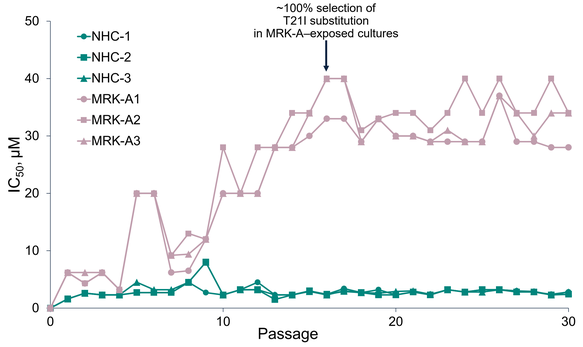
Methods

- Independent triplicate cultures of Vero E6 cells were plated in 24-well plates and infected with SARS-CoV-2 (WA-1) in the presence of media alone, NHC, or MRK-A
- The NHC or MRK-A compounds were added to the cell culture across a range of concentrations in a 3-fold dilution series based on each drug's half-maximal inhibitory concentration (IC_{50}), that is 27× IC_{50}, 9× IC_{50}, 3× IC_{50}, 1× IC_{50}, 0.33× IC_{50}, and 0.1× IC_{50}
- IC_{50} values were estimated based on the cytopathic effect (CPE) scoring (from 0 to 4) by the crossing point, with 50% inhibition
- Culture supernatants from wells with the highest drug concentration that exhibited a CPE score >2 were repassaged
- 30 passages were completed
- At each passage, full genome next-generation sequencing (NGS) was performed on viral RNA amplified by use of the Qiagen custom panel QIAseq DIRECT SARS-CoV-2 Kit using 150 bp Illumina paired-end sequencing

Results

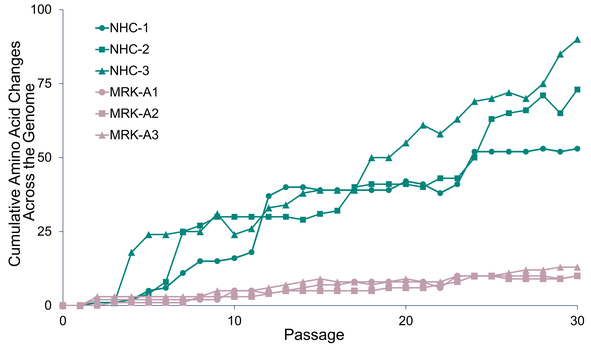
- The IC_{50} remained generally unchanged at each passage with NHC but generally increased at each passage with MRK-A (Figure 1)
- Random amino acid changes accumulated over time but were not associated with changes in susceptibility of SARS-CoV-2 to NHC after 30 passages (Figures 1, 2)
- None of the amino acid substitutions in the viral replicase complex genes (nonstructural protein [nsp] 8 to nsp14) was observed in >1 culture, consistent with the NHC mechanism of action (Table 1)

Figure 1. IC_{50} of SARS-CoV-2 at each passage in cultures exposed to NHC or MRK-A



MRK-A/NHC-1, 2, and 3 denote the triplicate cultures.
MRK-A, 3C-like protease inhibitor; NHC, N-hydroxycytidine.

Figure 2. Amino acid changes in SARS-CoV-2 at each passage in cultures exposed to NHC and MRK-A



MRK-A/NHC-1, 2, and 3 denote the triplicate cultures.
MRK-A, 3C-like protease inhibitor; NHC, N-hydroxycytidine.

Table 1. SARS-CoV-2 amino acid changes detected at >50% frequency at passage 30 in cultures exposed to NHC or MRK-A[a]

Gene region	NHC-1	NHC-2	NHC-3	MRK-A1	MRK-A2	MRK-A3
nsp3	N51D, S148P, K379R, S543P, K610E, Q842R, Y1185C, C1223R, K1290R, T1446A, G1681D, A1803V	K97R, E115G, G334N, V453I, N506S, A655V, M953I, Q1100R, V1385I, I1412M, G1433S, R1518G, G1643S K1861R, K1909R	G47D, V170I, L312F, V348I, I388T, S400G, K497R, K1130R, N1146S, D1208N, R1341W S1406F, I1413T, A1642V, V1770I, V1741I, S1782L, F1823S, H1841Y	K1330N, L1870F	I967V	E1799A
nsp5	P96L, V186I	T24I[b], Q69R, I259V	–	**T21I**[b], S301P	**T21I**[b], L75P, T304I	**T21I**[b], L27V
nsp8[c]	K37R	–	V160A, K196R	–	–	T141M
nsp12[c]	Y80C, H613Y, D711N	T85I, V166I, R457C, D717G	A199V, T319I, S451G, V675I	E744K	–	–
nsp13[c]	H290Y	V154I, A208T, V266I, V397A	D56G, S259L, T599I	–	L280F	G439E
nsp14[c]	A353V, V460M, S503L	G44S, P239L	K47R, R98K, I201M, P203S, V317I	–	–	–
Spike protein	IHVSGTNGT-68-76X, V83I, **R682Q**, S686N, **K986E**, V1060I, V1065M, D1259N	T20I, **W64R**, N74K, M177I, P479S, **T572I**, **K814R**, **K986E**, G1171S	L5F, **W64R**, H66R, V127I, F175S, H207Y, T250A, **T572I**, **R682W**, **K814R**, N969S, **K986E**, F1121V, T1136I	N74K, N211K	IHVSGTN-GT68-76X, E484D, S708F	L180S, E181K, S247R, S982L
Nucleo-capsid protein	P168S V246I, Q272R, P383S	S33G, I84V, N192S, A220T, **T334I**	G34K, S187L, M322I, **T334I**, V350I, D371N, Q380R	P80L	–	–

Bolding indicates a change observed in more than one culture.
[a]Only selected genes are shown in this table.
[b]The T24I/T21I substitution in nsp5 is involved in substrate binding to the 3C-like protease.
[c]Proteins involved in viral replication.
MRK-A/NHC-1, 2, and 3 denote the triplicate cultures.
MRK-A, 3C-like protease inhibitor; NHC, N-hydroxycytidine; nsp, nonstructural protein.

Table 2. Number of transition and transversion nucleotide errors in NHC- and MRK-A–passaged cultures[a]

Culture	C-U	G-A	U-C	A-G	G-U	U-A	A-C	U-G	C-A	C-G
	Transition errors				Transversion errors					
NHC-1	26	21	32	33	1	0	0	0	0	0
NHC-2	36	38	37	45	0	1	0	0	0	0
NHC-3	47	37	47	64	0	0	0	1	0	0
MRK-A1	6	1	2	0	0	2	1	1	1	0
MRK-A2	4	0	1	1	1	0	1	0	0	0
MRK-A3	6	2	1	0	0	1	2	0	0	1

[a]Nucleotide changes detected at >50% frequency at passage 30.
MRK-A/NHC-1, 2, and 3 denote the triplicate cultures.
A, adenine; C, cytosine; G, guanine; MRK-A, 3C-like protease inhibitor; NHC, N-hydroxycytidine; U, uracil.

- Most nucleotide changes observed in NHC cultures were transition errors not transversion errors, consistent with the mechanism of action of NHC (Table 2)

Conclusions

- No evidence of SARS-CoV-2 phenotypic or genotypic resistance to NHC was observed after 30 passages in cell cultures
- The pattern of changes observed across multiple proteins was random and consistent with the NHC mechanism of action
- None of the SARS-CoV-2 spike protein substitutions observed was reported to impact viral phenotypic changes or binding of neutralizing antibodies
- Resistance was readily selected in all 3 MRK-A control cultures and as associated with a previously reported T21I substitution on nsp5[4]
- These data are consistent with data from previous studies with other RNA viruses and further support the conclusion that molnupiravir/NHC has a high barrier to the development of resistance, which is expected to translate to durable clinical efficacy

References
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