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Molnupiravir; molecular and functional descriptors of mitochondrial safety

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ABSTRACT

Molnupiravir is an orally active nucleoside analog antiviral drug that recently was approved by the U.S. FDA for emergency treatment of adult patients infected with the SARS-CoV-2 (COVID-19) virus and at risk for severe progression. The active form of the drug, N-hydroxycytidine (NHC) triphosphate competes for incorporation by RNA-dependent RNA-polymerase (RdRp) into the replicating viral genome resulting in mutations and arrest of the replicating virus. Historically, some nucleoside analog antiviral drugs have been found to lack specificity for the virus and also inhibit replication and/or expression of the mitochondrial genome. The objective of the present study was to test whether molnupiravir and/or NHC also target mitochondrial DNA polymerase gamma (PolG) or RNA polymerase (POLRMT) activity to inhibit the replication and/or expression of the mitochondrial genome leading to impaired mitochondrial function. Human-derived HepG2 cells were exposed for 48 h in culture to increasing concentrations of either molnupiravir or NHC after which cytotoxicity, mtDNA copy number and mitochondrial gene expression were determined. The phenotypic endpoint, mitochondrial respiration, was measured with the Seahorse® XF96 Extracellular Flux Analyzer. Both molnupiravir and NHC were cytotoxic at concentrations of $\geq 10 \mu\text{M}$. However, at non-cytotoxic concentrations, neither significantly altered mitochondrial gene dose or transcription, or mitochondrial respiration. From this we conclude that mitochondrial toxicity is not a primary off target in the mechanism of cytotoxicity for either molnupiravir or its active metabolite NHC in the HepG2 cell line.

1. Introduction

Molnupiravir is an orally active broad spectrum antiviral agent that recently received emergency use authorization by the U.S. FDA for treating mild-to-moderate coronavirus disease in adult patients with positive test results for SARS-CoV-2 and who are at high risk for progression to severe COVID-19, including hospitalization or death, and for whom alternative COVID-19 treatment options authorized by the FDA are not accessible or clinically appropriate. Structurally, molnupiravir is the 5'-isopropyl ester prodrug of N⁴-hydroxycytidine (NHC), which is the active metabolite. Being a ribonucleotide analog, NHC is mistakenly incorporated by RNA-dependent RNA polymerase (RdRp) into the replicating viral genome leading to mutations and arrest (Kabinger et al., 2021; Pruijssers and Denison, 2019; Sheahan et al., 2020). This activity is not specific to the SARS-CoV-2 virus, but also to assorted other viruses including both SARS-CoV-1 and MERS-CoV (Sheahan et al., 2020).

This spectrum of activity extends beyond that of the virus to include the host cell; Zhou et al. demonstrate that NHC can be reduced to the

deoxyribonucleoside and incorporated by host cell DNA polymerase into and cause genomic mutations in human derived respiratory epithelial cells (Zhou et al., 2021). Another off target for molnupiravir or NHC may be the mitochondrial genome, as has been documented for a number of antiviral ribonucleoside analogs (Arnold et al., 2012; Birkus et al., 2002; Feng, 2018; Jin et al., 2017; Kakuda, 2000; Lewis et al., 2003). This includes the documented mitochondrial toxicity caused by 2',3'-dideoxycytidine in human derived HepaRG cells (Young et al., 2021). Notable examples of mitochondrial toxicity associated with nucleoside analogs are the nucleoside reverse transcriptase inhibitors (NRTI) prescribed for treating HIV infections in AIDS patients (Brinkman et al., 1998; Kakuda, 2000). Because of this concern for potential mitochondrial off-targets, we investigated whether molnupiravir or NHC at non-cytotoxic concentrations inhibit the replication or expression of the mitochondrial genome resulting in a loss of mitochondrial function.

2. Methods

All methods employed in this investigation were essentially the same

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as those used in our recent publication on molecular and functional measures of mitochondrial safety for Remdesivir (Bjork and Wallace, 2021).

2.1. Cell culture

Human hepatoma derived HepG2/C3A cells (ATCC; Manassas, VA) were grown in Eagle's Minimum Essential Media (EMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5%CO₂. The cells were passaged to assay plates and allowed to attach overnight prior to chemical exposure. Each replicate was a culture started from a single vial of frozen cells passage between 9 and 10 from the original stock.

2.2. Chemical exposure

Molnupiravir (EIDD-2801, MK-4482; Mol) was purchased from MedChemExpress (Item no. HY-135853). β-D-N⁴-hydroxycytidine (EIDD-1931; NHC) was purchased from Cayman Chemicals (item no. 9002958). 2'-3'-dideoxycytidine (ddC) was used as a positive control for inhibition of mtDNA synthesis and was purchased from Sigma (Item no. D6782). 2'-C-methyladenosine (2-C-MA), a positive control for inhibition of mitochondrial gene expression, was purchased from Santa Cruz Biotechnology Inc. (Item no. SC-283467). Stock solutions of Molnupiravir, NHC, and 2-C-MA were prepared in anhydrous DMSO at 1000× working concentration and diluted in growth media with a final DMSO concentration of 0.1% at all concentrations of the drugs. A 1000× stock solution of ddC was prepared in growth media and further diluted in media to exposure concentrations. On the day of the experiment, the media was replaced with exposure media and the cells exposed for 48 h.

2.3. Cytotoxicity

A cytotoxicity dose response was measured by the sulphorhodamine B (SRB) binding assay (Skehan et al., 1990) as previously described (Bjork and Wallace, 2021). Briefly, cells were seeded at 50% density on 48-well culture plates and exposed to increasing concentrations of the drugs for 48 h. The cells were rinsed and fixed in ice cold 1% acetic acid in methanol then stained with 0.5% SRB in 1% acetic acid. After rinsing and drying the plates, the cell bound SRB was solubilized in 10 mM Tris Base and the absorbance measured at 540 nm on a SpectraMax® M3 microplate reader (Molecular Devices, Inc.).

2.4. Mitochondrial DNA copy number (mtDNA)

Mitochondrial and nuclear (nDNA) DNA gene dose were measured by quantitative PCR (qPCR) of the 262–388 bp region of mitochondrial DNA and the nuclear encoded B2M gene, respectively (Malik et al., 2011). Quantification was made using a 5-point standard curve of gene specific DNA with FastStart Essential DNA Green Master mix (Roche, 06924204001). Dideoxycytidine (ddC) was selected as a positive control for inhibiting mitochondrial DNA replication (Jin et al., 2017). PCR reactions were performed on a LightCycler 96 system (Roche Molecular Systems). Values are reported as the ratio of mtDNA copies per nDNA copies.

2.5. Mitochondrial gene expression

Gene expression was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) as previously reported (Bjork and Wallace, 2021). Briefly, total RNA was extracted from cells exposed to increasing drug concentrations on 24-well plates using the RNeasy Plus Mini Kit (Qiagen, 74136) according to the manufacturer's protocol. The positive control for inhibition of mitochondrial gene expression was 25 μM (2'-C-methyladenosine; 2-C-MA) (Arnold et al., 2012). Reverse transcription was performed using 300 ng of total RNA with the

Table 1

Gene specific primer sequences and mRNA/DNA source sequence identification.

Gene Symbol	mRNA accession	Direction	Sequence
B2M	NG_012920.2	Forward	TGT TCC TGC TGG GTA GCT CT
		Reverse	CCT CCA TGA TGC TGC TTA CA
COX5B	NM_001862.3	Forward	TTG CTG CTA GTC GCG GAC GC
		Reverse	TCC CTC TCC AAC CCA GTC GCC
DDIT3	NM_001195053.1	Forward	AGT CAT TGC CTT TCT CCT TCG GGA
		Reverse	AAG CAG GGT CAA GAG TGG TGA AGA
MT-CO2 (COX2)	NC_012920.1	Forward	CGA GTA CAC CGA CTA CGG CGG
		Reverse	AGT CGC AGG TCG CCT GGT TCT
mtDNA 262–388	NC_012920.1	Forward	CAC TTT CCA CAC AGA CAT CA
		Reverse	TGG TTA GGC TGG TGT TAG GG
MT-ND2	NC_012920.1	Forward	CAC CCC TCT GAC ATC CGG CCT
		Reverse	TCC ACC TCA ACT GCC TGC TAT GAT
NDUFB3 v1	NM_002491.3	Forward	TTC CGG TTG GCT CCG GTT GCA
		Reverse	CGG CCC CAT GGA TCC CTT AGC

Omniscript RT Kit (Qiagen, 205113). Quantitative PCR (qPCR) was accomplished using the FastStart Essential DNA Green Master mix (Roche, 06924204001) on a LightCycler 96 system (Roche Molecular Systems Inc.) with calibration against a 5-point gene specific DNA standard curve. Gene specific primers (Table 1) designed as previously described were purchased from Integrated DNA Technologies; IDT, Coralville, IA).

2.6. Mitochondrial respiration

Mitochondrial respiratory function was assessed using the Seahorse XF Cell Mito Stress Test protocol on an Agilent Seahorse XFe96 analyzer as previously described (Bjork and Wallace, 2021). Briefly, the cells were plated at 10,000 cells per well on Seahorse 96-well culture plates and exposed to drugs for 48 h in EMEM growth media. On the day of the assay, the cells were acclimated to Seahorse XF DMEM assay medium (Agilent, 103575–100). The Seahorse Cell Mito Stress Test assay was performed according to the manufacture's protocol with sequential additions of oligomycin (Calbiochem, 495455), FCCP (Sigma, C2920) and rotenone/antimycin A (Sigma, R8875/A8674). Parameter calculations were made using the Seahorse XF Cell Mito Stress Test Report Generator application (Agilent).

2.7. Statistical analysis

Data were analyzed by Single Factor Analysis of Variance (ANOVA) followed by Dunnett's test for multiple comparisons against control (Dunnett, 1955). A probability of $P < 0.05$ was selected as the criterion for statistical significance.

3. Results

To test whether either molnupiravir or NHC interfered with mitochondrial genomics or function directly, we first established the highest drug concentrations that did not cause cell death. The purpose was to avoid results that were not primary, but secondary effects of cell morbidity on genomic stability or mitochondrial integrity. To address

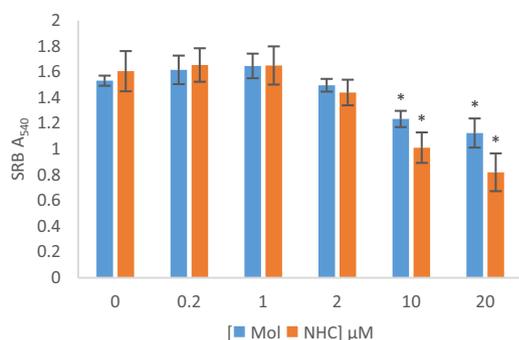


Fig. 1. Concentration-dependent Cytotoxicity of Molnupiravir and NHC – HepG2/C3A cells were exposed to increasing concentrations of either Molnupiravir or NHC for 48 hours in 24-well plates. At the end of the exposure the cells were fixed with methanol and stained with sulphorhodamine B (SRB). The bound SRB was solubilized in 10 mM Tris base and the absorbance measured 540nm. Values represent the mean \pm SD for 4 independent determinations. Asterisks (*) represent a statistically significant difference ($P < 0.05$) compared to the zero drug concentration.

this, we exposed HepG2 cells in culture for 48 h to increasing concentrations of either molnupiravir or NHC then measured protein content as an indirect measure of cell number or cytotoxicity (Skehan et al., 1990). The results are illustrated in Fig. 1. For both drugs, concentrations of 2 μ M and less did not affect cell viability as measured SRB. In contrast, 10 μ M and 20 μ M molnupiravir or NHC caused significant reduction in SRB staining (\geq c.a. 15%). Based on these results, we identified 2 μ M molnupiravir or NHC as a high but non-cytotoxic concentration. Effects on mitochondrial genomics or function observed at drug concentration exceeding 2 μ M were considered to be confounded by secondary effects of cellular and molecular instability associated with cell morbidity and death.

Characteristics associated with mitochondrial off-targets of inhibitors of DNA polymerase, such as nucleoside reverse transcriptase inhibitors (NRTI) and mitochondrial POLG, is the depletion of mitochondrial DNA that is accompanied by bioenergetic deficits (Young, 2017). Fig. 2A illustrates the lack of effect of molnupiravir or NHC on mitochondrial DNA copy number. Concentrations of either drug up to and including 20 μ M failed to alter the ratio of mtDNA/nDNA gene targets. Conversely, exposure of HepG2 cells to the positive control dideoxycytidine (0.4 μ M for 48 h) resulted in an 80% decrease in mitochondrial DNA copy number (Fig. 2B).

Not only did neither molnupiravir nor NHC affect mtDNA copy number, neither drug inhibited mtDNA gene expression (Fig. 3). In fact,

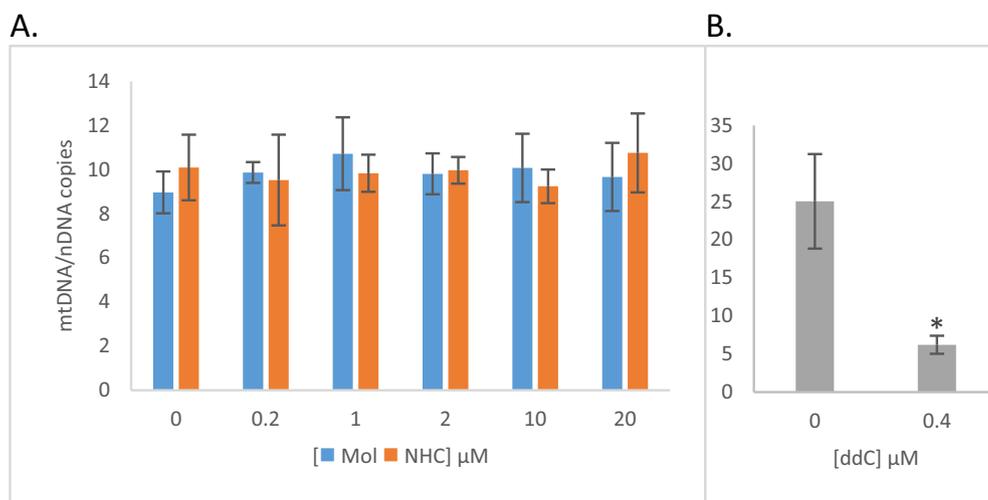


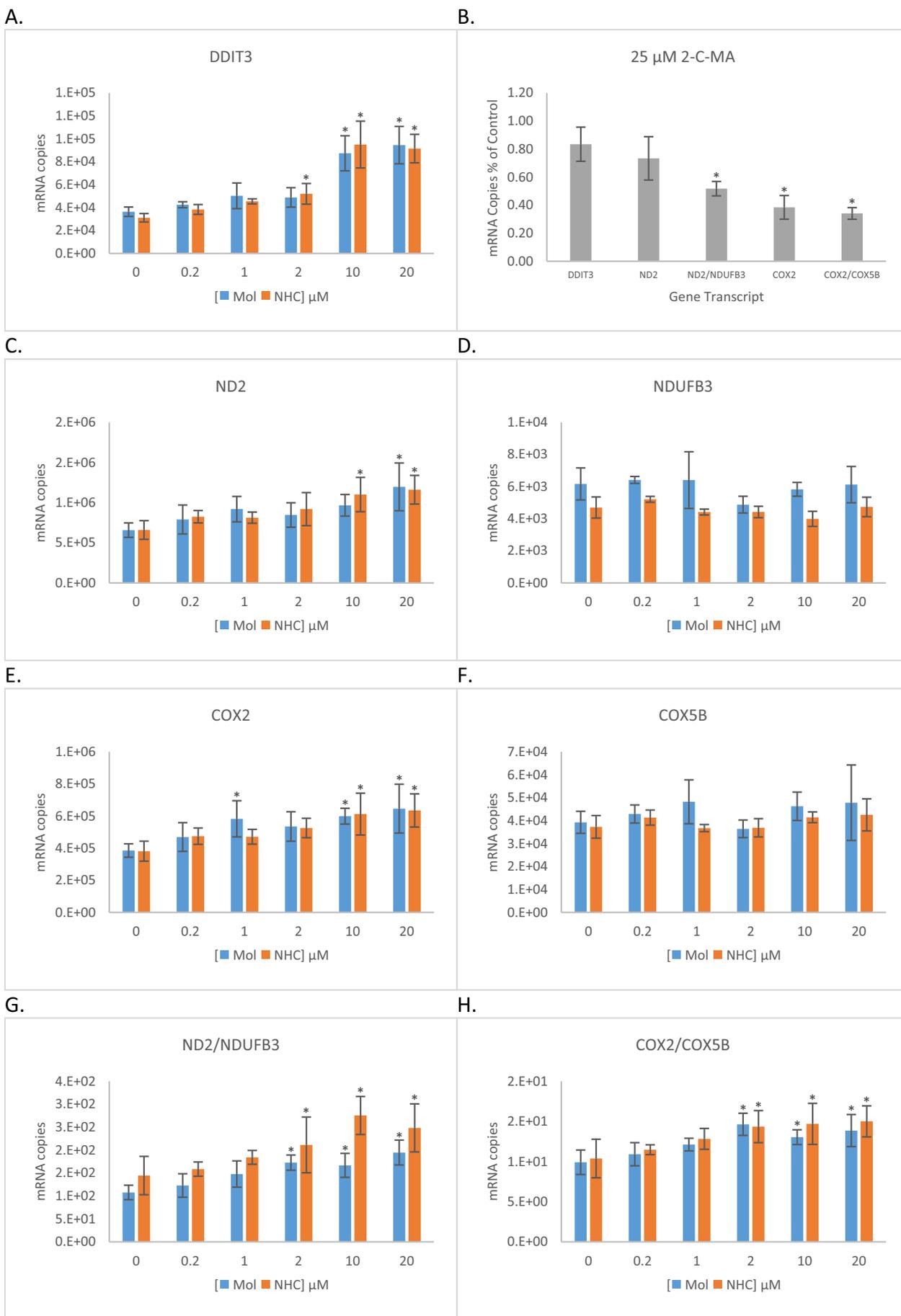
Fig. 2. Concentration-dependent Effect of Molnupiravir and NHC on Mitochondrial DNA Copy Number – A) HepG2 cells were exposed to increasing concentrations of either Molnupiravir or NHC for 48 hours. At the end of the exposure, DNA was extracted and quantified by qPCR using the recommended nuclear (nDNA) and mitochondrial (mtDNA) gene targets (Malik et al., 2011). B) HepG2 cells were exposed to sub-cytotoxic concentration of 2', 3' -dideoxycytidine (40 nM ddC) for 48 hours as a positive control for mtDNA depletion. For both panels, values represent the ratio of mtDNA/nDNA gene dose and are expressed as the mean \pm S.D. for 4 independent determinations. Asterisks indicate the statistically significant difference compared to zero drug concentration (Student's t-test $P < 0.05$).

the expression of mitochondrially encoded genes (ND2 and COX2) were both statistically increased in response to high drug concentrations, which we interpret to possibly reflect a compensatory response. When HepG2 cells were incubated for 48 h with 25 μ M 2-C-MA, the positive control inhibitor of mitochondrial gene expression, the ratio of mitochondrial to nuclear gene transcripts (ND2/NDUFB3 and COX2/COX5B) decreased by 40% to 60% compared to control (panel B). The pairing of these specific transcripts align by mitochondrial and nuclear encoded subunits of the same respiratory complex. 2-C-MA decreased the ratio of mitochondrial to nuclear transcripts for both complex I and IV. In contrast to what was observed for the positive control, neither molnupiravir nor NHC inhibited the expression mitochondrial encoded ND2 or COX2 transcripts nor did they reduce the corresponding ratio (Fig. 3, panels C–H).

Panel A demonstrates the concentration-dependent effect of molnupiravir and NHC of the expression of DDIT3, a transcript associated with the unfolded protein response indicative of cell injury (Bjork and Wallace, 2009; Los et al., 1999; Oyadomari and Mori, 2004). It is unknown what role the unfolded protein response possibly plays in the mechanism leading to cell death. It is interesting that for both drugs, this measure of cytotoxicity using Ddit3 expression is consistent with the concentration dependent loss of SRB binding (Fig. 1), reinforcing our determination of cytotoxic concentration.

It is interesting to note that even at cytotoxic concentrations, neither molnupiravir nor NHC affected transcription of the nuclear encoded NDUFB3 or COX5B genes (panels 3D and 3F). Conversely, both drugs caused an increase in expression of both mitochondrial encoded gene transcripts, ND2 and COX2 (panels 3C and 3E) as well as the corresponding ratios (panels 3G and 3H). Although not pursued, we speculate that this might reflect a compensatory response to cell injury, with either the mitochondria possibly being a primary off-target or the change being secondary to general cell injury. There were two anomalies to the general conclusion that neither molnupiravir nor NHC inhibited mitochondrial gene expression; Panel E shows that 1 μ M molnupiravir, but not NHC, causes a statistically significant increase in expression of ND2 and, Panel E demonstrates that 2 μ M molnupiravir or NHC increases the ratio for mitochondrial to nuclear encoded subunits to complex 1 (NDH) of the respiratory chain. In the absence of a consistent pattern, we suggest that these are statistical anomalies and not biological constructs. Regardless, the fact that none of these endpoints indicate a drug-induced decrease in gene expression refutes the original test hypothesis that the nucleoside analogs inhibit mitochondrial gene expression.

Investigation into a phenotype typical of mitochondrial toxicity was by measuring mitochondrial-specific cell respiration using the Cell Mito Stress Test protocol on an Agilent Seahorse XFe96 analyzer (Beeson



(caption on next page)

Fig. 3. Concentration-dependent Effect of Molnupiravir and NHC on Mitochondrial and Nuclear Gene Expression. Panels A and C-H) HepG2 cells were exposed in culture to increasing concentrations of Molnupiravir or NHC for 48 hours. RNA was extracted and RT-qPCR performed to quantify gene transcripts. Values represent mRNA copies normalized to 18s ribosomal RNA. B) HepG2 cells were exposed to a sub-cytotoxic concentration of 2'-C-methyladeosine (25 μ M 2-C-MA) as a positive control for mitochondrial RNA polymerase inhibition. The plots represent the mean \pm S.D. for 4 independent replications and asterisks indicate a statistically significant difference compared to the respective 0 μ M dose group (Dunnett's $P < 0.05$).

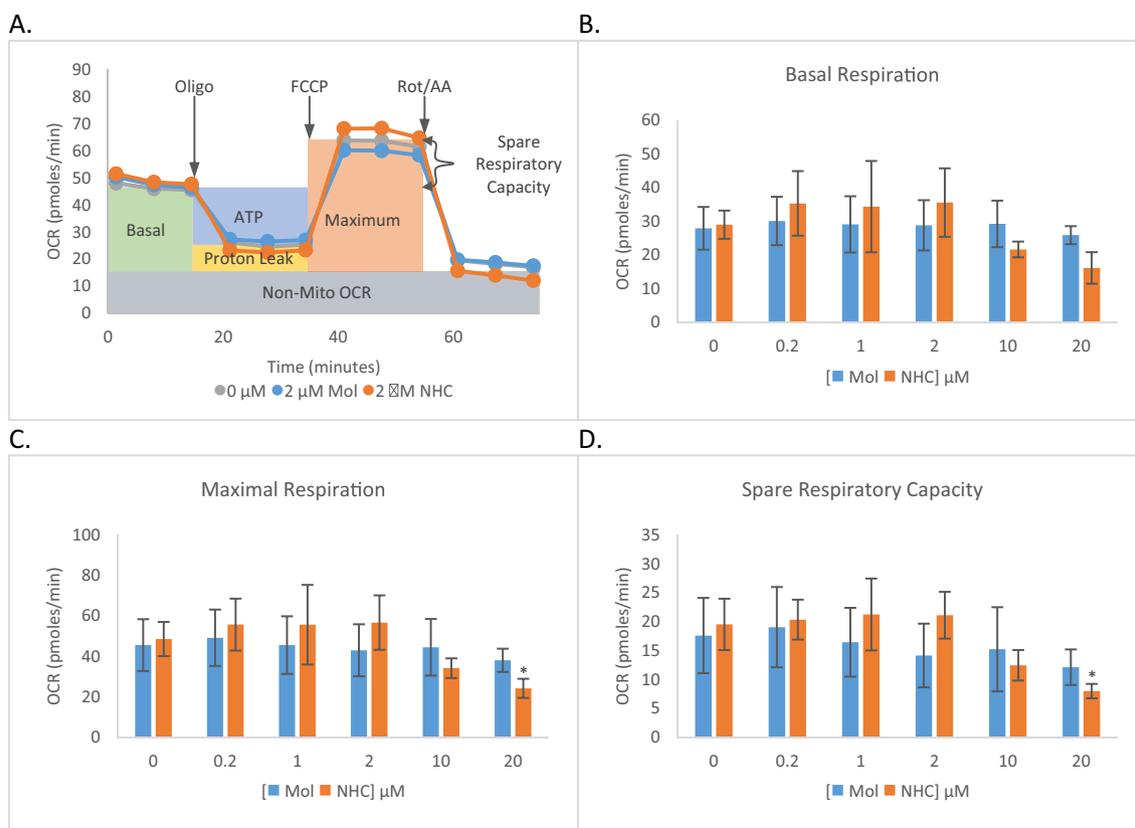


Fig. 4. Mitochondrial Respiration Parameters Associated with Molnupiravir or NHC Cell Exposure. Oxygen consumption rates (OCR), normalized to protein concentration as determined by the SRB assay, were measured for HepG2 cells exposed to increasing concentrations of Molnupiravir (Mol) or NHC for 48 h. Panel A illustrates the sequence of additions for the Seahorse XF Extracellular Flux Analyzer Mito Stress Test and the areas reflecting the corresponding respiratory parameters as defined in panels B, C and D.

Each panel represents a parameter calculated according to the Seahorse XF Cell Mito Stress Test Report Generator (Agilent) and each bar reflects the mean \pm S.D. for 4 independent experimental observations. Asterisks (*) indicate a statistically significant difference compared to the zero drug control (Dunnett's, $P \leq 0.05$).

et al., 2010; Bjork and Wallace, 2021; Nadanaciva et al., 2012). Fig. 4, panel B is a graphic representation of the assay, which involves measuring the oxygen consumption rate (OCR) following sequential additions to the sample (basal) of oligomycin, FCCP, and rotenone combined with antimycin A. In panel B, the tracings for the two drugs superimposed rather well with that of control. Panels A, C, and D describe the lack of effect of increasing concentrations of molnupiravir or NHC on basal and maximum respiration and spare respiratory capacity, respectively. Calculation of these parameters was according to the manufacturer's instructions, slightly modified and described in our recent publication (Bjork and Wallace, 2021). Although Fig. 4 illustrates the lack of effect of molnupiravir or NHC (0.2 μ M – 20 μ M) on only 3 of the respiratory parameters, we similarly did not observe an effect of increasing concentrations of either drug on any of the other parameters, which included proton leak, ATP production, or coupling efficiency (results not shown). Likewise, neither drug at concentrations 0.2 μ M to 20 μ M had an effect of extracellular acidification rate (ECAR; data not shown). Based on these results we conclude that neither molnupiravir nor NHC induced a phenotype of mitochondrial toxicity, even at high cytotoxic drug concentrations.

4. Discussion

Nucleoside analogs constitute a major class of antiviral drugs, the philosophical strategy being to compete for viral RNA polymerase-dependent incorporation into the viral genome. Depending on the nucleoside, the result is either the truncation or mutation of the replicating viral RNA (Prujssers and Denison, 2019). Unfortunately, these nucleotide analogs have varying specificity to the viral genome and also compete for the host cell DNA or RNA polymerases. A classic example are the nucleotide reverse transcriptase inhibitors that inhibit host cell mitochondrial DNA polymerase gamma (PolG) to inhibit replication of mitochondrial DNA (mtDNA) leading to lower mtDNA gene dose (Birkus et al., 2002). The result is a decrease in mitochondrial respiratory capacity and cell bioenergetic deficits.

Although there are no published reports of either Molnupiravir or NHC inhibiting mitochondrial DNA polymerase gamma (PolG) or RNA polymerase (POLRMT) activity, Sticher et al. reported that NHC-triphosphate is incorporated by POLRMT into a primer extension assay, albeit at 1/12th the efficiency as 3'-deoxycytidine triphosphate (Sticher et al., 2020). Associated with this was a concentration-dependent inhibition of both mitochondrial complex I and complex II activities in PC3 cells, with an IC50 of about 10 μ M and 5 μ M,

respectively. Curiously, incubating HepG2 cells with 10 μM NHC for 14 days did not alter mtDNA copy number, which might suggest that the effect of NHC on mitochondria owes to the expression (transcription or translation) and not the quantity of mtDNA.

Using sulphorhodamine B (SRB) staining, we observed a similar threshold for cytotoxicity in HepG2 cells as reported by Sticher et al. (Sticher et al., 2020). We also observed no effect of either molnupiravir or NHC on mtDNA copy number, even at cytotoxic drug concentrations. This is in spite of using different gene targets than those used by Sticher et al. (Sticher et al., 2020). Whereas Sticher et al. report a decrease by NHC in HepG2 cells on mitochondrial protein expression (Sticher et al., 2020), we observed no effect of either NHC or molnupiravir on mitochondrial gene expression. The inconsistency of these results may reflect either a specific effect of NHC on RNA translation or differential effect depending on the specific mitochondrial gene target.

The ultimate observation, however, is that neither molnupiravir nor NHC produced a functional phenotype for mitochondrial toxicity. Neither drug, at non-cytotoxic concentrations, significantly altered any parameters descriptive of mitochondrial respiration; basal, maximum, spare respiratory capacity, proton leak, or ATP production. The combined results provide strong evidence that mitochondrial off targets, whether it be genomic or functional, are not a primary concern in the mechanism of cytotoxicity for molnupiravir. For clinical perspective, Painter et al. (Painter et al., 2021) report that maximum plasma concentration of NHC in human patients receiving the recommended dose of 800 mg Molnupiravir (p.o., BID) was 2500–3000 ng/ml (0.75–1 μM), below that found to lack mitochondrial effects in this investigation. Consequently, mitochondrial toxicity is not expected to confound the safety profile of this newly authorized emergency treatment for COVID-19 infected patients.

Author credit

This research was conceived by KBW and executed by JAB. Both authors participated equally in the design, analysis and interpretation of the investigation.

Declaration

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Declaration of Competing Interest

None.

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