Antiviral activity of molnupiravir precursor NHC against SARS-CoV-2 Variants of Concern (VOCs) and its therapeutic window in a human lung cell model

Keywords: Molnupiravir, NHC, Anti-viral, ß-D-N4-hydroxicytidine, SARS-CoV-2

Running title: *In-vitro* activity of molnupiravir against SARS-CoV-2 Variants of Concern

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Synopsis

Background: The UK Medicines and Regulatory Healthcare Agency (MHRA) have recently licensed the anti-viral drug, molnupiravir, for use in patients with mild-moderate COVID-19 disease with one or more risk factors for serious illness. Treatment with anti-viral drugs is best initiated early to prevent progression to severe disease, although the therapeutic window for intervention has not yet been fully defined. **Objectives:** This study aimed to determine the activity of the molnupiravir (NHC) to different SARS-CoV-2 Variants of Concern (VoCs), and to establish the therapeutic window in human lung cell model. **Methods:** Dose response assays were performed in parallel to determine the IC50 (the concentration of drug required to inhibit virus titre by 50%) of NHC against different variants. Human ACE-2 A549 cells were treated with NHC at different time points either before, during or after infection with SARS-CoV-2. **Results**: Here we demonstrate that ß-D-N4-hydroxycytidine (NHC), the active metabolite of molnupiravir, has equivalent activity against four variants of SARS-CoV-2 in a human lung cell line ranging 0.04-0.16µM IC50. Furthermore, we demonstrate that *in-vitro* activity of the drug is reduced in cells exposed to drug 48 hours after infection. **Conclusions:** One of the main advantages of molnupiravir is that it can be administered orally, and thus given to patients in an out-patient setting. These results support giving the drug early on after diagnosis or even in prophylaxis for individuals with high risk of developing severe disease.

Introduction

SARS-CoV-2 emerged in China in late 2019 and has now caused more than 5 million deaths across the world [1]. In common with other coronaviruses, SARS-CoV-2 genomic variants are generated randomly through both single nucleotide polymorphisms and recombination resulting in insertions and deletions. These changes can then be subject to selection pressure in the host, including immune status and application of medical countermeasures. To date, several Variants of Concern (VOCs) have emerged with apparently increased transmissibility and reduced susceptibility to anti-viral antibodies. These VOCs include the Alpha variant (B.1.17), the Beta variant (B.1.351) and the Delta variant (B.1.617.2). Most recently, the Delta variant has predominated in the UK, and in much of the rest of the world.

While vaccination efforts have been largely successful in preventing severe disease, many people worldwide remain either unable or unwilling to be vaccinated. However, vaccination does not prevent household transmission [2]. Important groups of patients, such as those on immunosuppressive therapies, mount sub optimal responses to vaccines [3]. Therefore, effective treatments that are successful against multiple lineages of the virus are required. Targeting unique (to the virus) but conserved regions across variants, affecting functions of viral proteins such as the RNA dependent RNA polymerase (RdRp) (NSP12) is one such approach.

Molnupiravir is an anti-viral pro-drug originally developed against influenza virus currently undergoing clinical trials in humans for the treatment of COVID-19 [4,5]. Interim phase III results suggested the drug reduced the risk of hospitalisation or death by 50% with efficacy unaffected by the timing of symptom onset, underlying risk factors, or variant type (gamma, delta, and mu) [6]. As a result, the MHRA in the UK has granted emergency use of the drug for

64 treatment of mild-moderate cases of COVID-19 in patients with at least one risk factor for
65 severe disease [7].

66 Treatment of SARS-CoV-2 infection in a ferret model of disease with molnupiravir resulted
67 in reduced upper respiratory tract viral load and blocked transmission between animals [8]. The
68 drug was also shown to be effective against SARS-CoV-2 infection in mice [9]. A combined anti-
69 viral effect with suboptimal doses of molnupiravir with favipiravir has also been reported in a
70 hamster model, demonstrating a 5-log drop in viral titre and a near complete halt in
71 transmission. However, in contrast, 24 hours post-infection the drop was 2.5 logs. Combined
72 treatment was found to cause increased C to U changes in comparison to single treatment [10].
73 Most of these studies have however used an early variant of the virus, a lineage A virus rather
74 than assessed the efficacy of the drug in the more recent VOCs.
75 Molnupiravir is a pro-drug and is converted enzymatically *in-vivo* to its active form, thereby
76 improving its absorption in the gastrointestinal tract and bioavailability. The active drug of
77 molnupiravir is known as NHC or ß-D-N4-hydroxycytidine and is used for *in-vitro* studies. NHC
78 has been tested against lineage-A SARS-CoV-2 in primary human lung epithelial cells and Calu-
79 3 cells and found to have an IC50 of approximately 0.08µM in Calu-3 cells and no cytotoxicity
80 after 48 hours [11]. Another study using Calu-3 cells found an IC50 after 24 hours of 0.4uM [12].
81 Two independent studies have demonstrated efficacy against the Alpha and Beta variants in
82 Vero E6 and Calu3 cells and shown to be equivalent to an ancestral strain [13,14]. In hACE2-A549
83 cells, the drug has an anti-viral effect at concentrations between 0.3 to 3µM [15]. As yet no
84 published *in-vitro* studies compare the drug efficacy at inhibiting viral replication to the Delta
85 variant of SARS-CoV-2. To investigate whether NHC is effective at inhibiting variants of SARS-
86 CoV-2 with equal efficiency, a human lung epithelial cell model (hACE2-A549 cells) was

87 infected at the same time as treatment with varying concentrations of the drug or treated at

88 different times.

Materials and Methods

Compound

NHC (Alsachim) was supplied as a 1mg powder and was resuspended in 1ml DMSO to provide a 4.07mM stock solution. This was diluted in viral maintenance media (DMEM containing 2% FBS and 0.05mg/ml gentamicin) for experiments using a range of concentrations.

Cell culture

Human ACE2-A549 (hACE2-A549), a lung epithelial cell line which overexpresses the ACE-2 receptor, were the kind gift of Oliver Schwartz [16]. These were used to test the drug. These were cultured in DMEM with 10% FBS and 0.05mg/ml gentamicin with the addition of 10µg/ml Blasticidin (Invitrogen). Only passage 3-10 cultures were used for experiments. Vero/hSLAM cells (PHE) were grown in DMEM with 10% FBS and 0.05mg/ml gentamicin (Merck) with the addition of 0.4mg/ml Geneticin (G418; Thermofisher) at 37°C/5% CO_2.

Viral Culture

Virus stocks were grown in Vero/hSLAM cells using DMEM containing 2% FBS, 0.05mg/ml gentamicin and 0.4mg/ml geneticin and harvested 72 hours post inoculation. Virus stocks were aliquoted and stored at -80°C. The titre of stocks (PFU/ml) was determined by plaque assay. RNA from viral stocks were sequenced by Oxford Nanopore long read length sequencing on flow cells run on GridION.

Sequencing of viral stocks

Sequencing libraries for amplicons generated by ARTIC were prepared following the 'PCR tiling of SARS-CoV-2 virus with Native Barcoding' protocol provided by Oxford Nanopore Technologies using LSK109 and EXP-NBD196. The artic-ncov2019 pipeline v1.2.1 (https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html) was used to filter the passed fastq files produced by Nanopore sequencing with read lengths between 400 nt and 700 nt for ARTIC amplicons. This pipeline was then used to map the filtered reads on the reference SARS-CoV-2 genome (MN908947.3) by minimap2 and assigned each read alignment to a derived amplicon and excluded primer sequences based on the ARTIC V3 and V4 primer schemes in the bam files. Primer-trimmed bam files were further analysed using DiversiTools (http://josephhughes.github.io/DiversiTools/) with the "-orfs" function to generate the ratio of amino acid change in the reads and coverage at each site of protein in comparison to the reference SARS-CoV-2 genome (MN908947.3). The amino acids with highest ratio and coverage > 10 were used to assemble the consensus protein sequences. Pangolin (https://pangolin.cog-uk.io/) was used to confirm lineages of each viral stock used in experiments.

***In-vitro* cytotoxicity of NHC**

Human ACE2-A549 cells were plated at 2 x 10^4 cells per well in a clear bottomed white 96 well plate. Twenty-four hours later the medium was replaced with media containing NHC at different concentrations. At 72 hours post-exposure, cell viability was measured by CellTiter-Glo assay (Promega) as per the manufacturer's instructions.

137 **Anti-viral activity of NHC against SARS-CoV-2 Variants of Concern (VoCs)**

138 Human ACE2-A549 cells were grown to confluency and infected at an MOI of 0.1 in either
139 DMEM with 2%FBS and 0.05mg/ml gentamicin, or in the same media containing 0.01µM,
140 0.1µM, 1µM or 10µM NHC by allowing virus to adsorb to cells in a volume of 100µl for one
141 hour at 37°C, and then topping up to 500µl with the relevant media afterwards. A mock
142 infected control and a DMSO control were included in each experiment and experiments
143 were repeated a minimum of 3 times. After 72 hours, supernatants were collected and
144 stored at -80°C until viral titre was determined by plaque assay. The inhibitory potency of
145 NHC measured as the absolute IC50 was defined as the concentration of drug that resulted
146 in a 50% reduction in the number of plaques compared to untreated controls.

147

148 **Pre-exposure and Post-exposure to NHC**

149 For pre-exposure experiments, media was removed from cells and replaced with media
150 containing NHC two hours prior to infection. This was then removed for infection, which
151 was performed as described above. For post-exposure experiments, infections were
152 performed in media as described above. At two, four, 24 or 48 hours post-infection, media
153 was removed from cells. The cells were washed twice with PBS, and the media replaced
154 with media containing 0µM, 0.01µM, 0.1µM, 1µM or 10µM NHC NHC. After 72 hours post-
155 infection, supernatants were collected and stored at -80°C until viral titre was determined
156 by plaque assay.

157

158 **Statistical analysis**

159 A one-way ANOVA was used to evaluate the in-vitro cytotoxicity data. The absolute IC50

160 values were calculated using GraphPad Prism 9, using a non-linear 4-parameter logistic

161 regression in a dose-response curve.

162

Results

In order to find the appropriate concentration range of NHC in hACE2-A549 cells, to investigate the effect on viral biology, Cell-titer Glo Assay (Promega) were used to measure % ATP production in cells. These were treated with DMSO or different concentrations of NHC diluted in hACE2-A549 culture medium compared to mock untreated cells. DMSO, at the highest concentration used (0.25%) had no effect on cells ($p>0.05$). There was a slight increase in the ATP production in cells at the lowest concentration of drug, 0.01µM ($p=0.02$). The only concentration of drug to inhibit the ATP production was 10µM ($p>0.0001$) (Figure 1). Therefore, dose response assays to the drug could be conducted with concentrations of the drug up to 10µM.

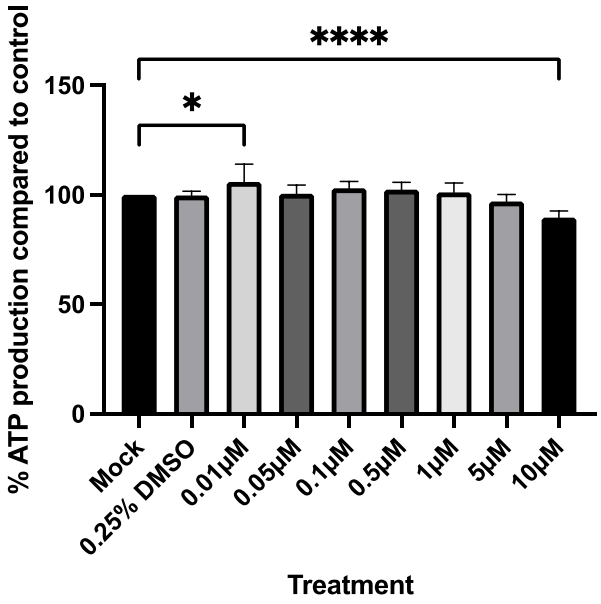

Figure 1. In-vitro cytotoxicity of NHC to hACE2-A549 cells. Cytotoxicity of different concentrations (in µM) was measured using the Cell-titer Glo Assay to measure the percentage ATP production in treated cells compared to mock treated cells (n=7). There was no significant difference in % ATP production of cells compared to control cells in most concentrations of NHC, except at 10uM ($p<0.0001$) and 0.1µM ($p=0.02$).

To determine the inhibitory activity of NHC against different VOCs and an ancestral B-lineage virus, dose response assays were performed by infecting hACE2-A549 cells at an MOI of 0.1 in media alone and in media containing 0.01, 0.1, 1, and 10µM NHC. After 72 hours incubation, cell supernatants were removed, and viral titres determined by plaque assay. The IC50 (the concentration of drug required to inhibit virus titre by 50%) was determined using non-linear regression with GraphPad Prism 9. The results demonstrated similar IC50 values for each variant and the ancestral strain of between 0.04 and 0.16µM concentrations (Table 1) (Figure2).

Variant	**IC50 (µM)**	**95% CI**
Liverpool/REMRQ0001/2020 (B lineage)	0.111	0.04-0.54
Alpha (B.1.1.7)	0.104	0.05-0.02
Beta (B.1.351)	0.134	0.05-0.03
Delta (B.1.617.2)	0.103	0.06-0.16

Table 1. IC50 values of NHC against variants in a hACE2-A549 human lung cell model. Inhibitory activity of NHC against an early variant of SARS-CoV-2 (Liverpool/REMRQ0001/2020) and Alpha (B.1.1.7), Beta (B.1.351) and Delta (B.1.617.2) variants of concern (VOCs). A non-linear regression was used to calculate the IC50 for each variant (n=4).

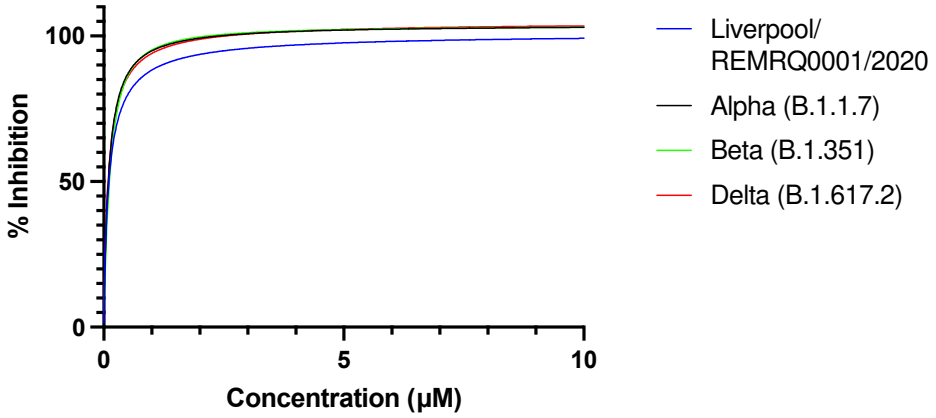

Figure 2. NHC is similarly active against both an early variant and VOCs. Inhibitory activity of NHC against an early variant of SARS-CoV-2 (Liverpool/REMRQ0001/2020) and Alpha (B.1.1.7), Beta (B.1.351) and Delta (B.1.617.2) variants of concern (VOCs). A non-linear regression was used to plot the dose response curves and calculate the IC50 for each variant (n=4).

To determine the effective treatment window, cells were both pre-treated with different concentrations of NHC two hours prior to infection and treated at two, four, 24- and 48-hours post-infection and compared to concurrent treatment/infection. Dose-response assays were performed on supernatants collected 72 hours post-infection. Viral titres remained similar if treatment was given prior to, or at the same time as infection and at time points of two, four- and 24-hours post-infection. However, if treatment was given at 48 hours post-infection, *in-vitro* activity of the drug was reduced for all variants tested (Figure 3).

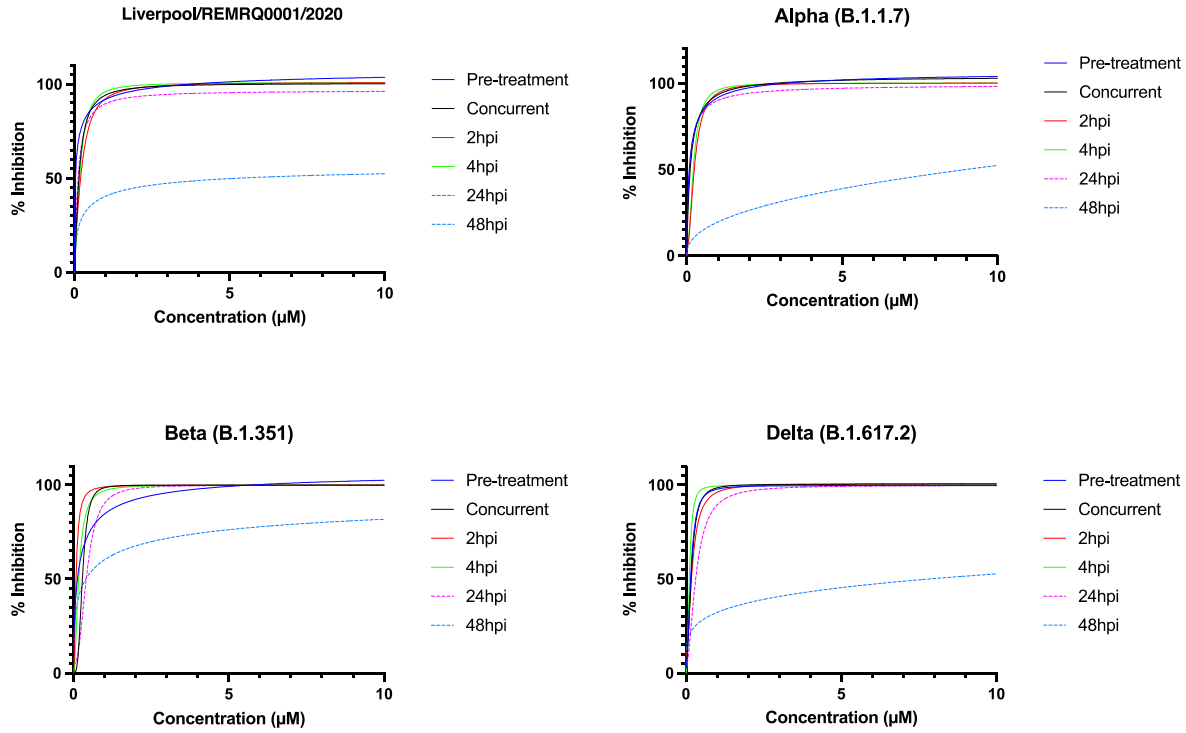

Figure 3. Effect of pre-treatment, concurrent treatment and treatment at two, four, 24 and 48 hours post-infection on inhibition of viral growth. Dose response assays were performed on supernatants collected 72 hours post-infection. Cells were either pre-treated with drug prior to infection, treated at the same time as infection, or treated at different time points post-infection. Treatment with drug inhibited growth of the virus to the same extent when given prior to infection, concurrently, or two, four- or 24-hours post-infection. When treatment was given 48 hours post-infection however, activity of the drug was reduced. A non-linear regression was used to plot the dose response curves and calculate the IC50 for each variant (n=3).

Discussion

These results support recent clinical trial data suggesting an inhibitory effect of molnupiravir on SARS-CoV-2 [6]. Molnupiravir, as a nucleoside analogue, acts by mimicking naturally occurring nucleosides to create error catastrophe during virus replication in the host. Therefore, we would expect this compound to act against all variants of the virus in a similar manner. This was tested in a cell culture system against four variants of SARS-CoV-2 (a B-lineage virus compared to alpha, beta and delta variants). The data indicated a similar pattern of growth inhibition for all four variants, suggestive of a common mechanism of action. Furthermore, we have explored the therapeutic window in which the drug is most active. We show that in infected cells the drug has reduced potency if given 48 hours post-infection. This data supports the results of the MOVe-In trial, where use of Molnupiravir in hospitalised patients was stopped prematurely since a statistically significant effect was deemed unlikely [17] and reinforces the choice to license the drug for use in mild-moderate out-patient cases.

However, *in-vitro* systems have several limitations in comparison to live models of infection, so results should be interpreted with care, although the mechanism of action will be intra-cellular. Use of molnupiravir in a Syrian hamster model infected with SARS-CoV-2 resulted in a drop in viral load and reduced lung pathology compared to controls [10,12]. Treatment 12 hours post-infection resulted in a protective effect [12] but not at 24 hours post-infection. Further work is required to delineate the true treatment window of the drug in humans with mild to moderate disease. Anti-viral drugs are best given early in infection to reduce viral load. *In-vivo* ferret models have shown that severity is linked to the size of the viral inoculum[18]. Therefore, molnupiravir likely acts to reduce disease by reducing viral load in patients, and potentially subsequently reducing transmission.

242 One of the main benefits of molnupiravir as opposed to remdesivir is that it can be
243 administered orally. However, as was seen with the Influenza anti-viral, Tamiflu, resistance to
244 anti-virals can develop rapidly [19]. A thorough analysis of the potential of SARS-CoV-2 to
245 develop resistance is necessary, though it is likely that any adaptation for resistance will
246 correspond with a reduction in fitness as seen with remdesivir [20]. Use of molnupiravir would
247 likely be most beneficial if used in combination with another treatment, preferably targeting
248 a different part of the viral life cycle as has been used with success for HIV treatment. Finally,
249 molnupiravir has broad spectrum activity, shown to be effective against RSV, Influenza, and
250 seasonal coronaviruses in *in-vitro* models. Here we have shown that the *in-vitro* activity of
251 NHC is retained across a broad range of variants tested, suggesting that the drug can be
252 deployed widely, and clinical effectiveness is unlikely to be adversely impacted by these
253 different viral strains.
254

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