Transfer and biotransformation of the COVID-19 prodrug molnupiravir and its metabolite β-D-N4-hydroxycytidine across the blood-placenta barrier

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Summary

Background Molnupiravir is an orally bioavailable prodrug of the nucleoside analogue β-D-N4-hydroxycytidine (NHC) and is used to treat coronavirus disease 2019 (COVID-19). However, the pharmacokinetics and transplacental transfer of molnupiravir in pregnant women are still not well understood. In the present study, we investigated the hypothesis that molnupiravir and NHC cross the blood-placenta barrier into the fetus.

Methods A multisite microdialysis coupled with a validated ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC−MS/MS) system was developed to monitor the dialysate levels of molnupiravir and NHC in maternal rat blood and conceptus (the collective term for the fetus, placenta, and amniotic fluid). Molnupiravir was administered intravenously (100 mg/kg, i.v.) on gestational day 16. To investigate the mechanism of transport of molnupiravir across the blood-placenta barrier, we coadministered nitrobenzylthioinosine (NBMPR, 10 mg/kg, i.v.) to inhibit equilibrative nucleoside transporter (ENT).

Findings We report that molnupiravir is rapidly metabolized to NHC and then rapidly transformed in the fetus, placenta, amniotic fluid, and maternal blood. Our pharmacokinetics analysis revealed that the area under the concentration curve (AUC) for the mother-to-fetus ratio (AUC_{fetus}/AUC_{blood}) of NHC was 0.29 ± 0.11. Further, we demonstrated that the transport of NHC in the placenta may not be subject to modulation by the ENT.

Interpretation Our results show that NHC is the predominant bioactive metabolite of molnupiravir and rapidly crosses the blood-placenta barrier in pregnant rats. The NHC concentration in maternal blood and conceptus was above the average median inhibitory concentration (IC_{50}) for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), suggesting a therapeutic effect. These findings support the use of molnupiravir in pregnant patients infected with COVID.

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Keywords: Molnupiravir; β-D-N4-hydroxycytidine; Blood-placenta barrier; SARS-CoV-2; Pharmacokinetics

Introduction

Molnupiravir is an orally bioavailable prodrug of the nucleoside analogue β-D-N4-hydroxycytidine (synonyms: N4-hydroxycytidine; NHC; EIDD-1931), which is a predominant bioactive metabolite of molnupiravir, and is widely researched as a broad-spectrum antiviral drug for treating viruses such as MERS-CoV, SARS-CoV, and SARS-CoV-2.[1] The mechanism of action of

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Research in context

Evidence before this study

Molnupiravir (200 mg) is an orally bioavailable prodrug of the nucleoside analogue β-D-N4-hydroxycytidine (NHC) and is used to treat coronavirus disease 2019 (COVID-19). However, the pharmacokinetics of molnupiravir in pregnant women are still not well understood. In the present study, we investigated the hypothesis that molnupiravir and NHC cross the blood-placenta barrier into the fetus. This transfer was postulated to be mediated by the equilibrative nucleoside transporter (ENT).

Added value of this study

A multisite microdialysis coupled with a validated ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) system was developed to monitor the dialysate levels of molnupiravir and NHC in maternal rat blood and conceptus (the collective term for the fetus, placenta, and amniotic fluid).

Implications of all the available evidence

Our results show that NHC is the predominant bioactive metabolite of molnupiravir and rapidly crosses the blood-placenta barrier in pregnant rats. The NHC concentration in maternal blood and conceptus was above the average median inhibitory concentration (IC_{50}) for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), suggesting a therapeutic effect. These findings support the use of molnupiravir in pregnant patients infected with COVID.

molnupiravir may involve the insertion of NHC into viral RNA, which replaces uracil during replication as NHC 5′-triphosphate, thus inhibiting RNA-dependent RNA-polymerase.[2,3] One *in vitro* study showed that the half maximal inhibitory concentration (IC_{50}) of NHC ranged between 0.08 μM and 0.3 μM (equivalent to 20.8–78 ng/mL) in several cell lines infected with SARS-CoV-2.[4] The United States Food and Drug Administration (US FDA) reported that the half effective concentration (EC_{50}) of NHC ranged between 0.67 and 2.66 μM in cell culture assays and showed similar activity against variants of concern (VOC) of SARS-CoV-2, including alpha (B.1.1.7), beta (B.1.351), gamma (P.1) and delta (B.1.617.2) strains.[5] Animal models have shown that NHC has poor bioavailability; however, molnupiravir increases the bioavailability of NHC by more than 90%.[6] Indeed, after oral administration of molnupiravir, NHC is rapidly formed to increase its bioavailability.[7–9]

Clinical data indicate that pregnancy is associated with higher clinical risk with COVID-19 infection compared to the general population.[10–12] Although the placenta normally acts as a barrier that prevents maternal infection of the fetus, contracting COVID-19 during pregnancy can result in vertical transmission to the fetus.[13–15] Clinical studies have shown that SARS-CoV-2 is highly expressed in the placenta and umbilical cord.[16] Therefore, identifying antiviral drugs that are suitable for pregnant women is urgently required.

NHC can be detected in the plasma of nursing pups from lactating rats administered molnupiravir.[5] However, similar data regarding molnupiravir or its metabolites in human milk are not available. Furthermore, molnupiravir may cause the risk of harm to the fetus after administration,[17] as administering doses that exceed eight times the normal dose have been observed to result in developmental toxicity, including lethality, teratogenicity, and other developmental abnormalities including reduced growth of the fetus in a pregnant rat model.[6,9] Although molnupiravir and NHC influence fetal growth, the biodistribution of molnupiravir and NHC across the placenta and into the fetus has not been investigated.

Drug absorption, distribution, and excretion with influx and efflux transport often depend on drug transporters, which are expressed in many tissues, including the intestine, liver, brain, and placenta.[18] The equilibrative nucleoside transporter (ENT) is an example of an influx transporter in the apical layer of placental cells.[19] A previous report indicated that NHC interacts with human ENT1 and ENT2, which influences the deposition of SARS-CoV-2.[20] However, direct evidence demonstrating the modulation of molnupiravir and NHC transport in the placenta and fetus is lacking.

In the present study, we investigated the hypothesis that molnupiravir and NHC penetrate the blood-placenta barrier and are transported to the fetus by the action of specific transporters. To investigate this hypothesis, a multisite microdialysis approach was developed using ultra high-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) to monitor molnupiravir and NHC in the maternal rat blood and conceptus (the collective term for the fetus, placenta, and amniotic fluid). We further investigated the role of ENT in the blood-placenta barrier transport of molnupiravir and NHC.

Methods

Chemicals and reagents

Molnupiravir and NHC were purchased from ChemScene (New Jersey, USA). Nitrobenzylthioinosine (NBMPR) was obtained from Cayman (MI, USA). MS grade acetonitrile was purchased from J.T. Baker, Inc. (Phillipsburg, NJ). Ammonium acetate was obtained from Sigma–Aldrich Chemicals (St. Louis, MO). Ultrapure water was used for sample preparation (Millipore, Bedford, MA). A standard stock solution of

molnupiravir and NHC (1 mg/mL) was dissolved in acetonitrile and stored at –20 °C for experimental use.

Animal experiments

The Institutional Animal Care and Use Committee of the National Yang Ming Chiao Tung University approved and supervised all animal experiments (IACUC no. 1110210). The animal surgical procedures followed the protocols approved by the IACUC of National Yang Ming Chiao Tung University. The guideline of animal surgical procedure was followed Guide for the care and use of laboratory animals[21] and ARRIVE.[22] Pregnant Sprague–Dawley rats at a gestational age of 16 days and weighing 300 ± 50 g were supplied by the Laboratory Animal Center of the National Yang Ming Chiao Tung University, Taipei, Taiwan. The animals had access to Laboratory Rodent Diet 5001 (PMI Feeds, Richmond, IN, USA) and water ad libitum. The animal facility maintained a 12-h light/dark cycle during the entire feeding process. Prior to surgery, the rats were fasted for 12 h. Anesthesia was induced by a single injection of urethane (1 g/kg, i.p.), and drugs were administered through a femoral vein using a polyethylene tube-50 (PE-50). The doses of molnupiravir (100 mg/kg, i.v.) and molnupiravir (100 mg/kg, i.v.) concomitantly administered with the ENT inhibitor NBMPR (10 mg/kg, i.v.) were used. Considering chemical solubility, molnupiravir (100 mg/kg, i.v.) was dissolved in normal saline for the concentration of 100 mg/mL, and NBMPR (10 mg/kg, i.v.) was dissolved in 10% dimethyl sulfoxide (DMSO) and 90% polyethylene glycol 400 (PEG-400) for the concentration of 10 mg/mL prior to drug administration.

Microdialysis experiment

The microdialysis system was composed of a microinjection pump (CMA/400, CMA, Stockholm, Sweden), a microfraction collector (CMA/142, CMA), and dialysate collection probes, which were designed and assembled in our laboratory.[23,24] The microdialysis probe was composed of a concentric silica capillary with a semipermeable dialysis membrane (Spectrum, New Brunswick, NJ, USA) with a fiber diameter of 200 μm and a molecular weight cutoff of 13 kDa. The active lengths of the blood and conceptus dialysis membranes were 1.2 cm and 0.6 cm, respectively. Perfusate was composed of an anticoagulant citrate dextrose (ACD) solution consisting of 3.5 mM citric acid, 7.5 mM sodium citrate, and 13.6 mM D-(+)-glucose and was delivered at a flow rate of 2.0 μL/min. The left femoral vein was catheterized by PE-50 tubing for the intravenous drug injection of molnupiravir (100 mg/kg) or concomitant administration of molnupiravir (100 mg/kg) and NBMPR (10 mg/kg), and six rats were used to confirm the reproducibility for each experimental group.[25,26] Initially, the blood microdialysis probe was positioned within the right side of the jugular vein toward the direction of the heart. Microdialysis probes were then inserted into the fetus, placenta, and amniotic fluid for sampling.[27] Dialysate samples were collected every 20 min for 6 h using a microfraction collector (CMA/142) and kept at –20 °C until further analysis by UHPLC–MSMS/MS.

Data analysis and statistics

Profiling solution software (version 1.1; Shimadzu, Kyoto, JPN) was used to evaluate the chromatograms. WinNonlin Standard Edition software (version 1.1; Scientific Consulting Inc., Apex, NC, USA) was used to calculate the main pharmacokinetic parameters of molnupiravir in blood. The results of one compartmental model were according to Akaike's information criterion (AIC).[28] To consider the biological barrier and biotransformation involved, a noncompartmental model was used for the remaining biological sites. In addition, the following parameters were calculated: the area under the concentration curve (AUC); initial drug concentration (C_0); maximum drug concentration (C_{max}); half-life ($t_{1/2}$); clearance (CL); mean residential time (MRT); biotransformation ratio of molnupiravir to NHC using the formula $[AUC_{NHC}/AUC_{molnupiravir}] \times 100\%$; and biodistribution ratio of blood to fetus, placenta, and amniotic fluid using the formula $[AUC_{fetus}/AUC_{blood}] \times 100\%$, $[AUC_{placenta}/AUC_{blood}] \times 100\%$, $[AUC_{amniotic\ fluid}/AUC_{blood}] \times 100\%$, respectively. Drug concentration-time curves were drawn using SigmaPlot (version 10.0; Systat Statistics, London, UK). Statistical contrasts were determined using one-way analysis of variance (ANOVA) and Tukey's post hoc test. The alpha criterion was set at 0.05. Data are expressed as the mean ± standard error of the mean. Statistical analyses were performed using SPSS Statistics (version 22.0, IBM Corp., Armonk, NY, USA). We compared the parameters at different biological sites. One-way ANOVA was selected to compare blood, fetus, placenta, and amniotic fluid. When comparing the molnupiravir alone group and the group involving the combined administration of molnupiravir and NBMPR, Student's t test and Tukey's post hoc test were performed.

Results

Optimization of UHPLC–MS/MS conditions

A UHPLC–MS/MS system coupled with microdialysis was developed (positive MRM-ESI mode) to analyze molnupiravir and NHC. The analytical method was based on the 2018 bioanalytical method validation guidelines announced by the US FDA[29] and provided high sensitivity and selectivity for the quantification of molnupiravir and NHC. Following optimization, mass transitions of molnupiravir and NHC were observed at 330.2–128 (m/z) and 260–128.05 (m/z), respectively. The collision energies were –15 eV and –11 eV for molnupiravir and NHC, respectively (Supplementary

Materials, Supplementary Figure S1). The quantitative determination limits of molnupiravir and NHC in rat dialysates were 2.5 and 10 ng/mL, respectively. Method validation data, including accuracy, precision, stability, probe recovery, and matrix effect, are presented in Supplementary Materials, Supplementary Tables S1–S5.

Fig. 1 shows the MRM chromatogram of molnupiravir and NHC in blood (Fig. 1A–C), the fetus (Fig. 1D–F), placenta (Fig. 1G–I), and amniotic fluid (Fig. 1J–L). Each figure also shows blank dialysates, dialysates spiked with standard concentrations of molnupiravir and NHC, and the sample dialysates. The retention times of molnupiravir and NHC were 1.35 and 5.96 min, respectively. The representative MRM chromatograms indicate that there was an interference signal in the blank matrix (Fig. 1).

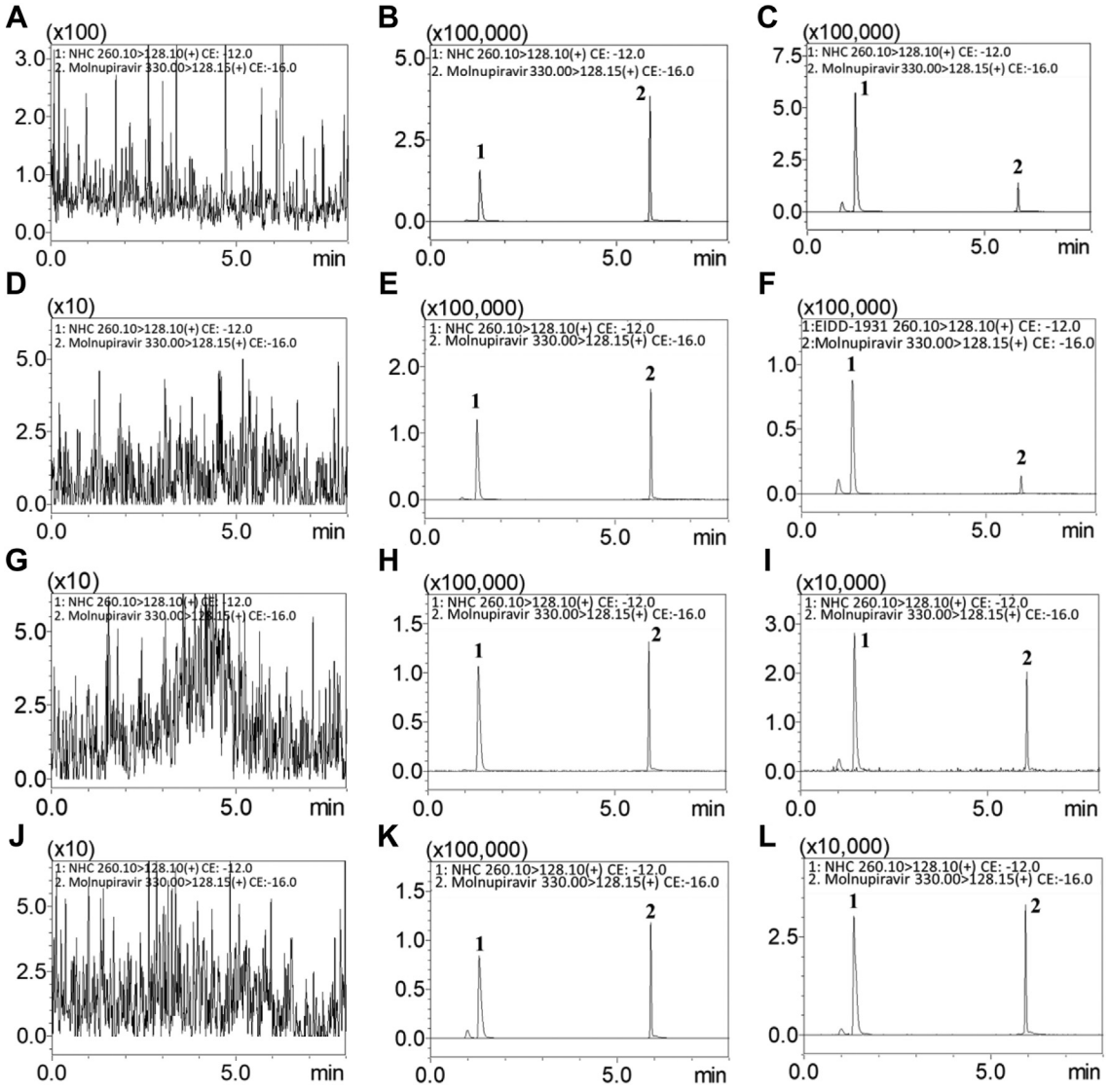

Fig. 1: Representative MRM chromatograms of (A) blank blood dialysate, (B) blank blood dialysate spiked with 250 ng/mL NHC and 50 ng/mL molnupiravir, (C) a sample of blood dialysate containing 960 ng/mL NHC and 18.2 ng/mL molnupiravir collected 140 min after molnupiravir administration, (D) blank fetus dialysate, (E) blank fetus dialysate spiked with 250 ng/mL NHC and 50 ng/mL molnupiravir, (F) fetus dialysate sample containing 185 ng/mL NHC and 3.02 ng/mL molnupiravir collected at 140 min, (G) blank placenta dialysate, (H) blank placenta dialysate spiked with 500 ng/mL NHC and 100 ng/mL molnupiravir, (I) placenta dialysate sample containing 126.2 ng/mL NHC and 15.1 ng/mL molnupiravir collected 80 min after molnupiravir administration, (J) blank amniotic fluid dialysate, (K) blank amniotic fluid dialysate spiked with 250 ng/mL NHC and 50 ng/mL molnupiravir, (L) amniotic fluid dialysate sample containing 10.05 ng/mL NHC and 1.48 ng/mL molnupiravir collected 60 min after molnupiravir administration (100 mg/kg, i.v.); peak 1 = NHC, peak 2 = molnupiravir.

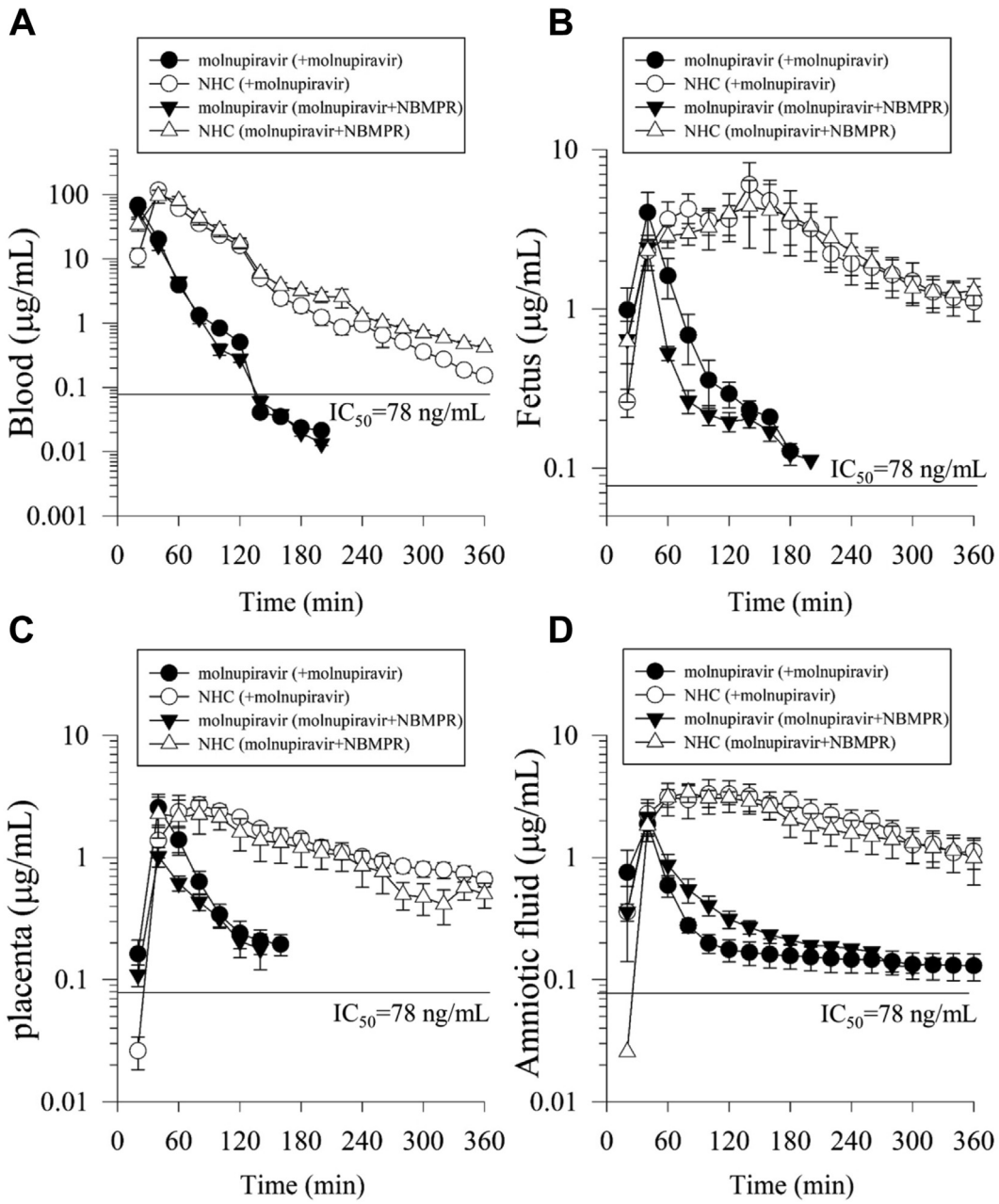

Fig. 2: Concentration-time curves of protein unbound dialysates of molnupiravir and NHC versus time in maternal rat blood, fetus, placenta, and amniotic fluid. (A) molnupiravir and β-D-N4-hydroxycytidine (NHC) in maternal rat blood, (B) fetus, (C) placenta, and (D) amniotic fluid after molnupiravir administration (100 mg/kg, i.v.) alone and administration of molnupiravir (100 mg/kg, i.v.) + NBMPR (10 mg/kg, i.v.). Data are expressed as the mean ± SEM (n = 6); nitrobenzylthioinosine = NBMPR, an inhibitor of ENT. The IC_{50} value was showed in figure.[4]

Biotransformation and biodistribution of molnupiravir and NHC

Analytes were detected using the validated analytical system described above. Fig. 2 shows concentration versus time curves of molnupiravir and NHC in maternal rat blood (Fig. 2A), the fetus (Fig. 2B), placenta (Fig. 2C), and amniotic fluid (Fig. 2D) after molnupiravir administration (100 mg/kg, i.v.). Our pharmacokinetic data indicated a rapid biotransformation and biodistribution of molnupiravir during the first 20 min into the placenta, fetus, and amniotic fluid. Molnupiravir was detected for up to 200 min in blood, and the $t_{1/2}$

ranged from 11 ± 2, 29 ± 5, 60 ± 25, and 828 ± 173 min in blood, fetus, placenta, and amniotic fluid, respectively. Furthermore, the elimination rate of NHC was slow and could be detected in the dialysates of all organs until 360 min post-administration (Fig. 2). The time for the maximum concentration of molnupiravir in the conceptus (C_{max}) was approximately 40 min. The T_{max} of NHC was 40, 123 ± 11, 90 ± 16, and 150 ± 33 min for blood, fetus, placenta, and amniotic fluid, respectively. The C_{max} of NHC was 116.5 ± 3.87, 7.20 ± 2.10, 2.98 ± 0.42, and 4.78 ± 0.83 μg/mL in blood, fetus, placenta, and amniotic fluid, respectively (Table 1).

The AUCs of molnupiravir in blood, fetus, placenta, and amniotic fluid were 3697 ± 532.2, 198.4 ± 43.89, 145.3 ± 35.21, and 279.4 ± 55.04 min μg/mL, respectively, and the AUCs of NHC in blood, fetus, placenta, and amniotic fluid were 7230 ± 1377, 1728 ± 595.6, 598.7 ± 41.42, and 900.6 ± 140.9 min μg/mL, respectively. The biotransformation rate from molnupiravir to NHC was expressed using the $AUC_{NHC}/AUC_{molnupiravir}$ of blood, fetus, placenta, and amniotic fluid, which were 1.69 ± 0.45, 16.89 ± 9.75, 5.94 ± 1.58, and 4.82 ± 0.52, respectively. These results demonstrated that the biotransformation ratios in the fetus, placenta, and amniotic fluid were significantly higher than those in the blood. The biodistribution ratios (AUC_{tissue}/AUC_{blood}) of molnupiravir transfer into the fetus, placenta, and amniotic fluid were 0.03 ± 0.01, 0.02 ± 0.009, and 0.05 ± 0.01, respectively, and the biodistribution ratios of NHC were 0.29 ± 0.11, 0.09 ± 0.01, and 0.19 ± 0.03, respectively (Table 1). These results demonstrated that NHC can cross the blood-placenta barrier into the amniotic fluid and fetus, with a transfer ratio between 2 and 5% molnupiravir and 9–29% NHC.

The transporter mechanism for molnupiravir and NHC

To investigate the mechanism by which transporters in the blood-placenta barrier of molnupiravir and NHC cross into the placenta, amniotic fluid, and fetus, the equilibrative nucleoside transporter inhibitor NBMPR (10 mg/kg, i.v.) was coadministered with molnupiravir (100 mg/kg, i.v.). The results demonstrated that the AUCs of molnupiravir and NHC in blood were 2979 ± 713.9 and 7039 ± 1478 min μg/mL, respectively, the AUCs in fetus were 98.91 ± 13.80 and 1557 ± 254.3 min μg/mL, respectively, the AUCs in placenta were 112.8 ± 25.05 and 569.9 ± 145.7 min μg/mL, respectively, and the AUCs in amniotic fluid were 166.4 ± 22.88 and 1139 ± 325.1 min μg/mL, respectively. Compared to molnupiravir alone, the AUCs of molnupiravir and NHC in the blood, fetus, placenta, and

Parameter	Blood		Fetus		Placenta		Amniotic fluid	
	Molnupiravir	NHC	Molnupiravir	NHC	Molnupiravir	NHC	Molnupiravir	NHC
Molnupiravir (100 mg/kg, i.v.)								
AUC_{inf} (min μg/mL)	3697 ± 532.2	7230 ± 1377	198.4 ± 43.89[a]	1728 ± 595.6[b]	145.3 ± 35.21[a]	598.7 ± 41.42[b]	279.4 ± 55.04	900.6 ± 140.9[b]
C_{max} (μg/mL)	219.2 ± 42.27	116.5 ± 3.87	3.54 ± 1.32[a]	7.20 ± 2.10[b]	2.56 ± 0.73[a]	2.98 ± 0.42[b]	2.11 ± 0.38[a]	4.78 ± 0.83[b]
$t_{1/2}$ (min)	11 ± 2	45 ± 5	29 ± 5	492 ± 305[b]	60 ± 25	138 ± 18[b]	828 ± 173	148 ± 52[b]
T_{max} (min)	–	40	40 ± 4	123 ± 11[b]	40	90 ± 16	40 ± 4	150 ± 33
CL (mL/min/kg)	21.89 ± 2.99	–	–	–	–	–	–	–
MRT (min)	16 ± 4	57 ± 6	42 ± 6	157 ± 10	70 ± 14	156 ± 10	104 ± 14	169 ± 8
$AUC_{NHC}/AUC_{molnupiravir}$	–	1.69 ± 0.45	–	16.89 ± 9.75	–	5.94 ± 1.58	–	4.82 ± 0.52
AUC_{tissue}/AUC_{blood}	–	–	0.03 ± 0.01	0.29 ± 0.11	0.02 ± 0.009	0.09 ± 0.01	0.05 ± 0.01	0.19 ± 0.03
Molnupiravir (100 mg/kg, i.v.) + NBMPR (10 mg/kg, i.v.)								
AUC_{inf} (min μg/mL)	2979 ± 713.9	7039 ± 1478	98.91 ± 13.80[c]	1557 ± 254.3[d]	112.8 ± 25.05[c]	569.9 ± 145.7[d]	166.4 ± 22.88[c]	1139 ± 325.1[d]
C_{max} (μg/mL)	260.6 ± 110.8	97.26 ± 24.95	2.41 ± 0.18[c]	4.69 ± 1.92[d]	1.02 ± 0.19[c]	2.95 ± 0.97[d]	2.14 ± 0.64[c]	4.21 ± 0.44[d]
$t_{1/2}$ (min)	12 ± 1	66 ± 3	26 ± 6	507 ± 178[d]	180 ± 85[c]	157 ± 56[d]	187 ± 50[c]	181 ± 56[d]
T_{max} (min)	–	43 ± 3	40	93 ± 16[d]	40	106 ± 12[d]	40	100 ± 14[d]
CL (mL/min/kg)	59.20 ± 22.57	–	–	–	–	–	–	–
MRT (min)	17 ± 1	56 ± 6	50 ± 4	762 ± 255	237 ± 89	251 ± 65	88 ± 9	294 ± 77
$AUC_{NHC}/AUC_{molnupiravir}$	–	2.98 ± 0.76	–	15.95 ± 1.71	–	7.01 ± 2.68	–	7.73 ± 2.68
AUC_{tissue}/AUC_{blood}	–	–	0.05 ± 0.02	0.27 ± 0.07	0.05 ± 0.01	0.14 ± 0.06	0.08 ± 0.02	0.19 ± 0.05

Abbreviations: AUC, area under the concentration-time curve; $t_{1/2}$, half-life; C_{max}, concentration maximum; CL, clearance; MRT, mean residence time. AUC_{tissue}/AUC_{blood} represents the rat blood-to-tissue transfer ratio. Data are expressed as the mean ± SEM (n = 6); nitrobenzylthioinosine (NBMPR; an inhibitor of ENT). [a]$p < 0.05$ compared with molnupiravir in blood dialysate in the molnupiravir (100 mg/kg, i.v.) alone group by one-way ANOVA with post hoc Tukey HSD test. [b]$p < 0.05$ compared with NHC in blood dialysate in the molnupiravir (100 mg/kg, i.v.) alone group by one-way ANOVA with post hoc Tukey HSD test. [c]$p < 0.05$ compared with molnupiravir in blood dialysate in the concomitant administered with molnupiravir (100 mg/kg, i.v.) + NBMPR (10 mg/kg, i.v.) group by one-way ANOVA with post hoc Tukey HSD test. [d]$p < 0.05$ compared with NHC in blood dialysate in the concomitant administered with molnupiravir (100 mg/kg, i.v.) + NBMPR (10 mg/kg, i.v.) group by one-way ANOVA with post hoc Tukey HSD test.

Table 1: **Pharmacokinetic parameters of molnupiravir and NHC in maternal rat blood, fetus, placenta, and amniotic fluid after treatment with molnupiravir (100 mg/kg, i.v.) and concomitant administration of molnupiravir (100 mg/kg, i.v.) + NBMPR (10 mg/kg, i.v.).**

amniotic fluid were not significantly affected by coadministration with NBMPR (Table 1).

The biotransformation ($AUC_{NHC}/AUC_{molnupiravir}$) of molnupiravir in the blood, fetus, placenta, and amniotic fluid was 2.98 ± 0.76, 15.95 ± 1.71, 7.01 ± 2.68, and 7.73 ± 2.68, respectively. The biodistribution ratios (AUC_{tissue}/AUC_{blood}) of molnupiravir and NHC were 0.05 ± 0.02 and 0.27 ± 0.07, 0.05 ± 0.01 and 0.14 ± 0.06, and 0.08 ± 0.02 and 0.19 ± 0.05 in the fetus, placenta, and amniotic fluid, respectively. Compared with molnupiravir alone, the biotransformation and biodistribution of molnupiravir and NHC in the blood, fetus, placenta, and amniotic fluid were not significantly affected by the administration of NBMPR (Table 1). Statistical analysis by one-way ANOVA and Tukey's post hoc test yielded p values in the various sites greater than 0.24, thus indicating no significant difference compared to when molnupiravir (100 mg/kg, i.v.) was administered alone.

Discussion

In the present study, we investigated the transfer of molnupiravir across the blood-placenta barrier using a multisite microdialysis monitoring approach. The biological samples collected from the semipermeable membrane and the dialysates without protein did not undergo an additional clean-up step during sample preparation. These dialysates were directly injected into the UHPLC–MS/MS, and an internal standard was not used.[23,24] By administering the compound intravenously, we circumvented the first-pass effect that occurs with oral administration. We used a dose of molnupiravir (100 mg/kg, i.v.) that was based on the clinical dose of molnupiravir (1600 mg/day, p.o.) in humans and equivalent bioavailability data.[9,30] We report that the concentration of molnupiravir in maternal rat blood decreases rapidly after administration, which is consistent with a previous report on the short half-life ($t_{1/2}$) of this antiviral drug and is a consequence of the rapid conversion of molnupiravir to NHC catalyzed by carboxylesterases in the body.[6]

Following intravenous administration of molnupiravir (100 mg/kg, i.v.), the C_{max} of molnupiravir and NHC in blood was 219.2 ± 42.27 and 116.5 ± 3.87 μg/mL, respectively. A previous report showed that NHC was potently antiviral with an average median inhibitory concentration (IC_{50}) between 0.08 μM and 0.3 μM (equivalent to 20.8–78 ng/mL) in several cell lines infected with SARS-CoV-2.[4] Thus, the administration of NHC achieves a clinically effective concentration for at least 6 h. Another recent report testing both single and multiple doses observed that NHC appeared rapidly in plasma, with a median T_{max} of 1 h and C_{max} effectively above the EC_{90} after dosing.[8,30] The C_{max} of molnupiravir and NHC in the fetus, placenta, and amniotic fluid was 3.54 ± 1.32 and 7.20 ± 2.10, 2.56 ± 0.73 and 2.98 ± 0.42, and 2.11 ± 0.38 and 4.78 ± 0.83 μg/mL, respectively. Following C_{max}, NHC levels decreased slowly in the fetus, placenta, and amniotic fluid, confirming that NHC maintains effective concentrations in these regions compared with maternal blood.[2]

Nevertheless, there are indications that molnupiravir poses a fetal risk when administered during pregnancy.[17] Doses eight times the expected exposure to NHC treatment (800 mg twice daily) was observed to cause developmental toxicity, including lethality, teratogenicity, and other developmental abnormalities in a rat model of pregnancy.[17] The US FDA and the European Medicines Association (EMA) also indicate that molnupiravir can cause fetal harm when administered to pregnant individuals in animal reproduction studies, so medical professionals providing prescriptions must communicate to the patient the known and potential benefits as well as the potential risks of the use of molnupiravir during pregnancy.[6,9] Our data show that NHC has a long half-life in the fetus, placenta, and amniotic fluid, specifically 492 ± 305, 138 ± 18, and 148 ± 52 min, respectively. The phenomenon of a long elimination half-life in the fetus may be due to the absence of renal elimination. Moreover, NHC is highly hydrophilic; therefore, it most likely crosses the placental barrier slowly and then crosses back in a slower manner. Thus, prolonged exposure of the fetus to NHC may explain why the administration of molnupiravir is linked to harmful outcomes of the fetus. However, this should be further confirmed by a toxicological study. In terms of therapeutic potential, our data demonstrate that the NHC concentrations in the conceptus were all higher than the IC_{50} between 0.08 μM and 0.3 μM (equivalent to 20.8–78 ng/mL) of NHC in several cell lines infected with SARS-CoV-2.[4]

To investigate NHC transport at the blood-placenta barrier, NBMPR, an equilibrative nucleoside transporter inhibitor, was administered. Our findings definitively indicated that drug transfer across the blood-placenta barrier was not significantly affected by the coadministration of NBMPR. We posit that the concentrative nucleoside transporters (CNTs) in placental expression during gestation are much higher than the expression of equilibrative nucleoside transporters,[31] which can cause NHC to not be significantly affected by the equilibrative nucleoside transporter inhibitor.

A post hoc power analysis was conducted for the contrast between the molnupiravir alone group and the molnupiravir plus ENT inhibition group in the conceptus. Based on the group sizes of 6, alpha 0.05, and a non-directional test, the power of molnupiravir in the two groups (with or without ENT inhibitors) was 83.3%, however, the general investigation groups of the post-hoc test did not reach 80% (power = 52%). Additionally, a retrospective power analysis was performed

using the observed effect size, sample size, and significance level chosen for the study. The analysis revealed that the calculated power is 50%. Due to the biotransformation of molnupiravir to NHC catalyzed by carboxylesterases, tissue distribution and the biological barrier, each biodistribution and transportation process can cause some variation for molnupiravir and its metabolite to reach conceptus. Therefore, future studies are merited to replicate the basic effect of the ENT inhibitor on molnupiravir levels with larger group sizes to overcome the evident individual variation in the biotransformation of molnupiravir to NHC and the transport of the blood-placenta barrier.

Considering the balance between therapeutic efficacy and adverse effects of the antivirus medicines remdesivir and molnupiravir in pregnant women, Merck Pharmaceuticals released the disclaimer that a high dose of molnupiravir (1000 mg/kg) can cause internal and skeletal malformations of the fetus, including abnormal eye socket, absent kidney, rib malformations, and thoracic and lumbar vertebra malformations.[6,17] At a lower dose, molnupiravir (500 mg/kg) decreased fetal body weight was observed with no effects on post-implantation loss or malformations related to molnupiravir. The dose of no-observed-adverse-effect level (NOAEL) for maternal and developmental toxicity is 250 mg/kg.[6,17] To avoid toxicity the dose of molnupiravir (100 mg/kg) in the current study was 0.4 times the NOAEL, which 0.2 times the dose associated with fetal weight loss, and 0.1 times the dose associated with fetal malformation. Regarding therapeutic potential, a previous antivirus study observed a dose-dependent reduction in the IC_{50} range of NHC of approximately 0.08–0.3 μM (equivalent to 20.8–78 ng/mL).[4] Our study demonstrates that the concentrations observed in the fetus, placenta, and amniotic fluid were all above the therapeutic concentration range after molnupiravir administration (100 mg/kg) (Fig. 2).

Compared to molnupiravir, a previous report demonstrated that a metabolite of remdesivir, GS-441524, was rapidly transferred to the placenta with a transfer ratio from mother to fetus of 0.51 ± 0.18, a transfer ratio from mother to placenta of 0.35 ± 0.09, and a transfer ratio from mother to amniotic fluid of 0.71 ± 0.19 after remdesivir administration (30 mg/kg, i.v.).[32] Furthermore, the EC_{50} of GS-441524 was observed to be 0.18 μM (equivalent to 46.8 ng/mL) in human airway epithelial cells (HAE).[33,34] A previous study revealed that the conceptus concentration was approximately the effective concentration at the therapeutic dose after remdesivir administration (30 mg/kg, i.v.).[32] Although molnupiravir and remdesivir have different therapeutic efficacies and associated adverse effects, our data support the notion that both antivirus drugs reach effective concentrations for SARS-CoV-2 at regular doses of remdesivir (30 mg/kg, i.v.) and molnupiravir administration (100 mg/kg, i.v.).

Conclusion

In conclusion, an optimized UHPLC–MS/MS method coupled to a microdialysis sampling system was developed to monitor molnupiravir and NHC in maternal rat blood, fetus, placenta, and amniotic fluid dialysates. Molnupiravir was rapidly metabolized to NHC within 20 min, penetrated the blood-placenta barrier, and entered the placenta, amniotic fluid, and fetus. NHC levels in the fetus and amniotic fluid remained elevated for a prolonged time. Following ENT inhibition, we observed no significant difference in drug transfer across the placenta compared with rats treated with molnupiravir alone. These null findings imply that another transporter may be involved in the transformation of the blood-placenta barrier of NHCs. This preclinical study provides important new information on the pharmacokinetics of the prodrug molnupiravir with therapeutic implications for the treatment of pregnant women infected with SARS-CoV-2.

Contributors

Methodology: CHC, WHL; Investigation: CHC, WHL, TYL, WYP; Visualization: TYL, WYP; Funding acquisition: MHY, THT; Project administration: MHY, THT; Supervision: THT; Writing—original draft: CHC, THT; Writing—review & editing: CHC, THT, JD; All authors have read and approved the final version of the manuscript.

Data sharing statement

The data and materials supporting the conclusions of this article are included in the Supplementary Materials files and also available from the corresponding author on request.

Declaration of interests

The authors declare no conflicts of interest related to this study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104748.

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