Preclinical assessment of nirmatrelvir penetration into cerebrospinal fluid and central nervous system cells and tissues

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Abstract

Three years after SARS-CoV-2 emerged as a global infectious threat, the virus has become endemic. The neurological complications such as depression, anxiety, and other CNS complications after COVID-19 disease are increasing. The brain, and CSF have been shown as viral reservoirs for SARS-CoV-2, yielding a potential hypothesis for CNS effects. Thus, we investigated the CNS pharmacology of orally dosed nirmatrelvir/ritonavir (NMR/RTV). Using both an in vitro and an in vivo rodent model, we investigated CNS penetration and potential pharmacodynamic activity of NMR. Through pharmacokinetic modeling, we estimated the median CSF penetration of NMR to be low at 18.11% of plasma with very low accumulation in rodent brain tissue. Based on the multiples of the 90% maximal effective concentration for SARS-CoV-2, concentrations in the CSF and brain may be inadequate to achieve exposure similar to that of plasma, which may have implications for viral persistence and neurologic post-acute sequelae of COVID-19.

Introduction

As of March 10th, 2023, global cases of coronavirus infectious disease 2019 (SARS-CoV-2; COVID-19) had reached 676,609,955 with 6,881,955 total deaths. Although SARS-CoV-2 is often referred to as a respiratory virus, in addition to the lung it has been found in tissues including the brain, liver, intestine, feces, heart, and kidneys of individuals with COVID-19. The impact of COVID-19 on human health has led to significant investment in new strategies including the development of new therapeutic agents to reduce the risk of infection, disease, and negative outcomes.

The primary oral antiviral treatment for COVID-19 is nirmatrelvir/ritonavir (NMR/RTV). This drug is a combination of a SARS-CoV-2 MSP5 protease inhibitor (PI) NMR, and RTV used in a low-dose as a pharmacokinetic (PK) enhancer to increase the concentrations of NMR in the blood via inhibition of hepatic phase 1 metabolism. NMR/RTV received FDA approval on May 25th 2023, as the first oral antiviral treatment for mild to moderate COVID-19 in adults who are at high risk for severe COVID-19.

Neurological complications associated with SARS-CoV-2 infection are not well understood. Post-acute sequelae of COVID-19 (PASC), also known as Long COVID, is a chronic syndrome that affects some individuals who have recovered from acute COVID-19 illness. While most people clear the virus, some experience persistent neuro-specific PASC (neuroPASC) symptoms (e.g., depression, anxiety, difficulty concentrating, central nervous system [CNS] disturbances) lasting months or even years after the infection. The etiology of neuroPASC is unclear, and the exact mechanisms of SARS-CoV-2 entry into the CNS are uncertain. Some theories for entry include infection of the endothelium, access through the blood-brain barrier (BBB), and through nervous tissue conduits that bypass the BBB. Given that cells in the CNS can be infected with SARS-CoV2, it is plausible that CNS infections lead to the neurological complications described by neuroPASC. Another theory is that neuroPASC is due to prolonged inflammation present in the CNS post-infection. Clinical data from autopsy sampling performed on the CNS of patients who died from COVID-19 found viral RNA, with patients having detectable CNS virus from 4-230 days after infection. Given data to support viral entry into the CNS, and the known neurological issues associated with neuroPASC, early and effective antiviral treatment of acute COVID-19 may offer hope in preventing or reducing neuroPASC occurrence and severity.

Currently, there are no data on NMR concentrations in the CNS. It is unknown if NMR can cross the BBB and achieve therapeutic concentrations necessary to treat SARS-CoV-2 infection in the CNS. Given the limited treatment options available for COVID, it is essential to evaluate whether current treatment can be maximized to ensure viral eradication. Treatment and prevention of neuroPASC caused by virus in the CNS would require therapeutic CNS NMR concentrations, which are a function of effective concentration goals (EC50 − 90), brain penetration and dose. Suboptimal drug concentrations in the CNS during acute treatment may unintentionally contribute to neuroPASC. A general principal for treatment of infectious diseases is the need for adequate drug concentration at site of infection. CNS penetration is dependent on many factors that control the ability and amount of a drug that can cross the BBB (e.g., lipophilicity, molecular weight, molecular charge, etc.). Thus, to reach effective drug concentrations in the CNS, strategies to raise the systemic drug levels by increasing dose, frequency or duration, or changing formulation or route of administration, may be necessary. However, increasing the drug dose may significantly increase the risk of systemic toxicity. Preclinical studies investigating penetration into reservoirs are necessary to determine if therapeutic concentrations are clinically achievable. The objective of this study was to use in vitro and in vivo preclinical models to determine NMR penetration into the CNS. Our approach was to use an in vitro system consisting of cells of the BBB to explore the ability of NMR/RTV to enter these cells, and in vivo measurements of NMR/RTV in cerebrospinal fluid (CSF) and other anatomical sites utilizing a rat model.

Methods

This study was conducted at the University of Nebraska Medical Center in Omaha, NE. All study methods were approved by the Institutional Animal Care and Use Committee (IACUC; Protocol #2006507) and conducted in an AAALAC-accredited animal facility.
Chemicals and reagents

Animals were administered NMR/RTV (NMR: Medkoo Biosciences, Catalog#555985 Lot#:C22R06B23, Morrisville, NC, USA. RTV: Medkoo Biosciences, Catalog#318671, Lot#: A22M08B04) for oral dosing. Artificial CSF (TOCRIS Biotechne, #3525) and normal saline (B/BRAUN, Lot#:R5200-01) was used as described in sampling methods below. LC-MS/MS standard curves were generated using commercially obtained NMR (Cayman Chemical, Lot#:0635075, Ann Arbor, MI, USA) with a purity of >98%. Nirmatrelvir-2H9 (2H9-PF-07321332, Lot#:NA-ALS-22-044-P3, Alsachim, Illkirch, France) was used as an internal standard for the NMR quantification. Formic acid, methanol and acetonitrile were obtained from Fischer Scientific (Waltham, MA, USA). Ultra-pure water was obtained from UNMC via a Barnstead GenPure xCAD Plus water purification system (Thermo-Fisher, Waltham, MA, USA). Frozen, non-mediated, non-immunized, pooled Sprague-Dawley rat plasma and pooled human CSF were used for calibration of standard curves (BioIVT, Westbury, NY, USA). For oral dosing, NMR and RTV were mixed into a premade vehicle formulation similar to previous methods21–23.

Cells and culture system

Human brain primary astrocytes (#1800), pericytes (#1200), and human neurons (#1520) were purchased from ScienCell Research Laboratories (SCRL), USA. Required media and growth supplements for the respective cells were also obtained from SCRL. Astrocytes were cultured in astrocyte media (AM) (Catalog#1801) and astrocyte growth supplement (AGS) (Catalog#1852); pericytes were cultured in pericyte media (PM) (Catalogue#1201), pericyte growth supplement (PGS) (Catalog#1252) and human neurons were seeded in neuronal media (NM) (Catalog#1521) with neuronal growth supplement (NSG) (Catalog#1562). Supplements, including FBS (Catalog#0010), and penicillin/streptomycin solution (P/S) (Catalog#0503) were also purchased from ScienCell. Frozen cells were revived and cultured according to the manufacturer's instructions. Astrocytes and pericyte cells were grown in either a 25cm, 75cm, or 150cm culture flask (TPP#90076) in accordance with experimental requirements. Culturing flask were pre-coated for human brain cells with bovine fibronectin at 2µg/ml (ScienCell#8248). The 6-well plates (TPP#92006) were coated with Poly-L-Lysine (Sigma #RNBL4935) for 10 minutes at room temperature for human neuron cells, washed with PBS, and air dried. Astrocytes and pericytes were harvested by trypsinization (0.25% trypsin, Lonza # CC-5012) from the asks having close to 90% conuency of growing cells and washed in DPBS (Dulbecco's # 1960454). Cells were prepared for counting by mixing 10µl of cell suspension with 10µl of trypan blue. 10µl of the mixture was read in a cell counter (Invitrogen Countess). Neuron cells were directly seeded on the pre-coated 6-well plates after thawing the frozen vials.

Cell seeding in 6-well plates.

All steps were carried out in a biosafety cabinet under aseptic conditions, similar to methods previously described24. Astrocytes with a cell count of 0.5x10^5/well were seeded into 6-well plates containing 2ml of astrocyte media in each well. Seeding was performed in triplicate for each drug or drug combination and incubated in 37°C cell culture incubator, as described in our previous work24. Pericytes with a cell count of 0.5x10^5/well were seeded into 6-well plates containing 2ml of pericyte media in each well. Cell seeding was performed in triplicate for each drug or drug combination and incubated in 37°C cell culture incubator. Neurons with a cell count of 0.3x10^5/well were seeded into 6-well plates containing 2ml of neuronal media in each well. Seeding was performed in triplicates for each drug or drug combination and incubated in 37°C cell culture incubator.

Drug formulation for in-vitro work

Powdered NMR (Medkoo Biosciences, Catalog#555985 Lot#:C22R06B23) was dissolved in 1mL of 100% DMSO to make a stock concentration of 4.4mg/mL. Powdered RTV (Medkoo Biosciences, Catalog#318671, Lot#: A22M08B04) was dissolved in 1mL of 100% DMSO to achieve the stock concentration of 2mg/mL. NMR and RTV were weighed and dissolved in 1mL of 100% DMSO to achieve 4.4 and 2mg/mL stock concentration for NMR and RTV, respectively.

Drug addition to cells and sample preparation

NMR and RTV, individually or in combination, were added to the cultured cells at 2200ng/mL and 1000ng/mL final concentration, respectively. The in vitro doses of NMR and RTV were selected based on previous studies25,26. After 24-hour incubation with drugs, astrocytes, pericytes, and neuron cells were washed once with PBS and harvested using a cell scraper (Corning #3010) in 500ul of 70% methanol. Samples were kept at -20°C prior to drug quantification.

Experimental design and animals

Male Sprague-Dawley rats (n = 10, mean weight = 306g) were obtained from Charles River (Raleigh, NC 27610). All catheters (cisternal and vein cannulation) for the animals were surgically implanted27,28 at Charles River prior to shipping. On arrival to the housing facility, animals were acclimated for 72hrs prior to starting study protocol. Catheter management was performed daily to ensure viable sampling. Animals were administered 30mg/kg NMR + 10mg/kg RTV twice a day (60mg/kg NMR and 20 mg/kg RTV total daily dose) daily for five days (as described
below). All NMR/RTV doses were administered orally via gavage. The dose chosen for this study allometrically scaled to a humanized equivalent of NMR/RTV based on fixed dosing (i.e., 60kg patient, 300mg NMR + 100mg RTV twice daily)\textsuperscript{23}. The five day duration of the study also aligns with the current FDA recommendation for treatment of COVID-19 with NMR/RTV in patients\textsuperscript{8}. Rats were housed in a light and temperature-controlled room for the duration of the study and allowed free access to water and food, except during sampling. Data were analyzed for all animals that entered the protocol. When animals contributed incomplete data (i.e., early protocol termination), all available levels were analyzed for PK. Concentrations below the lower limit of quantification were inputted as 0\textsuperscript{30}.

**Blood, CSF, PBMC, and tissue sampling and determination of NMR concentrations**

Blood samples were drawn from a single right-sided internal jugular vein catheter in a sedation-free manner when possible. Blood catheter lines were flushed with normal saline after each blood draw to prevent blood contamination. CSF was collected via an intracisternal catheter. Isoflurane gas was used for temporary sedation when needed (5% initially, followed by 1–3% maintenance). Within each 24hrs, planned sample collection was eight blood and two CSF samples per animal. An approximation of the full sampling strategy over the five day study can be found in Supplemental Fig. 1. Each sample obtained (0.25mL blood and 0.05-0.1mL CSF aliquots) was replaced with either an equivalent volume of normal saline or artificial CSF (as appropriate) to maintain euvolemia. Blood and CSF samples from NMR-treated animals were processed similar to our previous reports\textsuperscript{31–34}.

Upon completion of the protocol, rats were euthanized, and tissues (lungs, heart, kidney, brain, liver) were harvested. The tissues were rinsed with cold saline solution, blotted with paper towel, and snap-frozen. Rat tissues (lungs, heart, kidney, brain, liver) were analyzed for NMR content by preparing tissue homogenate samples. PBMC sampling was conducted on each rat prior to termination using mononuclear cell preparation tubes per manufacture protocol (BD Biosciences, Franklin Lakes, NJ).

Plasma, CSF, tissue, and PBMC concentrations of NMR were quantified with LC-MS/MS using individual standard curves for each matrix. Standard calibrators, quality controls, and samples were prepared in microcentrifuge tubes. Internal standard was added to track the analyte of interest through the extraction and instrumentation processes. NMR was extracted from 20µL of rat plasma, PBMCs or CSF with a stable labeled internal standard [2H9]-PF-07321332 (IS) by a protein precipitation using 50:50 ACN:MeOH to provide a protein free extract. CSF samples were treated with ammoniated methanol prior to extraction to ensure no analyte adsorbs to the tube wall as previously described\textsuperscript{33}. Supernatant was removed and diluted with mobile phase in a 96-well plate prior to injection. HPLC was used to separate the analytes from potential interferences on a C18 100x3.00mm column (MAC MOD, Chadds Ford, PA, USA) for stationary phase, using 60% acetonitrile, 0.1% formic acid in water as an isocratic mobile phase. Detection of NMR and the IS in plasma and CSF was done with an ABSciex 5500 Q-trap mass spectrometer (ABSciex, Framingham, MA, USA) in positive ion mode. PBMC levels were converted to µM concentrations based on the single cell volume for PMBCs\textsuperscript{35}.

Tissues were homogenized using a Precellys Evolution Cryolys homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Each tissue was homogenized with 0.5mL of 70:30 methanol:25 mM phosphate buffer. Calibration curves for the tissue homogenates were prepared as described above in the section on estimation of NMR in plasma. Tissues were quantified by weight (mg of drug/g of tissues), reported as mg/g, and converted to µg/mL as previously described\textsuperscript{31,36}.

PBMC cellular and tissue accumulation ratios (AR) were calculated as observed NMR PBMC and tissue concentrations to NMR plasma concentrations at the same time of collection\textsuperscript{37,38}.

**NMR PK and drug exposure**

The simplest base PK model considered was a 3-compartment model with an oral compartment, plasma compartment, and a CSF compartment. Three and four-compartment models with/without a lag constant were similarly fit using the nonparametric adaptive grid (NPAG) algorithm within the Pmetrics package version 1.5.0 (Los Angeles, CA) for R version 3.2.1 (R Foundation for Statistical Computing, Vienna, Austria)\textsuperscript{39,40}. Multiple different CSF models were considered where CSF intercompartmental clearance (CL)/transfer and CSF CL were added and omitted based on investigator judgement, and other PK CSF studies\textsuperscript{41–44}. A model comparison table can be found in Supplemental Table 1. The initial estimate of parameter weighting was accomplished using the inverse of the assay variance. Model performance was quantitatively described using observed vs. predicted concentrations to calculate bias, imprecision, and coefficients of determination\textsuperscript{45}. The final model was selected based on regression of observed vs. predicted concentrations, prediction bias, visual plots of parameter estimates, lowest −2LL/Akaike information criterion and rule of parsimony. We modeled the relative bioavailability (F) for each dose in a given rat to account for inter-occasion variability in concentrations among doses, by taking the maximum post-dose peak concentration observed for that rat over all doses and calculating F for each peak as the peak after that dose divided by the maximum peak. The dose which was followed by the maximum peak then had F = 1, and all other doses were F ≤ 1.
To compare NMR concentrations in animals to a putative pharmacodynamic (PD) endpoint, the concentration needed for three times the 90% maximal effective concentration (3xEC90) for the SARS-CoV-2 was utilized. The FDA integrated review from the clinical studies (EPIC-ER) showed 95% of participants had NMR trough concentrations ≥ 3xEC90. Therefore, the goal for the CSF was set to achieve the same exposure conditions as for plasma. The protein-binding adjusted EC90 (EC90Adjusted) concentration for plasma is 292ng/mL (585nM), and the EC90 unadjusted for protein binding (EC90unAdjusted) for CSF is 90.5ng/mL. Therefore, the 3xEC90 PD values would be 876 ng/mL for plasma and 271.5 ng/mL for CSF. This EC90 value is based on the study on bronchial epithelial cells infected with USA_WAI/2020 isolate.

Estimation of PK exposure and percent (%) CSF penetration

The best-fit model was used to calculate median maximum a posteriori probability Bayesian NMR plasma and CSF concentration estimates at 12-minute intervals over the 5-day study period using each animal's measured NMR concentrations, exact dose, and dosing schedule. From these concentrations we calculated the area under the concentration-time curve over the entire experiment (AUC0−24hrs) using "makeAUC" function within Pmetrics. The highest predicted CSF concentration [CMAX0−5days] from the 12-minute interval Bayesian estimates was determined to be each animal's CMAX0−5days.

Ratios of the estimated AUCcsf/AUCplasma and CMAXcsf/CMAXplasma were used to determine percent CSF penetration. AUC was standardized to AUC0−24hrs by dividing AUC0−5days by 5 (i.e., 5-days protocol) to provide an estimated AUC0−24hrs value. For CMAX0−5days, the highest predicted CSF concentration and corresponding plasma concentration were used calculate percent penetration. Only animals with CSF concentrations sampled were used for estimation of CSF penetration.

Statistical methods

Summary statistics were calculated using GraphPad Prism V7.02 (GraphPad Software Inc., La Jolla, CA). Nonparametric summary statistics were reported given the small sample size and distribution of data.

Results

In-Vitro Drug Uptake

The mean ± SD uptake of NMR alone compared with in the presence of RTV by neurons was 34.7ng/mL ± 0.88 and 122.8 ± 7.8ng/mL, respectively (P < 0.0001). The mean ± SD of RTV uptake in astrocytes and pericytes in the presence or absence of NMR was 419.7ng/mL ± 12.8ng/mL vs. 665.2ng/mL ± 28.3ng/mL for astrocytes (P < 0.0002) and 202.6ng/mL ± 11.5ng/mL vs 321.9ng/mL ± 72.6ng/mL vs. for pericytes (P < 0.05), respectively. Overall, the maximum NMR uptake was 5.5% (i.e., 2200ng/mL administered vs. 122.8ng/mL uptake: 122.8/2200 = 5.5%, Fig. 1a:neurons), as seen with neurons in the presence of RTV. The uptake for NMR increased >3.6-fold in neurons in the presence of RTV (34.7ng/mL to 122.8ng/mL). We observed <2% uptake of NMR by astrocytes or pericytes (Fig. 1a:astrocytes, pericytes) in the presence or absence of RTV. Further, we observed 42.0% uptake of RTV in astrocytes (Fig. 1b:astrocytes, 1000ng/mL administered vs. 419.7ng/mL uptake), and in the presence of NMR, RTV uptake significantly increased to 66.5% (1000ng/mL administered vs. 665.2ng/mL uptake).

Characteristics of animal cohort

A total of 10 rats received NMR/RTV and had plasma and CSF concentrations obtained throughout dosing and tissue samples collected at completion. Each day, rats had an average of 6.5 plasma concentrations and 1.8 CSF concentrations sampled over the 5-day protocol (Total: 327 plasma, 83 CSF concentrations). One animal had intracisternal catheter failure before the collection of any CSF samples.

NMR PK model and parameter estimates

The final PK model was a three-compartment oral absorption model with a F covariate (Supplemental Fig. 2), AIC = 771.4 (Supplemental Table 1). The final model's median PK parameter values are given in Table 1. The PK model was fit for purpose with low bias in both plasma and CSF (-0.0778 mg/L and - 0.0263 mg/L). Bayesian predictions from the final model explained the variation in the observed individual animal concentrations well (r2 = 0.76 and 0.51 for plasma and CSF, respectively [Supplemental Fig. 3]).

NMR PK exposures and percent (%) CSF penetration

The overall PK exposures for all rats are summarized in Table 2. The median (IQR) NMR penetration into the CSF was 18.1% (7.65–30.59) (calculated from CMAX) and 15.2% (7.55–29.92) (calculated from AUC). The complete list of NMR penetration into CSF for each animal can be found in Table 2. Further, Bayesian observed versus predicted concentration time profiles for plasma and CSF vs. EC90 and 3xEC90 values can be found in Fig. 2. The CSF Bayesian prediction concentration time profiles for all animals showed the median (IQR) percent of time CSF concentrations were ≥ 3xEC90unAdjusted was 16% (0-20.5) (Fig. 2b).
**Tissue and peripheral blood mononuclear cells (PBMC) NMR concentrations**

The overall tissue AR and tissue concentrations for NMR can be found in Fig. 3 and Supplemental Fig. 4. The highest median NMR tissue ARs were observed in the liver and kidney, while the lowest median NMR tissue AR was observed in brain tissue at 0.15 (0.03–1.12). Compared to all the tissues, the brain had the lowest median (0.00002383mg/g, IQR: 0.00001094–0.00004685) NMR concentrations, which were all <3×EC₉₀ regardless of adjustment for protein binding. For PBMCs, the median (IQR) value for the cellular AR for NMR was 0.998 (0.48–27.05).

**Discussion**

We found that NMR CSF concentrations in rats given oral NMR/RTV twice daily for five days were 15–18% of those in plasma, whether determined as a ratio of CMAX or AUC (Table 2). Further, we found that tissue penetration of NMR in brain of the rats was low, which was consistent with the NMR cell uptake in our in vitro model. To our knowledge, this is the first study to determine NMR CSF and CNS penetration utilizing in vitro and in vivo models and to quantitatively describe the transit of NMR from plasma to the CSF. Saleh and colleagues used physiologically based pharmacokinetic (PBPK) modelling to predict whether NMR, remdesivir, and molnupiravir achieve effective concentrations against SARS-CoV-2 in human brain cells. Their model predicted NMR concentrations exceeded the EC₉₀ values in brain extracellular fluid concentrations, which is similar to what we found in rat CSF. However, they did not evaluate 3×EC₉₀, or other multiplicative factors of the EC₉₀ values, reflecting levels of plasma exposure observed clinically. Exposure-response relationships for SARS-CoV-2 viral loads relative to EC₉₀ factors have not been evaluated in the CNS or other potential viral reservoirs. We utilized the concentration needed for 3×EC₉₀ for SARS-CoV-2 as our PD target for the CSF, based on the FDA review from EPIC-HR showing 95% of participants had NMR trough concentrations ≥3×EC₉₀ [46,47]. If the two EC₉₀ values utilized in the PBPK simulation study by Saleh and colleagues for the Delta variant are multiplied by a factor of 3 (0.149µM:~100ng/mL x 3 = 300ng/mL), the majority of time is spent below this PD goal. In our study, we found that the uptake of NMR and or RTV in the presence of the other drug differed significantly from RTV levels in the choroid plexus and brain were higher at 41.97% and moderate by pericytes at 20.2%. In the presence of NMR, RTV uptake increased to 66.65% in astrocytes and 32.19% in pericytes and neurons individually. Further studies will evaluate NMR/RTV penetration utilizing a more novel 4-cell in vitro model [2].

To evaluate specific drug uptake by relevant cells of the CNS rather than only CSF, we investigated the uptake of NMR and RTV in astrocytes, pericytes and neurons individually (Fig. 1). We found that the uptake of NMR and or RTV in the presence of the other drug differed significantly in neurons, astrocytes and pericytes. For neurons, the presence of RTV increased the uptake of NMR significantly. This boosting effect is likely a result of p-glycoprotein (P-gp) inhibition via RTV. The expression of P-gp in human brain capillary endothelial cells is well documented [57]. However, its expression in astrocyte, pericyte, and neurons is still under investigation [58–61]. No significant differences were noted in astrocytes and pericytes when NMR was administered alone or with RTV (Fig. 1a). Further studies will evaluate NMR/RTV penetration utilizing a more novel 4-cell in vitro model [2]. When comparing NMR to other PIs used to treat HIV, as a class, they achieve poor CSF exposure [62]. However, when co-administered with RTV (or other boosters), CSF penetration has been shown to increase [63,64]. For example, increased CSF concentrations of atazanavir were found when administered with RTV (7.9 to 10.3ng/mL) [65]. When looking at RTV in our in vitro model, uptake by astrocytes was high at 41.97% and moderate by pericytes at 20.2%. In the presence of NMR, RTV uptake increased to 66.65% in astrocytes and 32.19% in pericytes. RTV CSF distribution is low [66–68]. A study by Anthonypillai and colleagues in guinea pigs found that CSF levels of RTV were low, but RTV levels in the choroid plexus and brain were higher. They hypothesized this was due to RTV regulation in the CSF and choroid plexus by efflux transporters that may limit drug accumulation in the CSF. In our study, we found that RTV uptake in astrocytes and pericytes was affected by NMR (Fig. 1b). We believe this is likely due to NMR’s effect on certain transporters. Transporter inhibition studies are warranted to provide insight on the mechanisms behind the differences seen between cell lines.
We developed a 3-compartment PK model to predict individual animal concentration-time profiles for plasma and CSF, as shown in Fig. 2. This allowed us to accurately predict CSF and plasma exposures, which were used to calculate CSF penetration (Table 2). This also allowed us to make comparisons of our PK estimates with clinical and animal data. For example, the median half-life for NMR in the presence of RTV for the rats was 2.4x faster than what is seen in humans (2.55hrs vs. 6.05hrs)\textsuperscript{69}. This is expected as smaller animals clear most drugs faster given the principles of allometry\textsuperscript{21,70}. When comparing our NMR half-life to other animal models for NMR, we found that our half-life estimation was within the range of other oral rat PK studies (10mg/kg: 4hrs range:2.9–5.1, 10mg/kg: 2.8hrs ± 1.4hrs)\textsuperscript{21,22}. Our estimation for time at which CMAX is first observed (T_{\text{max}}), was similar to other rodent models (mean:1.84hrs vs. mean:1.5hrs)\textsuperscript{21,22}. The median relative bioavailability (F) value of 54.5% in our study was also consistent with other literature values estimated in rats for NMR (34%-50%)\textsuperscript{21}. We note our animals were not restricted of food or water, and this is likely why we saw variability in F between and within animals (Table 1b: range: 32%-62%). We compared our values for CMAX and AUC with clinical data from healthy volunteers. Rat geometric mean plasma values for CMAX (2.48µg/mL) and AUC\textsubscript{daily average} (20.25µg*hr/mL) compared well with healthy human geometric mean values of CMAX (2.21µg/mL) and AUC\textsubscript{0–12hrs} (23.01µg*hr/mL) supporting our allometric dose scaling strategies\textsuperscript{47}. Our AUC estimation was a daily average given the difficulty of standardizing twice-daily dosing in animals and the healthy volunteer data was based on an AUC of 0-12hrs. When comparing our rat CSF concentrations to the PBPK modeling performed by Saleh and colleagues, our CSF CMAX (median 0.41mg/L) is in agreement with what was projected in human brain extracellular fluid (~ 0.3-0.44mg/L, points extrapolated using graphgrabber2.02)\textsuperscript{53,71}. This shows the potential clinical application of our rat model as we were able to humanize our CMAX exposure in both plasma and CSF.

Our study has limitations. First, we did not design this study for animals infected with SARS-CoV-2 and thus could not assess viral loads in the CSF vs. CSF concentrations of NMR. Our findings indicate that CNS levels of NMR may not be adequate to achieve therapeutic concentrations, so plans for utilizing an infection model with the golden Syrian hamster model are ongoing\textsuperscript{72}. Second, our tissue concentrations represent total drug concentrations based on homogenized tissues. Understanding the dynamic relationship of unbound tissue concentrations vs. time or site-specific tissue concentrations would require microdialysis or other techniques. Further, it is unknown if CSF catheter placement could have influenced CSF penetration or if concentration-mediated changes to CSF transit occur. Future work to address concentration-mediated penetration utilizing a 4-cell in vitro model is planned\textsuperscript{24}. In addition, it is unclear how our animal model compares to active infection where inflammation could increase drug penetration through the BBB in active SARS-CoV-2 infection. Last, we quantified total NMR concentrations and did not quantify free drug (NMR is 69% protein bound)\textsuperscript{69}. The PD endpoints we utilized for CSF, plasma, tissues, and PBMC were adjusted and unadjusted for plasma protein-binding, depending on the matrix.

**Conclusion**

In summary, the data from our *in vivo* rat model demonstrates that NMR penetration into CSF and CNS tissues may be inadequate. Our *in vitro* model data shows minimal NMR uptake into cells relevant to the CNS. Collectively, these findings may have implications for viral persistence in these compartments and neurologic post-acute sequelae of COVID-19. These data motivate future investigations utilizing an infection model to understand the pharmacodynamic effects of NMR drug concentrations in the CNS on viral loads in the CNS.

**Declarations**

**Competing interests:** none

**Acknowledgements:** none

**Credit Author Statement**

**SNA:** Supervision, Conceptualization, Methodology, Visualization, Writing - Review & Editing, Resources, Funding acquisition, Validation, Project administration

**JRM:** Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing, Formal analysis.

**ATP:** Writing - Review & Editing, Validation, Project administration

**MN:** Writing - Review & Editing, Methodology, Analysis

**NJR:** Writing - Review & Editing, Methodology, Analysis

**KKS:** Writing - Review & Editing, Interpretation of Data

**MSH:** Writing - Review & Editing, Interpretation of Data
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**Tables**

**Table 1.** Median parameter values from final model (a) and individual animal NMR half-life and average bioavailability (b)

(a)

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<td>1.13</td>
</tr>
<tr>
<td>K30 (hr⁻¹)</td>
<td>0.24</td>
<td>43.73</td>
<td>0.01</td>
<td>11.96</td>
</tr>
<tr>
<td>Vc (L)</td>
<td>1.05</td>
<td>41.12</td>
<td>0.15</td>
<td>0.78</td>
</tr>
<tr>
<td>Vcsf (L)</td>
<td>3.46</td>
<td>63.98</td>
<td>6.49</td>
<td>5.56</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Rat</th>
<th>Half-Life (hrs)</th>
<th>Average Relative Bioavailability (F)*</th>
<th>Average T&lt;sub&gt;max&lt;/sub&gt; (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.87</td>
<td>0.58</td>
<td>2.17</td>
</tr>
<tr>
<td>2</td>
<td>3.86</td>
<td>0.58</td>
<td>3.63</td>
</tr>
<tr>
<td>3</td>
<td>2.46</td>
<td>0.46</td>
<td>2.25</td>
</tr>
<tr>
<td>4</td>
<td>1.32</td>
<td>0.55</td>
<td>1.56</td>
</tr>
<tr>
<td>5</td>
<td>3.23</td>
<td>0.54</td>
<td>1.22</td>
</tr>
<tr>
<td>6</td>
<td>1.46</td>
<td>0.48</td>
<td>1.65</td>
</tr>
<tr>
<td>7**</td>
<td>3.23</td>
<td>0.58</td>
<td>1.19</td>
</tr>
<tr>
<td>8</td>
<td>2.80</td>
<td>0.32</td>
<td>1.7</td>
</tr>
<tr>
<td>9</td>
<td>0.98</td>
<td>0.62</td>
<td>1.15</td>
</tr>
<tr>
<td>10.</td>
<td>2.65</td>
<td>0.41</td>
<td>1.9</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>2.55 (1.43-3.23)</td>
<td>1. (0.45-0.58)</td>
<td>1.675 (1.21-2.19)</td>
</tr>
<tr>
<td>Mean (SD)#</td>
<td></td>
<td></td>
<td>1.84 (0.73)</td>
</tr>
</tbody>
</table>

*Bioavailability was estimated after each dose given the variability of oral absorption, as described in Methods.

**Rat 7 only completed 1 day of treatment.

#calculated to compare to literature values

*Estimation to assess if the data are insufficient to precisely estimate the individual parameters.
Abbreviations: PK=pharmacokinetic, CV%=coefficient of variation percent, CL=NMR clearance, $V_c$=volume central compartment, $V_{csf}$=volume cerebral spinal fluid compartment, $K_{23}$=rate constant to cerebral spinal fluid from central compartment, $K_{30}$= elimination rate constant from CSF compartment, IQR= interquartile range, $T_{max}$=time at which CMAX was first observed.

Table 2. NMR plasma and CSF PK exposures estimated using Bayesian posteriors for $AUC_{0-\text{endoftreatment}}$ and $\text{CMAX}_{0-5\text{days}}$ and percent penetration of NMR into the CSF compared to blood

<table>
<thead>
<tr>
<th>Animal</th>
<th>$\text{CMAX}_{0-5\text{days}}$ (mg/L)</th>
<th>$AUC_{0-\text{endoftreatment}}$ (mg*h/L) Plasm</th>
<th>$AUC_{\text{daily_average}}$ (mg*h/L)</th>
<th>$\text{CMAX}_{0-5\text{days}}$ (mg/L)</th>
<th>$AUC_{0-\text{endoftreatment}}$ (mg*h/L) CSF</th>
<th>$AUC_{\text{daily_average}}$ (mg*h/L) CSF</th>
<th>% Penetration by CMAX CSF/Plasma</th>
<th>% Penetration by AUC CSF/Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.27</td>
<td>160</td>
<td>32</td>
<td>0.10581</td>
<td>7.11</td>
<td>1.42</td>
<td>4.66</td>
<td>4.44</td>
</tr>
<tr>
<td>2</td>
<td>3.66</td>
<td>189</td>
<td>37.8</td>
<td>0.252</td>
<td>12.5</td>
<td>2.5</td>
<td>6.89</td>
<td>6.61</td>
</tr>
<tr>
<td>3</td>
<td>2.02</td>
<td>92.7</td>
<td>18.54</td>
<td>0.6558</td>
<td>28.9</td>
<td>5.78</td>
<td>32.47</td>
<td>31.175</td>
</tr>
<tr>
<td>4</td>
<td>0.796</td>
<td>76.3</td>
<td>15.26</td>
<td>0.144</td>
<td>11.6</td>
<td>2.32</td>
<td>18.10</td>
<td>15.20</td>
</tr>
<tr>
<td>5</td>
<td>1.86</td>
<td>80.7</td>
<td>16.14</td>
<td>1.1699</td>
<td>52.21</td>
<td>10.44</td>
<td>62.84</td>
<td>64.7</td>
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<tr>
<td>6</td>
<td>2.218</td>
<td>128</td>
<td>25.6</td>
<td>0.560</td>
<td>30.2</td>
<td>6.04</td>
<td>25.27</td>
<td>23.59</td>
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<tr>
<td>7#</td>
<td>4.55</td>
<td>19.55</td>
<td>3.91</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>8</td>
<td>3.8579</td>
<td>99.8</td>
<td>19.96</td>
<td>1.107</td>
<td>28.6</td>
<td>5.72</td>
<td>28.70</td>
<td>28.66</td>
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<tr>
<td>9</td>
<td>4.8793</td>
<td>205</td>
<td>41</td>
<td>0.410</td>
<td>17.4</td>
<td>3.48</td>
<td>8.41</td>
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<tr>
<td>10</td>
<td>1.8627</td>
<td>128</td>
<td>25.6</td>
<td>0.2716</td>
<td>17.6</td>
<td>3.52</td>
<td>14.58</td>
<td>13.75</td>
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<tr>
<td>Median</td>
<td>2.24</td>
<td>113.9</td>
<td>22.78</td>
<td>0.41</td>
<td>17.6</td>
<td>3.52</td>
<td>18.1</td>
<td>15.2</td>
</tr>
<tr>
<td>(IQR)</td>
<td>(1.86-4.03)</td>
<td>(79.6-167.3)</td>
<td>(15.92-33.45)</td>
<td>(0.2-0.88)</td>
<td>(12.05-29.55)</td>
<td>(2.41-5.91)</td>
<td>(7.65-30.59)</td>
<td>(7.55-29.92)</td>
</tr>
<tr>
<td>Median</td>
<td>2.22</td>
<td>128</td>
<td>25.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(IQR)*</td>
<td>(1.86-3.76)</td>
<td>(86.7-174.5)</td>
<td>(17.34-34.9)</td>
<td>(1.73)</td>
<td>(1.956)</td>
<td>(1.73)</td>
<td>(1.956)</td>
<td>(1.73)</td>
</tr>
</tbody>
</table>

*Excluding rat 7 as no CSF was obtained from this animal, #calculated to compare to clinical data

Abbreviations: CMAX= maximum concentration, AUC= area under the curve, CSF= cerebral spinal fluid, $T_1/2$= half-life, IQR=interquartile range, SD=standard deviation

**Figures**
Figure 1

In-vitro analysis of NMR and RTV penetration into three different human brain cells

Legend: (a) Evaluation of NMR uptake by cells in the absence or presence of RTV and (b) intracellular RTV uptake in the absence or presence of NMR. The p-values (*) indicate, *=<0.05, **=<0.0002 and ****=<0.0001.

Abbreviations: NMR=nirmatrelvir, RTV=ritonavir
Legend: Plasma (la black) and CSF (lb red) Bayesian observed versus predicted plots for all animals compared to EC$_{90\text{Un adjusted}}$ values (dotted black line). The black and red lines represent the predictions where the filled circles represent the observed collected concentrations. A median of 16% of all the predicted CSF concentrations in rats were >3xEC$_{90\text{Un adjusted}}$.

Abbreviation: NMR=nirmatrelvir, CSF=cerebrospinal fluid, EC$_{90}$= 90% maximal effective concentration (unadjusted for protein binding given CSF, 90.5ng/mL=0.0905mg/L and 271.5ng/mL=0.2715mg/L)
Figure 3

Legend: Tissue and PBMC accumulation ratios for NMR. The highest median NMR tissue AR was observed in the liver and kidney, while the lowest median NMR tissue AR was observed in brain tissue.

*Rat 7 only completed 1 day of treatment, no PMBC levels available. Rats 1 and 2 do not have lung or heart NMR concentrations due to tissue processing complications.

#Calculated as a ratio of observed plasma NMR levels vs. tissue/PBMC levels at equivalent time of sampling

^Calculated using plasma level predictions vs. observed concentrations due to plasma NMR levels being BLOQ

Abbreviations: AR=accumulation ratio, BLOQ=below level of quantification, IQR=interquartile range

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalFiguresandTables.docx