Pantothenate kinase activation restores brain coenzyme A in a mouse model of pantothenate kinase associated neurodegeneration


1Department of Infectious Diseases, St. Jude Children’s Research Hospital, Memphis TN 38105. 2Department of Chemical Biology and Therapeutics, St. Jude Children’s Research Hospital, Memphis TN 38105. 3Department of Structural Biology, St. Jude Children’s Research Hospital, Memphis TN 38105. 4St. Jude Graduate School of Biomedical Sciences, St. Jude Children’s Research Hospital, Memphis TN 38105. 5Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, TN 38163. 6CoA Therapeutics, Inc., a BridgeBio Pharma, Inc. company, 3160 Porter Dr., Palo Alto, CA 94304

Correspondence to: Richard E. Lee, Department of Chemical Biology and Therapeutics, St. Jude Children’s Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105. Email: Richard.Lee@stjude.org; Phone: 901-595-6617.

Running Title: Correcting brain CoA deficiencies

Key Words: Pantothenate kinase associated neurodegeneration; coenzyme A; pantothenate kinase; blood brain barrier; BBP-671

Manuscript statistics
Number of text pages: 23
Number of tables: 4
Number of figures: 6
Number of references: 49
Abstract words: 228
Introduction words: 861
Discussion words: 595

List of abbreviations
CoA, Coenzyme A
CSF, cerebrospinal fluid
PANK, pantothenate kinase
PKAN, pantothenate kinase associated neurodegeneration

Requested section
Drug Discovery and Translational Medicine
ABSTRACT

Pantothenate kinase associated neurodegeneration (PKAN) is characterized by a motor disorder with combinations of dystonia, parkinsonism and spasticity, leading to premature death. PKAN is caused by mutations in the \textit{PANK2} gene that result in loss or reduction of PANK2 protein function. \textit{PANK2} is one of three kinases that initiate and regulate coenzyme A biosynthesis from vitamin B5 and the ability of BBP-671, an allosteric activator of pantothenate kinases, to enter the brain and elevate coenzyme A was investigated. The metabolic stability, protein binding and membrane permeability of BBP-671 all suggest it has the physical properties required to cross the blood brain barrier. BBP-671 was detected in plasma, liver, cerebrospinal fluid and brain following oral administration in rodents demonstrating the ability of BBP-671 to penetrate the brain. The pharmacokinetic and pharmacodynamic properties of orally administered BBP-671 evaluated in cannulated rats showed that CoA concentrations were elevated in blood, liver and brain. BBP-671 elevation of whole blood acetyl-CoA served as a peripheral pharmacodynamic marker and provided a suitable method to assess target engagement. BBP-671 treatment elevated brain coenzyme A concentrations, and improved movement and body weight in a PKAN mouse model. Thus, BBP-671 crosses the blood brain barrier to correct the brain CoA deficiency in a PKAN mouse model resulting in improved locomotion and survival, providing a preclinical foundation for the development of BBP-671 as a potential treatment for PKAN.

SIGNIFICANCE STATEMENT

The blood brain barrier represents a major hurdle for drugs targeting brain metabolism. This work describes the pharmacokinetic/pharmacodynamic properties of BBP-671, a pantothenate kinase activator. BBP-671 crosses the blood brain barrier to correct the neuron-specific CoA deficiency and improve motor function in a mouse model of pantothenate kinase associated neurodegeneration. The central role of CoA and acetyl-CoA in intermediary metabolism suggests that pantothenate kinase activators may be useful in modifying neurological metabolic disorders.
Introduction

Pantothenate Kinase Associated Neurodegeneration (PKAN) is a rare, autosomal recessive disorder caused by mutations in the \textit{PANK2} gene which encodes pantothenate kinase 2 (PANK2) (Zhou et al., 2001). PKAN is a debilitating and life-threatening condition characterized by dystonia, dysarthria and progressive extrapyramidal decline affecting movement, balance, speech, vision, cognition and behavior (Hogarth et al., 2017). Deposition of iron in the basal ganglia as detected by magnetic resonance imaging (MRI), together with gene sequencing, are diagnostic for the disease. Earlier disease onset, more rapid progression to loss of ambulation, and premature death have been observed in patients with two null alleles for \textit{PANK2}, although for many patients there is no clear genotype-phenotype correlation (Chang et al., 2020; Hayflick 2003; Hayflick et al., 2003; Pellecchia et al., 2005). The majority of the mutations associated with PKAN result in the expression of truncated or mutant PANK2 proteins with little or no catalytic activity (Zhang et al., 2006). There are three \textit{PANK} genes that encode four closely related mammalian isozymes of PANK that govern intracellular coenzyme A (CoA) concentrations: PANK1\textsubscript{α}, PANK1\textsubscript{β}, PANK2, and PANK3 (Leonardi et al., 2005). All three genes are expressed in the brain, with PANK2 being a dominant central nervous system (CNS) isoform in humans (Leonardi et al., 2007). CoA is a major acyl group carrier in biology and participates as a key cofactor and regulator of oxidative metabolism of fatty acids, amino acids and ketones for energy production and growth (Garcia et al., 2012; Leonardi et al., 2010; Leonardi et al., 2005; Subramanian et al., 2020; Zhang et al., 2007). PKAN symptoms are thought to arise from impaired CoA homeostasis in the brain that compromises important neuronal processes including iron metabolism, synaptic transmission, synaptic vesicle cycling, neuron projection development and protein quality control (Bettencourt et al., 2016).

The pantothenate kinases (PANK) catalyze the first step and control the rate of CoA biosynthesis. CoA synthesis is the only metabolic fate of pantothenate, also known as pantothenic acid or vitamin B\textsubscript{5}, an essential component of the human diet. BBP-671 activates the PANKs and
thereby increases the pool of intracellular free CoA (Subramanian et al., 2023), making it available for ligation with metabolic intermediates that are components of many mitochondrial and intracellular reactions, such as the citric acid cycle and the synthesis and oxidation of fatty acids. The PANK isoform expression levels and their potent feedback inhibition by acyl-CoAs control and stabilize the intracellular CoA content in cells and tissues (Dansie et al., 2014; Hong et al., 2007; Rock et al., 2000; Subramanian et al., 2016; Zhang et al., 2005). BBP-671 binding renders the PANKs refractory to feedback inhibition by acyl-CoAs, resulting in increased CoA production in both the liver and CNS (Subramanian et al., 2023). Therefore, the use of BBP-671 to increase CoA levels offers the potential to effectively alleviate brain CoA-dependent deficiencies and restore essential neuronal functions that become compromised by the reduction of PANK2 activity in PKAN.

The Pank2 gene was genetically inactivated in mice in the first effort to model PKAN disease (Kuo et al., 2007). The Pank2−/− animals had normal lifespan and did not have a movement disorder but instead exhibited retinal degeneration and azoospermia. Brain CoA deficiency was not found in adult animals (Leonardi et al., 2007) but a transient brain CoA deficiency occurred during postnatal development of Pank2−/− mice (Garcia et al., 2012; Subramanian et al., 2020). Wild-type mice maintained on a pantothenate-deficient diet for 8 months eventually developed a movement disorder and azoospermia (Kuo et al., 2007), linking these two phenotypes to CoA deficiency. Later examination of selected brain regions of Pank2−/− adult animals found evidence in the globus pallidus for perturbation of gene expression related to iron and dopamine metabolism, as well as altered mitochondrial functional capacity (Jeong et al., 2019). Investigation of PKAN therapies in Pank2−/− mice is challenging due to the mild biochemical phenotypes and the lack of a movement disorder in the animals. Neurons have been identified as the most affected cells in post-mortem brains of PKAN patients (Bettencourt et al., 2016). Therefore, a conditional mouse model with deletions of both Pank1 and Pank2 genes in neurons was developed to obtain a durable brain CoA deficiency and elicit a PKAN-like movement phenotype (Sharma et al., 2018).
The $Pank1^{fl/fl},Pank2^{fl/fl}$ SynCre$^+$ neuronal knockout mice established a clear connection between brain CoA deficiency and movement dysfunction (Sharma et al., 2018). This symptomatic PKAN mouse model provided tractable phenotypic readouts for the development of a small molecule that penetrates the blood-brain barrier, reverses the brain CoA deficiency and improves movement dysfunction.

There are no approved therapies for the treatment of PKAN. In this study, the $Pank1^{fl/fl},Pank2^{fl/fl}$ SynCre$^+$ mouse model of brain CoA deficiency to evaluate the potential of BBP-671 as a treatment for PKAN. BBP-671 was detected in rodent brain and cerebrospinal fluid (CSF), and treatment of the PKAN mouse model with BBP-671 restored brain CoA levels, and improved movement and growth. A Phase I study of orally-dosed BBP-671 in healthy adults (clinicaltrials.gov identifier NCT04836494) detected BBP-671 in CSF showing that it crossed the blood brain barrier (Gretler et al., 2022). Together, these data support the continued development of BBP-671 as a potential PKAN treatment.

**Materials and Methods**

**PKAN Mouse Model.** The generation, mating and phenotyping of the $Pank1^{fl/fl},Pank2^{fl/fl}$ SynCre$^+$ neuronal knockout mouse model has been described in detail (Sharma, et al., 2018). The SynCre transgene originated in B6.Cg-Tg(Syn1-cre)671Jxm/J transgenic mice (The Jackson Laboratory) that express the Cre recombinase driven by the synapsin1 (Syn) promoter. $Pank1^{fl/fl},Pank2^{fl/+}$ SynCre$^+$ (or $Pank1^{fl/+},Pank2^{fl/fl}$ SynCre$^+$) females were mated with $Pank1^{fl/fl},Pank2^{fl/fl}$ SynCre$^0$ males to obtain $Pank1^{fl/fl},Pank2^{fl/fl}$ SynCre$^+$ progeny that have both $Pank1$ and $Pank2$ conditionally deleted in neuronal tissues. Genotyping was performed using the primers and methods as described (Sharma et al., 2018). Control littermate mice had the $Pank1^{fl/fl},Pank2^{fl/fl}$ SynCre$^0$ genotype. Animal rooms were maintained at room temperature 72 ± 2 °F, humidity 50% ± 10%, and a 14-h light/10-h dark cycle with the dark cycle starting at 20:00. Water was supplied ad libitum. PKAN animals were entered into study on a rolling basis as these
emerged from the breeding program. Where indicated, wild-type C57BL/6J male mice, age 8 weeks, were purchased from Jackson Laboratory (Bar Harbor, Maine), and acclimatized for 1-2 weeks. In the course of this study, and where indicated, animals were dosed daily for a different number of times prior to analyses to investigate steady-state drug levels and mimic how the drug will be chronically dosed in human studies. All procedures were performed according to protocols 323 and 556 as approved by the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee.

**Analytical Techniques.** BBP-671 (1-(4-(6-chloropyridazin-3-yl)piperazin-1-yl)-2-(4-cyclopropyl-3-fluorophenyl)ethan-1-one) was synthesized and purified as described (Subramanian et al., 2023). BBP-671 was measured in either plasma or CSF by adding 20 µL of sample to 100 µL acetonitrile containing 0.6 µM warfarin to a final concentration of 0.5 µM. The samples were incubated on ice for 30 min. Samples were centrifuged at 3500 x g for 10 min to pellet debris, and the supernatant was transferred to a glass vial. A BBP-671 standard curve was created by spiking in known concentrations of BBP-671 into 20 µL of plasma from a control animal and following the above procedure (Fig. S1). Flash frozen tissue (30 mg) was homogenized in 2 mL of 80% methanol containing 0.1 µM warfarin and incubated at −80 °C for 4 h. Samples were spun at 3500 x g for 10 min to pellet debris, the supernatant was transferred to a glass tube and dried using a Savant SPD1010 Speed-Vac (Thermo Scientific) overnight. Samples were resuspended in 400 µL of 80% acetonitrile containing a final concentration of 0.5 µM warfarin and transferred to a glass vial. BBP-671 was analyzed using a Shimadzu Prominence UFLC attached to a QTrap 4500 equipped with a Turbo V ion source (Sciex). Samples (5 µL) were injected onto an XSelect® HSS C18, 2.5 µm, 3.0 x 150 mm column (Waters) using a flow rate of 0.25 mL/min. Solvent A was 0.1% formic acid in water, and Solvent B was acetonitrile with 0.1% formic acid. The HPLC program was the following: starting solvent mixture of 50% B, 0 to 0.5 min isocratic with 50% B; 0.5 to 1.5 min linear gradient to 95% B; 1.5 to 20 min isocratic with 95% B; 20 to 21 min linear gradient
to 50% B; 21 to 25 min isocratic with 50% B. The QTrap 4500 was operated in the positive mode, and the ion source parameters were: ion spray voltage, 5500 V; curtain gas, 30 psi; temperature, 450 °C; collision gas, medium; ion source gas 1, 30 psi; and ion source gas 2, 40 psi. The MRM transition for BBP-671 was m/z 375.2/199.1 and for warfarin was m/z 309.1/163.0, both with a declustering potential, 65 V and collision energy, 30 V. The system was controlled by the Analyst® software (Sciex) and analyzed with MultiQuant™ 3.0.2 software (Sciex). The LC-MS/MS methods used by the various analytical laboratories that supported this study all used the same MRM for BBP-671 detection, although the columns, gradients, instruments, and the specific instrument setups varied among the laboratories.

Total tissue CoA was measured as described (Frank et al., 2019; Sharma et al., 2018). Briefly, either brain or liver tissue (30-40 mg) was homogenized in potassium hydroxide to hydrolyze the thioesters and then the CoA was derivatized with monobromobimane (mBBr). The CoA-bimane was purified on a SPE 2-(2-pyridyl) ethyl column and analyzed by HPLC with absorbance detection at 393 nm. The retention time was determined by running a CoA standard before each set of samples. Total CoA was quantified using a standard curve (0-400 μM) generated with known amounts of CoA (Frank et al., 2019).

Whole blood aliquots (50 μL) were added to a 1.7 ml epi-tube that was coated with EDTA, flash frozen in liquid nitrogen and stored at -80 °C for one week before processing. The samples were thawed on ice, and 300 μL of 80% methanol + 50 nM [13C2]acetyl-CoA was added, vortexed for 10 sec and centrifuged at 10,000 x g for 10 min. Aliquots of the supernatant (20 μL) were analyzed by liquid chromatography-coupled mass spectrometry (LC-MS/MS) using a Shimadzu Prominence UPLC attached to a QTrap 4500 equipped with a Turbo V ion source (Sciex). Samples were fractionated on an Acquity UPLC C18, 2.5 μm, 3 x 150 mm column at 40 °C (Waters) using a flow rate of 0.4 mL/min. Solvent A was 100 mM ammonium formate, pH 5.0 + 2% acetonitrile and 0.1% tributylamine, and Solvent B was 95% acetonitrile + 10 mM ammonium formate, pH 6.3, and 0.1% tributylamine. The HPLC program was the following: starting solvent mixture of 100% A/0% B; 0 to
2 min isocratic with 0% B; 2 to 20 min linear gradient to 50% B; 20 to 24 min linear gradient to 95% B; 24-29 min isocratic with 95% B; 29-30 min linear gradient to 0% B; and 30-35 min isocratic with 0% B. The QTrap 4500 was operated in the positive ion mode, and the ion source parameters were: ion spray voltage, 5500 V; curtain gas, 30 psi; temperature, 450 °C; collision gas, medium; ion source gas 1, 25 psi; ion source gas 2, 30 psi; declustering potential, 60 V; and collision energy, 45 V. The multiple reaction monitoring transitions were: acetyl-CoA; m/z 810.1/303.1; and [13C2]acetyl-CoA m/z 812.1/305.1. The system was controlled by the Analyst® software (Sciex) and analyzed with MultiQuant™ 3.0.2 software (Sciex).

The St. Jude Chemical Biology and Therapeutics Analytical Technologies Center measured brain protein binding using equilibrium dialysis (Di et al., 2011; Liu et al., 2005), and BBP-671 and control compound concentrations were measured by LC-MS/MS. The Center also performed the parallel artificial membrane permeability assay to rank blood brain barrier penetration (PAMPA-BBB) using the system and standards provided by pION (Tsinman et al., 2011). Quintara Discovery Inc performed plasma protein binding assays in human, mouse (CD-1), rat (Sprague Dawley), dog (beagle), and monkey (cynomolgus) (van Liempd et al., 2010; Hong and Gao, 2001; Wan and Tehngren, 2006), and microsomal stability with male or female liver microsomes from human, mouse (CD-1), rat (Sprague Dawley), dog (beagle), and monkey (cynomolgus). The concentration of BBP-671 was 1 µM, and the microsomes were incubated at 37 °C with 0.5 mg/mL liver microsomes and 1 mM NADPH in 100 mM potassium phosphate, pH 7.4, with 3.3 mM MgCl2. At each time point (0, 15, 30 and 60 min), the incubation was stopped by adding 150 µL of cold quenching solution (100% acetonitrile, 0.1% formic acid) and the mixtures were shaken briefly and centrifuged at 4,000 x g at 4 °C. The supernatants (80 µL) were transferred to a clean 96-well plate, and n = 3 samples were analyzed for BBP-671 by LC-MS/MS.

**Pharmacokinetics/Pharmacodynamics.** The BBP-671 dose-response in C57BL/6 mice was carried using 0.1, 1, 3, or 30 mg/kg once daily for 7 days, and the samples collected 4 h after the
last dose. The pharmacokinetic and pharmacodynamic correlation between plasma, liver, brain and CSF concentrations of BBP-671, and liver and brain concentrations of total CoA were evaluated following the administration of 10 mg/kg BBP-671 once daily via oral gavage to intracisternal cannulated (INTRCIST) male Sprague-Dawley rats for 5 consecutive days. Plasma, CSF, left liver lobe and brain samples were collected for analyses at 0.25-, 0.5-, 1-, 2-, 4-, 6-, and 8-hours post-dose on day 5 of the study. There were no abnormal cage-side observations during the study. The in-life procedures were carried out at Siri Pharma Labs (San Jose, CA), and samples were shipped to St. Jude for total CoA and BBP-671 determinations. Pharmacokinetic parameters for BBP-671 were calculated using Phoenix WinNonlin 8.3 (https://www.certara.com/software/phoenix-winnonlin/). Male Sprague-Dawley rats, beagle dogs and cynomolgus monkey were dosed orally and intravenously with BBP-671 at WuXi AppTec and the pharmacokinetic parameters determined. C57BL/6J mice (8-9 weeks old) were dosed with 1 mg/kg BBP-671 in 0.5% Methocel once daily for 3 days, and whole blood samples were collected to determine whole blood acetyl-CoA amounts between 0-8 h after the dose. There were three mice per time point. Whole blood acetyl-CoA was also measured in C57BL/6J mice maintained on a diet containing 22.5 ppm BBP-671 for four weeks. Oral dog pharmacokinetics were performed by Preclinical Research Services and the mouse pharmacokinetics experiment was performed by SAI Life Sciences.

**BBP-671 Treatment and Mouse Phenotyping.** SynCre+ and SynCre− mice were randomly enrolled into the treatment or control arms of the BBP-671 trial as they emerged from the breeding program. Animals were maintained on a purified chow diet containing 17.9% protein, 62.4% carbohydrate, 6% fat as described (Subramanian et al., 2023). BBP-671 was formulated at 75 ppm with the chow. The Pank1fl/fl, Pank2fl/fl SynCre+ mice were either provided with the chow containing 75 ppm BBP-671 or the matched control chow lacking BBP-671 at P14 and were weaned at P25. The plasma and tissue compound concentrations were measured in animals
supplied with BBP-671 in chow for 30 days with an estimated dose of 10 mg/kg/day based on food consumption from P14 to P45. Estimation of daily dosage was based on prior food intake measurements using C57BL/6J mice, age 8-10 weeks, that consumed 3-4 g chow/day. Chow was provided ad libitum continuously to pups and dams starting at P14 during the last week of the postnatal nursing/chow adaptation period until weaning of the pups at P21, and after weaning chow was supplied to pups only. Untreated animals received the same handling and the same chow without BBP-671 supplement. At P45, mice were evaluated in the open field test, euthanized, plasma was collected in EDTA, and flash frozen for analysis. Livers, forebrains and hindbrains were collected, and flash frozen immediately with liquid nitrogen. Open field locomotion tests were performed as described (Sharma et al., 2018). Briefly, the mice were placed in an open rectangular arena (36.8 cm × 43.2 cm) for 5 min during the light phase, and motor activity was evaluated using a video tracking system. Each subject was placed in the center of the arena, and their movement tracked using HVS Image video camera. The total distance traveled and the percentage of time in motion were calculated using the associated 2100 Plus software (HVS Image, San Diego, CA, USA).

Statistics. Statistical tests were performed using GraphPad Prism software version 9.1.2 (https://www.graphpad.com/scientific-software/prism/). An unpaired parametric t test was used when comparing two sample populations. The sample sizes, the means, and the P values are reported in the figures or legends.

Results

Therapeutic Strategy. The basis for the therapeutic approach is that BBP-671 functions as an activator of all PANK isoforms by locking the proteins in an active conformation that prevents feedback inhibition by acetyl-CoA, the most abundant physiological PANK inhibitor (Fig. S2). Control of the cellular CoA concentration is cell autonomous and is governed by the level of PANK.
isoform expression combined with the feedback inhibition of the isoforms by acetyl-CoA (Fig. S2A).

In PKAN, the PANK2 protein is dysfunctional leading to decreased cellular total CoA biosynthesis. A reduction in CoA biosynthesis, either transient or consistent, can have a negative impact on motor function, mitochondrial energy production and intermediary metabolism where it functions as the major acyl group carrier (Fig. S2B). BBP-671 administration activates the endogenous PANK1α/β and/or PANK3 isoforms in human PKAN preventing their feedback inhibition by acetyl-CoA, and by other acyl-CoAs, to boost cellular CoA concentrations and restore critical CoA-dependent metabolic functions (Fig. S2C). The activation effect was demonstrated in vitro using enzymatic pantothenate kinase assays in the presence of increasing acetyl-CoA concentrations with or without added BBP-671 (Fig. S2D). Purified recombinant pantothenate kinase proteins were incubated at 37 °C with radiolabeled pantothenate and the formation of radiolabeled phosphopantothenate was quantified as a function of dose and time (Sharma et al., 2018; Subramanian et al., 2023). The significant increase in the IC\textsubscript{50} for acetyl-CoA in the presence of BBP671 shows that all the three isoforms of PANK become refractory to acetyl-CoA inhibition. These \textit{in vitro} experiments illustrate that BBP-671 attenuates acetyl-CoA inhibition of PANK1β, PANK2 and PANK3. PANK3 is expressed ubiquitously in tissues, including brain (Dansie et al., 2014; Leonardi et al., 2007), and the activation of this isoform provides a route to increasing cellular CoA concentrations when alternate PANK isoforms are compromised.

\textbf{Pharmaceutical Properties of BBP-671.} BBP-671 was designed to have properties favorable for human pharmaceutical use. BBP-671 binds to PANK3 and stabilizes the active conformation with a 97 pM affinity coupled with a 21 min residence time (Subramanian et al., 2023). BBP-671 has relatively high plasma protein binding across different species (Table S1), which is typically the case for drugs that readily penetrate cell membranes (Hitchcock and Pennington 2006; Rankovic 2015). The top CNS drugs have a specific chemical profile that includes a high cLogP value and low polar surface area, which are hydrophobic properties that increase protein binding (Hitchcock
BBP-671 has higher metabolic stability in human microsomes than in rodent microsomes (Fig. S3A and S3B) suggesting the potential for an increased half-life and a lower clearance rate in humans compared to rodents (Figs. S3C and S3D). The pharmacokinetic behaviors of single dose BBP-671 in mouse (Fig. 1A) and rat (Fig. 1B) were determined. BBP-671 plasma levels were below detection in both species 24 h after administration of the compound by either intravenous or oral routes. Initial human dosing recommendations were derived from the analysis of pre-clinical pharmacokinetic data from multiple animal species (Jones et al., 2013; Petersson et al., 2022). Therefore, the pharmacokinetic parameters for single-dose BBP-671 delivered either orally or intravenously were determined in mouse (Subramanian et al., 2023), rat, dog and monkey (Table S2). The oral bioavailability, clearance rate and volume of distribution of BBP-671 in the four species suggested that BBP-671 has pharmacokinetic properties suitable for human use and provides data to support the design of human clinical trials.

The therapeutic strategy requires that BBP-671 be administered daily in human patients for extended periods of time. In several of our experiments the animals were dosed repeatedly (3-7 times) prior to the measurements as described in Methods to investigate steady-state drug levels and more closely mimic how the drug will be dosed in humans. BBP-671 plasma and tissue concentrations and tissue CoA levels all returned to baseline levels within 24 h after administration after repeated doses and we did not find any evidence for tolerance or drug retention.

**Blood Acetyl-CoA, a Pharmacodynamic Marker.** A challenge in assessing BBP-671 target engagement in humans is that CoA biosynthesis is cell autonomous and invasive brain/liver biopsies are not desirable in clinical trials. However, CoA does exist in blood cells; therefore, a method to measure whole blood acetyl-CoA in mice was developed to determine if this parameter has the potential to be used as a biomarker for BBP-671 target engagement in humans. Acetyl-CoA was chosen as the analyte because it is the most abundant thioester in cells and is more
efficiently detected by mass spectrometry compared to non-esterified CoA (Subramanian et al., 2021). Mice were treated by oral gavage with 1 mg/kg of BBP-671, and the levels of whole blood acetyl-CoA were measured over 8 h (Fig. 2A). Blood acetyl-CoA was significantly elevated at 2 and 4 h post-dose, then decreased to pre-dose concentrations at 8 h as the drug left the blood compartment (Fig. 2A). In a second experiment, mice were maintained on chow containing 22.5 ppm (~3 mg/kg/day) of BBP-671 for 1 week, and the blood acetyl-CoA concentrations were measured (Fig. 2B) to approximate the method of drug delivery to be used in the PKAN mouse model. Acetyl-CoA was significantly higher in blood obtained from BBP-671-treated mice compared to the control untreated mice and the levels following single dose or continuous exposure in chow were equivalent. These data support the utility of measuring acetyl-CoA in whole blood to assess BBP-671 target engagement. In a Phase 1 study in healthy adults, BBP-671 increased whole blood acetyl-CoA levels (Gretler et al., 2022), demonstrating the utility of blood acetyl-CoA as a pharmacodynamic marker for BBP-671 activity in humans.

**In Vitro BBP-671 Brain Permeability.** The physicochemical properties of BBP-671 (cLogP, no hydrogen bond donors, etc.) is in line with the properties of effective drugs that target the human central nervous system (Hitchcock and Pennington 2006; Rankovic 2015). These chemical properties suggested that BBP-671 would efficiently diffuse across the blood brain barrier into the CSF and brain tissue. The permeability of BBP-671 was evaluated using a parallel artificial membrane permeability assay (Table 1). The high permeability of BBP-671 compared with the standard panel suggests BBP-671 will rapidly penetrate the blood brain barrier. BBP-671 was ranked as having intermediate brain protein binding based on its comparison to lidocaine (low), indomethacin (intermediate), and fluoxetine (high) brain protein binding standards (Table 2). These data predict that BBP-671 has sufficient exposure in brain to target PANKs.
**In Vivo BBP-671 Brain Penetration.** The response of brain CoA concentrations to increasing oral doses of BBP-671 were first determined in C57BL/6J control mice and correlated with plasma concentrations of BBP-671 prior to testing the efficacy in the PKAN model. Groups of control mice were treated with single ascending doses of BBP-671. Compound and tissue CoA concentrations were measured 4 h after administration. Concentrations of BBP-671 in plasma (Fig. S4A), liver (Fig. S4B) and forebrain (Fig. S4C) increased proportionally to the drug dose. Liver total CoA was not impacted by the lowest dose of BBP-671 (0.1 mg/kg) and was doubled compared to the control at the 3-10 mg/kg BBP-671 dose (Fig. 3A). In comparison, the highest amount of forebrain CoA was achieved at the 10 mg/kg BBP-671 dose which resulted in about a 60% increase above the pre-treatment basal level in the control mice (Fig. 3B).

The pharmacokinetic/pharmacodynamic behavior of BBP-671 in mice showed that BBP-671 has high oral bioavailability and elevates liver and brain tissue total CoA (Subramanian et al., 2023). This work was expanded to INTRCIST rats to determine if BBP-671 could be detected in CSF as a marker for brain penetration and to confirm the behavior of BBP-671 in another pre-clinical model. The 8-h time course for plasma and CSF concentrations of BBP-671 are shown in Fig. 4A. BBP-671 was detected in the CSF where it had a lower $C_{\text{max}}$ but the same half-life as BBP-671 in plasma. The protein content of CSF is about 0.5% of that in plasma meaning that a much higher percentage of the drug in CSF is unbound (Muller et al., 1991). Based on the plasma protein binding data (Table S1) and the plasma and CSF and plasma drug concentrations at $C_{\text{max}}$ (Table 3) we calculate a partition coefficient for unbound BBP-671 (CSF/plasma) of 0.5. The tissue pharmacokinetic properties of BBP-671 in the liver and brain were calculated (Table 4). BBP-671 entry into the brain was delayed compared to liver but the half-lives of BBP-671 in tissues was longer than in plasma, reflecting BBP-671 binding to and slow release from the intracellular PANKs. Liver BBP-671 concentrations were relatively constant between 14 min and 4 hr (Fig. 4B), whereas brain BBP-671 levels were highest between 1 and 2 h after BBP-671 administration (Fig. 4C). These observations are consistent with the rapid exposure of liver to the drug followed by the
slower diffusion of BBP-671 across the blood brain barrier and into brain tissue. The impact of BBP-671 on tissue CoA concentrations was not immediate, and reflected the time required to synthesize additional CoA by the biosynthetic pathway after the activation of PANK, the first step in CoA biosynthesis (Leonardi et al., 2005). Liver CoA began increasing at 1 h after the BBP-671 dose and reached a $C_{\text{max}}$ of 142 pmol/mg tissue in 2 h (Fig. 4B). The increase in CoA concentrations in brain ($C_{\text{max}} = 78$ pmol/mg tissue) lagged behind liver (Fig. 4C), consistent with the pharmacokinetic profile of BBP-671 in liver and brain (Fig. 4B and 4C). In both tissues, total CoA was elevated at 8 hours although the plasma levels of BBP-671 were rapidly falling. This observation was in agreement with the slow dissociation of BBP-671 from the intracellular PANKs (Subramanian et al., 2023) and the respective tissue CoA degradation rates (Orsatti et al., 2021). Tissue CoA concentrations return to their steady-state set points by 24 h after a single administration of BBP-671 (Subramanian et al., 2023) (Fig. 4).

**BBP-671 Efficacy.** The $Pank1^{fl/fl},Pank2^{fl/fl} SynCre^+$ mouse model of PKAN (Sharma et al., 2018) carries conditional deletions of both $Pank1$ and $Pank2$ genes in neurons only, driven by the selective transcription of the synapsin1 ($Syn1$) promoter in neurons (Zhu et al., 2001). This mouse was chosen to model brain CoA deficiency in pre-clinical translational experiments because neurons have been identified as a focal target of disease in PKAN (Bettencourt et al., 2016). The PKAN mouse model demonstrates a consistent, selective CoA deficiency in brain, rapid disease progression with reduced growth rate, shortened lifespan and a rapid onset of a movement disorder similar to aspects of PKAN disease in patients (Sharma et al., 2018).

The PKAN mice were maintained on chow supplemented with 75 ppm BBP-671 ($\sim$10 mg/kg/day based on food consumption) for 30 days. Doses were chosen to achieve at least a 30% increase in brain CoA in the context of reduced PANK1 and PANK2 expression which otherwise would contribute to CoA biosynthesis. The P14 start of treatment was chosen because consistent locomotion does not occur in mice until P15 (Hill et al., 2008). Chow was provided ad libitum
continuously to pups and dams starting at P14 during the last week of the postnatal nursing/chow adaptation period until weaning of the pups at P21, and after weaning chow was supplied to pups only. Untreated animals received the same handling and the same chow without BBP-671 supplement. BBP-671 was measured in plasma, liver, forebrain and hindbrain of the treated PKAN animals on the last day of the 30-day treatment at 4 h after the end of the dark cycle (Fig. 5A). Total liver CoA was not different between the matched wild-type control and untreated PKAN mouse livers (Fig. 5B), consistent with the genetic defect being targeted to neurons. As expected, BBP-671 treatment increased the liver CoA concentration in the PKAN animals (Fig. 5B), similar to the liver CoA response to BBP-671 in C57BL/6J wild-type mice (Fig 2A) (Subramanian et al., 2023). In contrast, the tissue CoA concentrations in both the forebrains (Fig. 5C) and hindbrains (Fig. 5D) of the untreated PKAN mice were significantly reduced by about 15-30%, consistent with the selective ablation of the *Pank1* and *Pank2* genes in neurons. BBP-671 therapy significantly elevated the forebrain CoA concentration (Fig. 5C) and restored the hindbrain CoA levels (Fig. 5D) in the treated PKAN mice.

PKAN mice were smaller and weighed less than their littermate controls (Fig. 6A). BBP-671 treatment led to a significant increase in body weight, but the animal weights did not return to control values after 30-days of treatment (Fig. 6A). The BBP-671-treated PKAN mice had improved survival (Fig. 6B); however, this difference was not statistically significant because all animals were sacrificed at P45, prematurely terminating the survival curves. Importantly, the movement disorder was significantly improved, when measured as either the total distance traveled during a 5-minute period (Fig. 6C) or the percentage of time the animals spent moving (Fig. 6D). These data show that BBP-671 treatment enabled significant restoration of movement in the *Pank*<sup>fl/fl</sup>/*Pank*<sup>fl/fl</sup> *SynCre*<sup>+</sup> model of brain CoA deficiency. PKAN is characterized by a progressive difficulty in movement, and these data suggest an improvement in an assessment that is clinically relevant for PKAN patients.
Discussion

The positive results of treating the symptomatic $\text{Pank1}^{\text{fl/fl}},\text{Pank2}^{\text{fl/fl}} \text{SynCre}^+$ mouse model of PKAN suggest that BBP-671 has the potential to improve the movement disorder associated with the human disease. Dystonia and progressive loss of motor control are important clinical features of PKAN, typically affecting the lower limbs and often with orolingual involvement (Iankova et al., 2021). Pathologic volume reduction in the globus pallidus of PKAN patients was correlated with dystonia scores and affirmed this brain nucleus as a center of severe malfunction (Roa-Sanchez et al., 2021). General cerebral white matter alterations, involving the superior cortico-striate and thalamic tracts, corpus callosum and cerebellar tracts have also been observed in patients (Rivera et al., 2022). These latter studies indicated that the disease was not confined to the globus pallidus but rather involved a general impairment of the motor system, because the white matter changes correlated with the severity of symptoms (Rivera et al., 2022). In humans, $\text{PANK2}$ is highly expressed in brain, and based on the analysis of multiple $\text{Pank}$ isoform single and double knockout mice, the elimination of a PANK isoform in a tissue where it is substantially expressed results in lower concentrations of total tissue CoA (Garcia et al., 2012; Leonardi et al., 2010; Sharma et al., 2018; Subramanian et al., 2020). The reduction of brain CoA by accelerating CoA degradation in neurons also reduces motor coordination in mice (Shumar et al., 2015). BBP-671 is a high affinity activator of all PANK isoforms, and its ability to raise brain CoA concentrations and improve movement in $\text{Pank1}^{\text{fl/fl}},\text{Pank2}^{\text{fl/fl}} \text{SynCre}^+$ mice supports that CoA has a role in regulating the function of the motor system and suggests that correcting a CoA deficit may be beneficial for treatment of human neurometabolic imbalance. Interestingly, overexpression of PANK, or supplementation with CoA precursors, rescues the phenotype of a genetic PINK1-deficient Parkinson’s disease model (Huang et al, 2022). In addition, alteration of brain CoA oxidative status, or re-distribution of CoA molecular species or precursors, has been linked to Huntington’s and Alzheimer’s as well as Parkinson’s disease in postmortem patient brains (Moiseenok and Kanunniko, 2023).
BBP-671 may be an investigational tool to gain further insight into the tissue-selective physiological networks of CoA-dependent processes. CoA is essential for the operation of the mitochondrial tricarboxylic acid cycle and is the predominant acyl group carrier in metabolism (Leonardi et al., 2005; Zhang et al., 2007). Reduced mitochondrial function and reduced energy generation are associated with low tissue CoA (Leonardi et al., 2010; Zhang et al., 2007). PKAN symptoms likely arise from a metabolic imbalance(s) due to low CoA in areas of the central nervous system that control motor function. This view is supported by functional MRI in PKAN patients with dystonic hand movements that revealed reduced connectivity between the basal ganglia and cortical motor-related areas of the brain (Stoeter et al., 2021). In comparison, symptomatic mouse models of dystonia have identified the basal ganglia and cerebellum as nodes in an integrated network that is dysfunctional, and accompanied by abnormal glutamate regulation (Wilson and Hess 2013). MRI analysis of brain metabolites in the \textit{Pank1}^{fl/fl},\textit{Pank2}^{fl/fl} SynCre\textsuperscript{+} PKAN mice show a marked reduction in cerebral glutamate/glutamine levels that is restored by BBP-671 treatment (Li et al., 2022). The impaired movement and neurometabolic imbalances in human PKAN and the \textit{Pank1}^{fl/fl},\textit{Pank2}^{fl/fl} SynCre\textsuperscript{+} model connect the movement disorder with aberrant cerebral glutamate homeostasis and neuronal CoA deficiency. Correction of the brain CoA deficiency with BBP-671 resulted in substantial improvement in both locomotion and in brain glutamate deficiency and provides the preclinical foundation for the development of BBP-671 as a potential PKAN therapeutic.

**Acknowledgements.** We thank Jina Wang, Karen Miller, Katie Creed and Lois Richmond for their expert technical assistance, and Lei Yang and Yong Li in the Chemical Biology and Therapeutics Analytical Technologies Center for their expert technical assistance.

**Data Availability Statement.** The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.
Author contributions.


Conducted experiments: Subramanian, Frank, Yun, Tangallapally.

Contributed new reagents or analytic tools: Tangallapally, Henry.

Performed data analysis: Subramanian, Frank, Sukhun, Wade, Harden, Rao.

Wrote or contributed to the writing of the manuscript: Subramanian, Frank, Sukhun, Wade, Harden, Rao, Yun, Tangallapally, White, Lee, Sinha, Rock, Jackowski.
References


Footnotes

Funding. This work was supported by a research grant from CoA Therapeutics Inc., a BridgeBio Pharma, Inc., company, and ALSAC, St. Jude Children's Research Hospital.

Figure Captions.

Fig. 1. Single dose pharmacokinetic behavior of BBP-671 in rodents.  (A) Plasma BBP-671 concentrations in mice (3 per point) administered BBP-671 by IV, 2 mg/kg (black) or PO, 10 mg/kg (red) routes.  Calculated mouse PK parameters are listed in Table S2.  (B) Plasma BBP-671 concentrations in rats dosed either by IV, 1 mg/kg (black) or PO, 10 mg/kg (red) routes.  Calculated rat PK parameters are listed in Table S2.  Three animals per point.  Data are mean ± SE.  ND means not detected.

Fig. 2. Whole blood acetyl-CoA concentrations following BBP-671 administration.  (A) C57BL/6J mice (3 per time point) were administered compound (1 mg/kg; PO) and blood acetyl-CoA concentrations ± SE were measured as a function of time by LC-MS/MS. Total 21 animals.  (B) C57BL/6J mice (6 per condition) were maintained for 5 days on chow containing 22.5 ppm BBP-671 (3 mg/kg/day), and the blood acetyl-CoA concentrations were measured by LC-MS/MS.  The P value (red) was calculated using an unpaired t-test to compare untreated control values ± SE with BBP-671 treated mice. Total 12 animals.

Fig. 3. Dose response for BBP-671 in C57BL/6J mice.  Mice (3 per dose) were administered PO, 0.1, 1, 3 and 10 mg/kg formulated in 0.5% Methocel suspension. Total tissue CoA ± SE was measured 4 h after the dose.  (A) Liver.  (B) Forebrain.  The P values (red) were calculated using an unpaired t-test to compare untreated control values with BBP-671-treated mice. Total 15 animals.

Fig. 4. Pharmacokinetics of BBP-671 and tissue CoA concentrations in INTRCIST cannulated male Sprague-Dawley rats (3 per point).  BBP-671 (PO, 10 mg/kg) was administered and compound levels were measured in plasma, CSF, liver and brain at the indicated time points. Total CoA was measured in liver and brain at each time point.  (A) BBP-671 levels in plasma
(closed circles) and CSF (open circles). (B) BBP-671 levels in liver (red) and total CoA in liver (black). (C) BBP-671 levels (red) and total CoA (black) in brain. The data represents mean ± SE. Total 24 animals.

Fig. 5. BBP-671 and CoA concentrations in \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{+} \) (PKAN) mice at postnatal day 45. (A) Concentrations of BBP-671 in the plasma, liver, forebrain and hindbrain of \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{+} \) treated with 75 ppm BBP-671 (10 mg/kg/day) in chow. In panels B-D, littermate \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{0} \) mice were the control group. (B) Total liver CoA concentration in control and \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{+} \) mice treated with or without BBP-671. (C) Total CoA concentrations in the forebrain of \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{+} \) mice treated with or without BBP-671. (D) Total CoA concentrations in the hindbrain of \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{+} \) mice treated with or without BBP-671. The \( P \) values (red) were calculated using an unpaired t-test to compare control mice values with BBP-671-treated mice. The number of mice in each group is shown in parenthesis Blue dots indicate male mice and red dots indicate female mice. Values calculated ± SE. Panel A, 46 animals; panel B, 55 animals; and panels C and D, 28 animals.

Fig. 6. Body weight, survival and movement in \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{+} \) (PKAN) mice at postnatal day P45. (A) Body weight of control and \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{+} \) mouse following treatment with 75 ppm BBP-671 (10 mg/kg/day) in chow. Littermate \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{0} \) mice were the control group. (B) Kaplan-Meyer plot of \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{+} \) mouse survival with or without BBP-671 treatment. The experiment was stopped at day 45, which means that the difference in life span not significant based on the log-rank (Mantel-Cox) test (\( P=0.2633 \)) and the Gehan-Breslow-Wilcoxon test, (\( P=0.1723 \)). (C) Distance traversed by control and \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{+} \) mice treated with BBP-671. (D) Percent time moving for control and \( \text{Pank1}^{fl/fl}, \text{Pank2}^{fl/fl}, \text{SynCre}^{+} \) mice treated with BBP-671. The \( P \) values (red) were calculated using an unpaired t-test to compare control mice values with BBP-671-treated mice. The number of mice
in each group is shown in parenthesis. Blue dots indicate male mice and red dots indicate female mice. Total 80 animals. ± SE.
### TABLE 1
Parallel artificial membrane permeability assays

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pe&lt;sup&gt;a&lt;/sup&gt; (10&lt;sup&gt;-6&lt;/sup&gt; cm/s)</th>
<th>Retention (%)</th>
<th>Expected Pe&lt;sup&gt;b&lt;/sup&gt; (10&lt;sup&gt;-6&lt;/sup&gt; cm/s)</th>
<th>Permeability Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBP-671</td>
<td>113 ± 7.69</td>
<td>50.3 ± 1.15</td>
<td></td>
<td>High</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>14.2 ± 1.34</td>
<td>13.7 ± 3.06</td>
<td>25</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Progesterone</td>
<td>98.4 ± 20.0</td>
<td>91.3 ± 5.13</td>
<td>200</td>
<td>High</td>
</tr>
<tr>
<td>Propranolol HCl</td>
<td>43.1 ± 9.80</td>
<td>82.7 ± 3.51</td>
<td>80</td>
<td>High</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0.171 ± 0.296</td>
<td>1.33 ± 2.31</td>
<td>&lt; 1</td>
<td>Low</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data were from three experiments. ± SD.
<sup>b</sup>Reference values taken from Tsinman et al. (2011).

### TABLE 2
Brain protein binding assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>Unbound Fraction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference Value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBP-671</td>
<td>0.0459 ± 0.003</td>
<td></td>
<td>Intermediate</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>0.550 ± 0.313</td>
<td>0.334 ± 0.100</td>
<td>Low</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.0595 ± 0.006</td>
<td>0.052 ± 0.012</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>0.0021 ± 0.0002</td>
<td>0.00094</td>
<td>High</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data were from three experiments ± SD.
<sup>b</sup>Reference values taken from Di et al. (2011) and Liu, et al. (2005).
TABLE 3
Plasma pharmacokinetic parameters of BBP-671 measured in cannulated rats

<table>
<thead>
<tr>
<th>Sample</th>
<th>t_{1/2} (h)</th>
<th>T_{max} (h)</th>
<th>C_{max} (mM)</th>
<th>AUC_{last} (h*mM)</th>
<th>AUC_{inf} (h*mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1.6 ± 0.1</td>
<td>2.0</td>
<td>5.9 ± 0.4</td>
<td>23.4 ± 0.4</td>
<td>24.6 ± 0.2</td>
</tr>
<tr>
<td>CSF</td>
<td>1.6 ± 0.3</td>
<td>2.3 ± 1.5</td>
<td>0.12 ± 0.01</td>
<td>0.54 ± 0.02</td>
<td>0.58 ± 0.02</td>
</tr>
</tbody>
</table>

*Data were from 3 rats per time point ± SD

TABLE 4
Tissue pharmacokinetic parameters for BBP-671 measured in cannulated rats

<table>
<thead>
<tr>
<th>Sample</th>
<th>t_{1/2} (h)</th>
<th>T_{max} (h)</th>
<th>C_{max} (pmol/mg)</th>
<th>AUC_{last} (hr*pmol/mg)</th>
<th>AUC_{inf} (hr*pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2.3 ± 0.1</td>
<td>0.5 ± 0.4</td>
<td>12.5 ± 0.8</td>
<td>67.8 ± 2.7</td>
<td>78.6 ± 2.1</td>
</tr>
<tr>
<td>Brain</td>
<td>2.4 ± 0.5</td>
<td>2.0</td>
<td>6.5 ± 1.1</td>
<td>24.2 ± 2.1</td>
<td>28.2 ± 0.8</td>
</tr>
</tbody>
</table>

*Data ± SD Were from 3 rats per time point.
Fig. 1
Fig. 2
Fig. 3
Fig. 5
Fig. 6