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# Correction of a genetic deficiency in pantothenate kinase 1 using phosphopantothenate replacement therapy

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ABSTRACT

Coenzyme A (CoA) is a ubiquitous cofactor involved in numerous essential biochemical transformations, and along with its thioesters is a key regulator of intermediary metabolism. Pantothenate (vitamin $B_5$) phosphorylation by pantothenate kinase (PanK) is thought to control the rate of CoA production. Pantothenate kinase associated neurodegeneration is a hereditary disease that arises from mutations that inactivate the human *PANK2* gene. Aryl phosphoramidate phosphopantothenate derivatives were prepared to test the feasibility of using phosphopantothenate replacement therapy to bypass the genetic deficiency in the *Pank1*$^{-/-}$ mouse model. The efficacies of candidate compounds were first compared by measuring the ability to increase CoA levels in *Pank1*$^{-/-}$ mouse embryo fibroblasts. Administration of selected candidate compounds to *Pank1*$^{-/-}$ mice corrected their deficiency in hepatic CoA. The PanK bypass was confirmed by the incorporation of intact phosphopantothenate into CoA using triple-isotopically labeled compound. These results provide strong support for PanK as a master regulator of intracellular CoA and illustrate the feasibility of employing PanK bypass therapy to restore CoA levels in genetically deficient mice.

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## 1. Introduction

Coenzyme A (CoA) is an essential cofactor that plays a central role in intermediary metabolism in all organisms. It functions as a carboxylic acid substrate carrier and supports a multitude of essential biochemical transformations, including the activities of the citric acid cycle, sterol biosynthesis, amino acid metabolism, and the synthesis and degradation of fatty acids and complex lipids [1]. CoA is synthesized in a five-step process from pantothenic acid (Pan), cysteine and ATP (Fig. 1). Pan is also known as vitamin $B_5$, and mammals must obtain Pan from their diet and/or intestinal flora [1]. The first committed and key regulatory step of CoA biosynthesis is the phosphorylation of Pan to phosphopantothenate (Ppan) catalyzed by the pantothenate kinases (PanK, EC 2.7.1.33) [1,2,3]. Ppan and other pathway intermediates are not detected in radiolabeled mouse hepatocytes [4] indicating that Ppan is rapidly converted to CoA [2,5–7]. Most bacteria, fungi and lower metazoans have one gene encoding PanK, whereas mammals have three genes that express four catalytically active isoforms: PanK1α, PanK1β, PanK2 and PanK3 [3]. The four human and mouse PanK isoforms share a homologous carboxy-terminal catalytic domain, but differ in their amino-termini which direct the isoforms to different cellular compartments [8]. All cell types studied to date express several PanK isoforms [9] providing some degree of functional redundancy. The global chemical inhibition of CoA synthesis at the PanK step in mice results in a severe phenotype that leads to death [4]. The roles of individual PanKs were explored by creating global deletions of the *Pank1*, *Pank2* and *Pank3* genes in mice [10]. *Pank1* is highly expressed in liver [9], and the *Pank1*$^{-/-}$ mice have reduced hepatic CoA content and exhibit fasting hypoglycemia and steatosis [11]. The murine *Pank2* gene is highly expressed in testes [12], and *Pank2*$^{-/-}$ mice exhibit azoospermia [13]. Unlike the human *PANK2*, the murine *Pank2* is not the most abundant isoform expressed in brain [12], and is located in the cytosol rather than the mitochondria [8]. The *Pank1*/*Pank2* double knockout mice have low brain CoA levels, and exhibit hind limb dragging transiently during the pre-weaning period, but die within 2 weeks after birth [10].

In humans, PANK2 is the principle isoform expressed in the brain [12], and mutations in the *PANK2* gene result in the debilitating neurologic disorder called PKAN (*P*antothenate *K*inase *A*ssociated *N*eurodegeneration, OMIM ID: 234200) [14]. PKAN is the most common form of neurologic degeneration with brain iron accumulation (NBIA), a group of clinical disorders marked by progressive abnormal involuntary movements, alterations in muscle tone, and postural disturbances. PKAN is inherited as an autosomal recessive genetic condition. Two distinct manifestations of the disease are observed: (i) classic PKAN patients have more rapid progression of symptoms within the first 10 years of life and typically do not survive past age 20, and (ii) atypical PKAN does not present symptoms until the second or third decade of life with disease progression occurring much slower than the classic PKAN [15]. Several *PANK2* mutations result in a frame shift or premature

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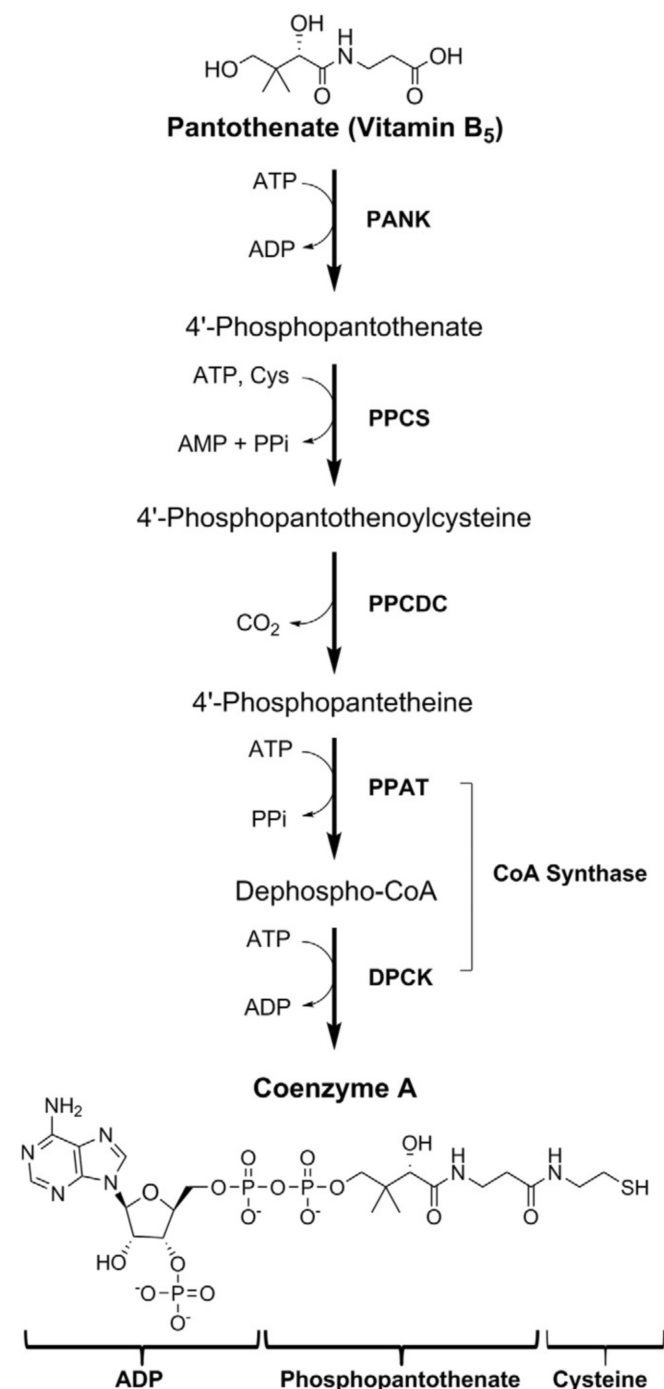


**Fig. 1.** The CoA biosynthetic pathway. The commitment step is the phosphorylation of pantothenate (vitamin $B_5$) by pantothenate kinase (PANK) to 4′-phosphopantothenate. This is followed by condensation with cysteine catalyzed by 4′-phosphopantothenoylcysteine synthase (PPCS) and then decarboxylation to form 4′-phosphopantetheine by 4′-phosphopanthenoylcysteine decarboxylase (PPCDC). 4′-Phosphopantetheine is adenylylated to dephospho-CoA by phosphopantetheine adenylyltransferase (PPAT), then phosphorylated by dephospho-CoA kinase (DPCK) at the 3′-OH of the ribose to form CoA.

stop codon in the coding sequence, and these proteins are predicted to be inactive because of the loss of the core catalytic domain sequence and are associated with early onset disease [16]. CoA synthase catalyzes the last two steps in the CoA biosynthetic pathway and NBIA patients with mutations in the *COASY* gene were recently identified [17]. Thus, disease pathogenesis is hypothesized to result from insufficient cellular CoA due to reduction of its biosynthesis. The mechanistic connections between CoA deficiency, neurodegeneration and iron accumulation in the brain are not understood. The *Pank2*$^{-/-}$ mice do not exhibit brain iron accumulation or a phenotype resembling the movement disorder that is characteristic of human PKAN patients [13].

The goal of this project was to determine if PanK bypass therapy is a viable approach to correct CoA levels in CoA-deficient animals. The rationale for this approach is based on prior research that strongly supports PanK as the rate-controlling step in cellular CoA biosynthesis [1, 7]. If this is correct, all intracellular Ppan will be efficiently and completely converted to CoA. However, if PanK is not the sole regulatory point in the pathway or if CoA degradation via nudix hydrolases plays a determinant role [18–20], then bypassing PanK may not have the desired impact on elevating CoA levels. Phosphorylated metabolic intermediates such as Ppan cannot be delivered to cells without chemical modification due to their instability in systemic circulation and limited cell membrane permeability. There have been a number of approaches developed for the protection of the phosphate moiety with substituents that are removed by intracellular enzymes [21–25]. The aryl phosphoramidate protection strategy was selected for this study because this approach has led to the development of several cell-penetrant antiviral and anticancer nucleoside analogs, and the biochemical pathway that releases the protected phosphomonoester is known [21–27]. We find that aryl phosphoramidate Ppan derivatives increase intracellular CoA levels in *Pank1*$^{-/-}$ mouse embryo fibroblasts and restore hepatic CoA levels in genetically deficient *Pank1*$^{-/-}$ mice. These results verify the key regulatory role of PanK in CoA homeostasis and show that Ppan bypass therapy is feasible.

## 2. Experimental procedures

### 2.1. Ppan derivatives

Compounds were synthesized and supplied by Retrophin, Inc. The details of chemical syntheses and compound characterizations were published previously [28].

### 2.2. Efficacy testing in vitro

Compounds were tested for toxicity and for metabolic conversion to CoA following addition to cultured mouse primary fibroblasts. Fibroblasts were isolated from E12.5 *Pank1*$^{-/-}$ (KO) and *Pank1*$^{+/+}$ (WT) embryos and cultured at 37 °C in an atmosphere of 5% $CO_2$, 95% air in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone), glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 μg/ml) and β-mercaptoethanol (55 μM). Compounds dissolved in vehicle were added to triplicate cultures of fibroblasts at 200 μM final concentration followed by incubation at 37 °C for 24 h. Vehicle alone (≤0.4% dimethylsulfoxide) was added to untreated control cultures. Liquid media were removed from cultures, and adherent cells (densities ranging from $3 \times 10^6$ to $9 \times 0^6$ per 150-mm dish) were washed once with phosphate-buffered saline (PBS) pH 7.4 and prepared either for quantification of CoA or determination of total viable cell number. Adherent cells in duplicate cultures were washed once briefly with ice-cold (4 °C) water and immediately processed for extraction, derivatization and CoA quantification by high-pressure liquid chromatography (HPLC) as discussed below. For determination of viable cell counts, cells were detached from the dish using [0.25% trypsin + ethylenediaminetetraacetic acid] (Hyclone) and incubated for 10 min at room temperature. The action of trypsin was stopped by the addition of equal volume of DMEM containing 10% fetal bovine serum. Cells in suspension (total and viable numbers) were quantified in a NucleoCounter (New Brunswick Scientific) in buffers supplied by the instrument manufacturer. Toxicity was indicated by viability ≤80% or total cell number ≤ 50% compared to vehicle only-treated control cultures. Toxic compounds were not advanced to in vivo testing.

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