The essential metabolic cofactor coenzyme A (CoA) plays a central role in a variety of metabolic processes, with an estimation that up to 9% of all enzyme activities use CoA in some form. In addition, it also serves as the carrier of the β-alanine moiety of the parent molecule has been exchanged for γ-aminobutyric acid (GABA, especially as antimicrobial agents). Since pantothenic acid (Pan, vitamin B₅, 3) is the precursor of de novo CoA biosynthesis in all organisms (Figure 1), there has been much interest in the development and therapeutic use of Pan analogues, especially as antimicrobial agents. Homopantothenic acid, also called hopantronic acid or hopantenate (HoPan, 1), among several other names, is a Pan analogue in which the β-alanine moiety of the parent molecule was exchanged for γ-aminobutyric acid (GABA, the precursor of the γ-aminobutyric acid [GABA, a neurotransmitter] and reveals the functional implications of mutations in the human PPCS that have been linked to severe dilated cardiomyopathy. Taken together, this study demonstrates that the molecular mechanism of action of HoPan is more complex than previously thought, suggesting that the results of studies in which it is used as a tool compound must be interpreted with care. Moreover, our findings provide a clear framework for evaluating the various factors that contribute to the potency of CoA-directed inhibitors, one that will prove useful in the future rational development of potential therapies of both human genetic and infectious diseases.
result in large decreases in CoA levels in isolated hepatocytes, and HoPan administration (100 µg/g/day) proved to be lethal within 5 days for males and 15 days for females in mice maintained on a diet without any Pan supplement. This correlated with a significant decline in total and free CoA in especially the livers of treated animals. Importantly, the toxicity was reversed when HoPan was coadministered with Pan. This is consistent with HoPan’s inhibition of CoA’s biosynthesis from Pan and also explains why previous studies—in which standard Pan-rich diets were used—showed no toxic effects for HoPan treatment.

Following this foundational study, HoPan has become the main small molecule tool compound for modulating CoA levels in a variety of model systems, including the fruit fly *Drosophila melanogaster*, mice, and various cell cultures, specifically in studies of rare genetic neurodegenerative diseases linked to errors in CoA biosynthesis.14–17 Chief among these is pantothenate kinase associated neurodegeneration (PKAN), a disease caused by certain mutations in PANK2, the gene encoding the human mitochondrial PanK isoform PANK2. In this context HoPan seems to be an especially appropriate tool compound based on the understanding that it reduces CoA levels by inhibiting PanK.

However, several unrelated findings, when taken together, have suggested that HoPan’s MoA may be more complex. First, the study that identified HoPan as a PanK inhibitor also showed that murine hepatocytes are able to transform [3H]HoPan into radiolabeled 4′-phospho-hopantenate (P-HoPan, 2), suggesting that HoPan is also a substrate of PanK (Figure 1).15 Second, we previously demonstrated that addition of pantethine—the disulfide of the CoA degradation product pantetheine (PantSH, 6)—reverses HoPan-mediated CoA depletion in *D. melanogaster*.14 A simple explanation for this observation is that the *D. melanogaster* PanK (dPanK, also known as fumble or Fbl) is not inhibited by HoPan and that dPanK (like other homologous type II PanKs and microbial type I PanKs) is able to phosphorylate PantSH to form 4′-phosphopantetheine (P-PantSH, 7) in a CoA salvage pathway (Figure 1).16,18–21 Formation of P-PantSH in this manner would bypass the phosphopantothenoylcysteine synthetase (PPCS) and phosphopantothenoylcysteine decarboxylase (PPCDC) steps of CoA biosynthesis, and would consequently show rescue if either of these activities is the molecular target of HoPan. Alternatively, if PantSH is degraded to Pan, the elevated levels of the natural PanK substrate may also provide rescue of HoPan’s inhibition of dPANK. To be able to distinguish between these various hypotheses would require a kinetic analysis of dPANK’s activity toward Pan and PantSH and the impact of HoPan thereon. However, no detailed comparative kinetic analysis of any eukaryotic PanK has been reported to date.

These findings prompted us to revisit the mechanism by which HoPan causes the lowering of CoA levels. In particular, HoPan’s apparent phosphorylation by PanK would suggest it has a MoA similar to that of the natural product antibiotic CJ-15,801, a fungal metabolite that selectively inhibits the growth of *Staphylococcus aureus* following its phosphorylation by *S. aureus* PanK and subsequent inhibition of its PPCS (Figure 1).

In this alternative mode of action, HoPan would therefore not target PanK for inhibition but require it for metabolic activation; such a proposal would be consistent with all the previously published data.

We therefore set out to use biochemical, structural, and genetic studies to elucidate the molecular mechanism of action (MOA) of HoPan with the goal of providing clarity regarding the results of those studies in which it is used as a tool compound. In addition, this work afforded valuable insights into the PPCS enzyme, as well as the potential of HoPan and compounds that share its MOA to serve as leads in our ongoing efforts to develop new selective CoA-targeting antimicrobial agents.

### RESULTS AND DISCUSSION

**Evaluating the Interaction of HoPan with PanK: Inhibitor or Substrate?** All previous studies that evaluated the impact of HoPan on the activity of the PanK enzymes of target organisms of interest were executed using discontinuous assays that rely on the turnover of radioactive substrates (either

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**Figure 1.** Coenzyme A (CoA) biosynthesis and the proposed molecular targets of HoPan. In humans and *D. melanogaster* CoA is biosynthesized *de novo* from Pan in a five-step pathway (boxed in green) catalyzed by pantothenate kinase (PanK), phosphopantothenoylcysteine synthetase (PPCS), phosphopantothenoylcysteine decarboxylase (PPCDC), and the bifunctional CoA synthase (CoAsy) that has both phosphopantetheine adenyltransferase (PPAT) and dephospho-CoA kinase (DPCK) activity. It can also be salvaged from pantetheine (PantSH), a CoA degradation product (pathway boxed in orange), through dual action of many PanKs. The possible targets for HoPan’s inhibition of CoA biosynthesis are indicated; this study aims to distinguish between these possibilities.

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radiolabeled Pan or ATP). More specifically, it was shown that HoPan is an apparent competitive inhibitor of all mouse PanK isoforms when using [13C]Pan as a substrate and tracking the formation of the radiolabeled 4′-phosphopantothenate (P-Pan) product.13 However, such an assay cannot distinguish between compounds acting as classical competitive inhibitors on the one hand, or as competitive substrates on the other (Figure 2A). In the latter case, the observed reduction in the rate of P-Pan formation would be due to HoPan also being turned over; the observed \( K_i \) would be the PanK enzyme’s \( K_M \) for HoPan. Since the same study showed that murine hepatocytes can transform HoPan into P-HoPan, we hypothesized that it was more likely that HoPan’s apparent inhibition of PanK’s Pan phosphorylation activity was due to it acting as a competing substrate of the enzyme.

To establish whether HoPan’s interaction with eukaryotic PanKs is that of a substrate or inhibitor, we prepared heterologously expressed and purified human and fruit fly PanKs to be used in in vitro activity assays. For these tests, we used the two main cytosolic isoforms of human PanK (hPANK1\(\beta\) and hPANK3) and the Fumble D (FblD) splicing variant of the single fruit fly PanK (dPANK), which represents the minimally catalytically active form of the enzyme in this organism.22 Importantly, the key catalytic and other sequence motifs of eukaryotic PanKs are largely conserved, suggesting a similar active site architecture—and therefore substrate specificity profile—for these enzymes (Figure 2B and Supporting Information Figure S1). For the activity determination, a standard continuous kinase assay was used that couples the formation of ADP to the oxidation of NADH, the latter of which is followed spectrophotometrically (Figure 2A). As such, the assay is naive of the substrate used and can be applied to determine the activity of the enzyme toward any substrate, or even mixtures of competing substrates.

The results, shown in Figure 2C and summarized in Table 1 clearly indicate that HoPan is a substrate of all the tested PanKs, with specificity constants \( (k_{cat}/K_M) \) no more than 2-fold lower than that observed for the natural substrate, Pan. This difference was mainly due to increased \( K_M \) values; at saturating concentrations, the enzymes showed no distinction in the rate of turnover of the two compounds. Both hPANK3 and dPANK/FblD enzymes showed a degree of cooperative behavior toward HoPan but not Pan (as evidenced by sigmoidal activity profiles that gave Hill coefficients of \( \sim 1.3– \)

Figure 2. Activity characterization of human and fruit fly PanK isoforms. (A) Schematic representation of the two PanK activity assays that are usually used: a discontinuous radiolabeled assay that determines the amount of radiolabeled P-Pan formed from radiolabeled ([13C] Pan) following separation of the product and unreacted substrate, which is blind to the enzyme acting on other substrates, and a spectrophotometric assay that couples the conversion of ATP to ADP to the oxidation of NADH using the pyruvate kinase (PK) and lactate dehydrogenase (LDH) catalyzed reactions, which measures kinase activity toward all substrates without distinction. (B) Active site of hPANK3 with the PantSH analogue N7-Pan (stick structure with carbon atoms in white) bound (PDB: 3SMS). The two subunits of the dimeric enzyme are shown in different shades of orange; the loop covering the active site is not shown for the sake of clarity. Residue E138 involved in catalysis and R207 involved in positioning the substrate are indicated. Also shown is the PanK reaction product P-Pan (stick structure with carbon atoms in yellow) obtained by overlaying the structure of hPANK3 bound to P-Pan and ADP (PDB: SKPZ\footnote{Supporting Information Figure S1}). (C) Activity profiles obtained for (from left to right) hPANK1\(\beta\), hPANK3, and dPANK/FblD with Pan, HoPan, and PantSH as substrates. The symbols in the activity profiles are the average values obtained at the indicated concentration in at least three independent experiments. The error bars indicate the SEM of the averaged data. The solid line represents the fit to the Michaelis–Menten or Hill equations (see Table 1). For dPANK/FblD, different batches of enzymes gave different levels of specific activity (suggesting poor stability); in its case, the rates are therefore reported relative to the amount of protein used to highlight this variability.

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Interestingly, for both hPANK3 and dPANK/FblD, the obtained kinetic parameters for Pan, with dPANK/FblD showing full cooperativity and a 3-fold increase in its specificity constant compared to hPANK1/2, indicates that, once formed, P-HoPan is a moderately potent competitive inhibitor of these enzymes. The analysis was performed twice for each enzyme using different batches of enzymes in each case, giving an average K<sub>i</sub> value of 13.4 ± 3.6 μM for hPPCS and 20.9 ± 1.1 μM for dPPCS (avg ± range/2). This indicates that, once formed, P-HoPan is a moderately potent competitive inhibitor of these enzymes.

**Cocrystallization with P-HoPan and AMPPNP Captures hPPCS in a Fully Ordered Conformation.** The discovery of P-HoPan’s inhibition of PPCS raises the question of the molecular mechanism by which this inhibition is achieved, considering the close structural similarity between P-HoPan and the P-Pan substrate. Unfortunately, none of the available PPCS structures could provide any insight in this regard due to a lack of appropriate complex structures and because in all these structures several portions of the active site remained disordered. In the case of hPPCS, only the apo structure has been determined, with large portions of the active site region found in a disordered state (PDB: 1P9O). While X-ray structures of a site-directed mutant of the *Saccharomyces cerevisiae* PPCS (Cab2) revealed the binding mode of the Pan substrate (PDB: 6AI9), the intermediate P-Pan-CMP (PDB: 6AIK), and the P-PanCyP product (PDB: 6AIM), no structures were obtained in complex with the unreacted P-Pan substrate in the presence of nucleotide. Similarly, none of the available structures of bacterial PPCS enzymes—those of the *Escherichia coli* PPCs (ecPPCS) and the *Mycobacterium smegmatis* CoaBC (bifunctional PPCS/PPCDC fusion)—could shed light on a possible inhibition mechanism for P-HoPan.

We therefore determined the X-ray structure of hPPCS in complex with P-HoPan and the nonhydrolyzable ATP analogue AMPPNP to a resolution of 1.9 Å (Supporting Information Table S1). The final structure was a functional dimer (Figures 4A), with electron densities for the cofactor AMPPNP and the ligand P-HoPan observed in both monomers (Figure 4B). Both the ligands were found to be positioned perpendicular to each other (Figure 4B), a reaction
geometry reminiscent of aminoacyl-tRNA (aa-tRNA) synthetases (Supporting Information Figure S3). This suggests that although the folds are evolutionarily diverse, the reaction mechanisms of both types of enzymes are very closely related. Importantly, the obtained structure is the first of any PPCS where all the active site loops as well as the N-terminal regions were completely ordered (Figure 4A,C,D). This allows for several important structural insights to be gained. Specifically, the conformation of the dimerization loop (loop1, residues A188−A201) was well-defined in the ternary complex of hPPCS (Figure 4A,C). This loop crosses over to the other monomer and completes the active site of the adjacent subunit, acting as a lid that covers the solvent accessible nucleotide binding pocket (Figure 4A). The active site pocket that harbors ATP and the other substrates is sandwiched between helices α7 and α2 and by strand β7 (topology numbering is in accordance with apo hPPCS, Figure 4D). Like the bacterial and yeast PPCSs, one side of the nucleotide pocket is composed of mostly charged residues (contributed by both the subunits) that stabilize the triphosphate group of AMPPNP, while the other side adopts a shape that complements its adenine moiety (Figure 4D).

Figure 3. P-HoPan inhibits human and fruit fly PPCS enzymes. (A) Mechanism of eukaryotic PPCS enzymes, showing formation of the 4′-phosphopantothenoyl adenylate (P-Pan-AMP, $8$) intermediate from P-Pan in the first step with concomitant release of pyrophosphate (PPi) and the reaction of the intermediate with $\gamma$-cysteine and release of AMP in the second step to form the product P-PanCys. The possible inhibition of PPCS by P-HoPan was studied by tracking the rate of PPi release using an assay that couples its formation to the oxidation of NADH. (B) On the left, progress curves for hPPCS with 150 μM P-Pan, 1 mM γ-cysteine, and 1 mM ATP and increasing concentrations of P-HoPan (concentrations in μM indicated on the right of each curve). Symbols indicate the average of duplicate readings, and the error bars show range/2. On the right, fractional activity ($v_i/v_0$) of hPPCS at each P-HoPan concentration is calculated from the progress curves, allowing for an IC50 value to be determined. The IC50 value was converted to the indicated Ki value using the Cheng-Prusoff equation for competitive inhibitors (eq 4). The Ki value is the average from two independent experiments, the error is the range/2. (C) As for B, but with dPPCS.
P-HoPan Binding Leads to a Nonreactive Complex That Traps hPPCS in a “Precatalytic” State. The highly ordered nature of the hPPCS−P-HoPan−AMPPNP ternary complex suggested that P-HoPan interacts with the enzyme differently than its substrate, P-Pan. Analyzing the P-HoPan binding mode, we find that the phosphate and hydroxyl groups of P-HoPan are stabilized by the residues S61, S62, G63, R65, and N258 which is present on a loop near the N-terminus between α2 and β7 (Figure 4D). P-HoPan is also covered by a flexible flap (ligand capping loop) that creates a hydrophobic core for the stabilization of the 4′-phosphopantoyl moiety. This flap is comprised of seven residues (259–265) that were found to be disordered in the apo state, only achieving a well-defined conformation upon P-HoPan binding to hPPCS (Figure 4D, Figure 5A). While this flap is a common feature in all PPCS enzymes, it is longer (13 residues) in prokaryotes than in eukaryotes, forming a more compact pocket in the latter (Figure 5B). Importantly, P-HoPan’s carboxylate group, which is the potential site of reaction, is anchored by a hydrogen bonding interaction with the backbone amide of both A180 and V181. These residues form part of the “AAV(A/S)D” motif present at the C-terminus of β6, which is conserved in all PPCS enzymes (Figure 5C). This anchoring interaction is the key to explaining why P-HoPan acts as an inhibitor rather than a substrate of PPCS.
Comparison of the ternary complex of hPPCS with the structures of the yeast PPCS homologue Cab2 with P-Pan as well with its intermediates bound shows that, with respect to the β- and γ-phosphates of the nucleotide, P-Pan’s carboxylate is positioned in an orientation that is ideal for nucleophilic attack at the α-phosphate, which will allow formation of the P-Pan-AMP intermediate (Figure 5 D,E). In contrast, the carboxylate group of P-HoPan, due to the presence of the extra −CH2− group and the anchoring interaction, is positioned in an opposite conformation (Figure 5 D,E).

Consequently, the P-HoPan carboxylate cannot perform the nucleophilic attack on the α-phosphate of ATP to form the analogous P-HoPan-AMP intermediate; such an attack becomes conformationally too difficult to achieve within the constraints of the enzyme’s active site architecture. Consequently, a stable nonreactive complex is formed, trapping the enzyme in a “precatalytic” state and leading to the observed inhibition of PPCS activity. P-HoPan therefore inhibits PPCS by a new mechanism that may hold potential for the discovery of inhibitors of other therapeutically relevant ligases.

Figure 5. Comparison of the hPPCS ternary complex with E. coli PPCS and Cab2 structures reveals the inhibition mechanism of P-HoPan. (A) Electron density of the ordered flexible flap (residues 259–265) involved in capping the ligand generated from the mFo−DFc map contoured at 2.5 σ. (B) Comparison of the substrate-binding site in hPPCS (pink), E. coli PPCS (ecPPCS; gray), and Cab2 (green). In the case of ecPPCS, the loop is longer (from residue D354 to N367) than in hPPCS and Cab2. P-HoPan, the P-Pan-CMP intermediate, and P-Pan are shown in yellow, gray, and green ball and stick view. (C) Sequence logo representation of an alignment of selected PPCS sequences (human, mouse, Arabidopsis thaliana, D. melanogaster, Oryza sativa, S. cerevisiae i.e Cab2), Mycobacterium tuberculosis, Streptococcus pneumoniae, S. aureus, and E. coli); only a portion of the sequence is shown to highlight the consensus motifs and conserved residues—including the “AAV(A/S)D” motif associated with nucleotide binding and proposed to be involved in binding of cysteine (red box). The overall height of the letter stack indicates the sequence conservation at that position, while the height of each letter within the stack indicates the relative frequency of each amino acid at that position. Numbering according to the hPPCS sequence. Gaps in the alignment, where present, were omitted for clarity. Figure prepared using Weblogo.23 (D) Overlay of AMPPNP and P-HoPan in the hPPCS structure (this study) with P-Pan-CMP intermediate (dark gray ball and stick structure) from ecPPCS (PDB: 1U7Z).25 (E) Overlay of AMPPNP and P-HoPan in the hPPCS structure (this study) with P-Pan (cyan ball and stick structure) from the Cab2 structure (PDB: 6AI9).27 The different conformations of the P-Pan and P-HoPan carboxylate groups is highlighted by the dashed line ellipse. (F) The cysteine binding site was modeled in the ternary hPPCS complex using AutoDock 4.2. The P-Pan-CMP intermediate was probed into the active site using the coordinates of the structure of Cab2 with the intermediate bound (PDB: 6AIK).27 The intermediate and cysteine are shown in gray and yellow ball and stick view, respectively. The residues shown in pink sticks are from one monomer, while those shown in blue are from the neighboring monomer’s dimerization loop.
Figure 6. HoPan targets PPCS in D. melanogaster Schneider’s S2 cells. (A) Relative cell counts in cultured Drosophila S2 cells treated with control dsRNA (directed against hMAZ, a nonrelevant human gene) or with dsRNA against dPANK/Fbl, and subsequent rescue through the addition of 100 μM P-PantSH or P-Pan (300 μM, 200 μM, or 100 μM). (B) Effect on histone acetylation (relative to tubulin) in the samples shown in A. (C) Relative cell counts in untreated control cells or cells treated with 1 mM HoPan, and the subsequent rescue of the effects of HoPan treatment by addition of either 100 μM P-PantSH or 300 μM P-Pan. (D) Effect on histone acetylation (relative to tubulin) in the samples shown in C. (E) Relative cell counts of S2 cells overexpressing a control construct (GFP), or overexpressing either dPANK/Fbl or dPPCS, and the e addition of either 100 μM P-PantSH or 300 μM P-Pan. (F) Effect on histone acetylation (relative to tubulin) in the cells under the indicated conditions as shown in E. Data represent mean ± s.d. (n ≥ 3). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, two-tailed unpaired Student’s t test.

P-HoPan Bound Structure Provides Insight into Cysteine Binding and the PPCS Catalytic Mechanism and Allows for Mapping of Deleterious Mutations. The ternary complex of hPPCS allowed us to model the cysteine binding site, an important prerequisite for proposing a catalytic mechanism of this enzyme. Previous reports have stressed that the enzyme uses a ping-pong mechanism to catalyze the conversion of P-Pan into P-PanCys; i.e., cysteine enters the active site only after formation of the acyl-cytidylate/adenylate intermediate.26,28 However, the exact location of the cysteine binding site remained elusive.

To delineate the cysteine site, we overlaid the coordinates of the P-Pan-CMP bound Cab2 structure on those of the hPPCS ternary complex. This allowed for the intermediate-bound state of hPPCS to be recreated. When cysteine was docked using this structure as a template, its conformation was predominantly stabilized by residues inside a pocket that is lined by the conserved "AAV(A/S)D" motif (Figure 5C,F). The stabilizing interactions included hydrogen bonding interactions with the conserved residues T41 (at β1-strand), A178 and A179 (at a hydrophobic loop), and Y155 (at helix α5). On the basis of previously performed mutagenesis and HPLC-based assays on E. coli PPCS, it has been proposed that residues A178 and A179 of the conserved "AAV(A/S)D" motif (Figure 5C) participate in the final step of the reaction, i.e., the attack of cysteine on the reaction intermediate to form PPC.28 Moreover, the –NH₂ group of the modeled cysteine is properly positioned for this nucleophilic attack, pointing directly toward the carbonyl group of the intermediate (Figure S5F). We therefore believe that the modeled conformation of cysteine is a true representation of its occupancy inside the pocket. However, our model is in contrast to the mechanism proposed for Cab2, which suggests that the cysteine binds inside the pocket via interaction with K195 and K231 (K226 and K281 in Cab2 PPCS).27 We believe that such a conformation is not conducive to formation of the amide bond as an attack of the amine on the carbonyl group from the front side would be sterically unfavorable (Figure S5F). On the basis of the proposed cysteine binding site, we developed a proposal for the PPCS catalytic mechanism that is supported by the currently available structure data (Supporting Information Figure S4).

Finally, the obtained hPPCS ternary structure also allows for the mapping of two mutations and a deletion (A180P, E233V, and A107−111) in hPPCS that have been linked to severe dilated cardiomyopathy.29 Of these, the A180P and E233V mutations were categorized as highly deleterious, but the previously available apo structure of hPPCS (PDB: 1P9O) with its largely disordered active site did not provide insight into the functional relevance of these mutants. A description of the location and functional implication of these mutants is provided in the Supporting Information, and in Supporting Information Figure S5.

Validating HoPan’s Proposed Molecular Mechanism of Action in S2 Insect Cells. We next turned to validating the molecular MOA revealed for HoPan in a whole cell model. This was done using D. melanogaster Schneider’s cells (S2 cells), as these have been used extensively for studying CoA levels and their relationship to neurodegenerative phenotypes,
and since the levels of dPANK/Fbl and dPPCS can be modulated in these cells using RNA interference (RNAi)-mediated down-regulation, or by overexpression.\textsuperscript{3,4,5,13,15,33--35} Importantly, the S2 cells are also sensitive to HoPan treatment.

The conclusion drawn from the \textit{in vitro} data—that P-HoPan inhibits PPCS—predicts that P-PantSH will rescue a HoPan induced phenotype but that P-Pan (downstream from dPANK/Fbl but upstream from PPCS) will not (Figure 1). First, we determined whether P-Pan is taken up by S2 cells. RNAi was used to generate dPANK/Fbl depleted S2 cells. Consistent with previous reports,\textsuperscript{14,15,34} dPANK/Fbl-depleted cells show a decreased cell count and decreased histone-acetylation levels (Figures 6A and 6B and Supplementary Figure S6). This phenotype is rescued by the addition of either P-PantSH\textsuperscript{15} or P-Pan (Figure 6A), indicating that both compounds enter S2 cells to enable continuation of CoA biosynthesis in the absence of dPANK activity.

Next, S2 cells were treated with 1 mM HoPan, which is known to reduce cell count and histone acetylation.\textsuperscript{14,15} While addition of 100 µM P-PantSH nearly completely reversed the impact of HoPan inhibition on cell count (Figure 6C) and histone acetylation (Figure 6D, Supplementary Figure S6), P-Pan was not able to show significant rescue as it did in the RNAi experiment, with only a small effect at a high concentration (300 µM). These results are consistent with an inhibitory action of P-HoPan on PPCS. Moreover, overexpression of dPANK/Fbl also did not result in any significant rescue of the cell count (Figure 6E) and histone acetylation (Figure 6F, Supporting Information Figure S6) phenotypes in S2 cells treated with increasing concentrations (up to 2.0 mM) of HoPan. In contrast, overexpression of dPPCS did show some rescue of the cell count (Figure 6E), and histone acetylation was significantly improved compared to the control (GFP overexpression; Figure 6F, Supporting Information Figure S6). Taken together, these results support the conclusion that the molecular target of HoPan is PPCS and not PanK as has previously been asserted.

**CONCLUSIONS**

The pantothenate analogue HoPan is a well-known modulator of CoA levels in various systems and disease models that was thought to act by inhibiting PanK, the first enzyme of CoA biosynthesis. This study shows that PanK in fact serves as a metabolic activator of HoPan, phosphorylating it and turning it into an inhibitor of the next CoA biosynthetic enzyme, PPCS. This molecular MOA explains why PanK was previously considered to be HoPan’s target: by acting as an alternative substrate of PanK, it competes with Pan (the enzyme’s natural substrate) for PanK-mediated phosphoryl transfer, thereby effectively reducing the rate of P-Pan formation. In this manner, the negative impact on CoA biosynthesis is compounded: first, by reducing the rate of P-Pan formation (albeit transiently, i.e., as long as unphosphorylated HoPan is present) and, second, by inhibiting PPCS. It is likely that the HoPan-induced phenotypes are a result of this combinatorial inhibition of CoA biosynthesis, as the determined K\textsubscript{c} values for P-HoPan’s inhibition of PPCS alone is relatively modest.

Due to its dependence on PanK for metabolic activation, and its competition with Pan for this transformation, HoPan’s effective potency is expected to be highly dependent on the amount of Pan present in the environment. Indeed, the conflicting reports on HoPan’s toxicity in various animals and humans can be reconciled with a knowledge of the circulating levels of Pan in each case, and/or whether Pan was withheld from or added to the diet.\textsuperscript{12,13,36,37} Consequently, contrary to the conclusion of some of these early studies that HoPan induces a Pan deficiency, it is more likely that an underlying Pan deficiency resulted in an apparent increase in HoPan’s potency. One could therefore reasonably conclude that HoPan may be a potent inhibitor under conditions where Pan availability is limited. For example, plants rely on their own \textit{de novo} biosynthesis of Pan, and HoPan treatment of cucumber, tomato, rice, and maize was found to suppress root and shoot growth and, at higher concentrations, also decreased seed germination.\textsuperscript{38} On the other hand, as Pan availability does not limit CoA synthesis in the heart, one would predict that HoPan would not show the same cardiac effects as those associated with the PPCS mutations in humans.\textsuperscript{39}

Our results show that the inhibitory potential of HoPan—and any other inhibitor with a similar MOA—can be predicted by considering four factors: first, the potency of the metabolically activated inhibitor acting on PPCS \textit{in vitro}; second, the concentration levels of Pan in the environment where inhibition is to occur; third, the specific activity of the PanK enzyme toward the inhibitor compared to Pan; and fourth, the availability of mechanisms to bypass PPCS inhibition, e.g., a functional CoA salvage pathway (Figure 1). These factors and their interplay provide a framework for directing the rational development of inhibitors and modulators of CoA biosynthesis. This will be aided by the costructure obtained in this study that uncovered previously unknown details about the mechanism of PPCS enzymes—including important insights into its elusive cysteine binding mechanism.

Taken together, the molecular MOA elucidated for HoPan here indicates that the underlying basis for the phenotypes it induces is more complex than originally thought. Moreover, our study significantly advances our understanding of small molecule inhibitors of CoA biosynthesis, of the diversity in the mechanisms by which they exert an effect on the pathway, and of the mechanistic and kinetic characteristics of PanK and PPCS—two CoA pathway enzymes that are associated with human genetic diseases and that are also targeted for the development of new antimicrobial drugs. These insights are likely to prove highly valuable in our search for new therapies in these areas.

**METHODS**

**General Materials and Methods.** Unless otherwise stated, all chemicals and reagents were from Merck-Millipore/Sigma-Aldrich. All NMR analyses were performed using 300 MHz Varian VNMRs, 400 MHz Varian Unity Inova, or 600 MHz Varian Unity Inova instruments at the Central Analytical Facility (CAF) of the University of Stellenbosch. Chemical shifts (δ) are given in parts per million, with the abbreviations indicating multiplicity (s = singlet, t = triplet, q = quartet, m = multiplet), the coupling constant (J) in Hz, and integration of the observed signals. All restriction enzymes were purchased from New England Biolabs or Thermo Scientific (FastDigest).

**P-HoPan Synthesis.** Phospho-hopantenate (P-HoPan, 2) was synthesized by adaptation of the method used to synthesise P-Pan (4).\textsuperscript{30} Sodium \textgamma-aminobutyrate (694 mg, 4.85 mmol) was combined with pantolactone (694 mg, 5.33 mmol) and heated overnight at 140°C before being lyophilized. The reaction mixture was subsequently dissolved in 5 mL of distilled water passed through an Amberlite (IR120 H\textsuperscript{+} form) column (0.6 g). The collected product solution was dissolved in 5 mL of distilled water passed through an Amberlite (IR120 H\textsuperscript{+} form) column (0.6 g). The collected product solution was dissolved in 5 mL of distilled water passed through an Amberlite (IR120 H\textsuperscript{+} form) column (0.6 g). The collected product solution was dissolved in 5 mL of distilled water.
methanol/acetonitrile/distilled water 5:2:1:1) to give HoPan as a yellow oil (888 mg, 3.8 mmol, yield 78%). HoPan was combined with an equivalent of NaOH (1 M solution) and dried by lyophilization before being dissolved in 15 mL of dry N,N-dimethylformamide (DMF) and esterified with benzyl bromide (471 µL, 3.97 mmol) by heating the mixture at 70 °C overnight while stirring. The solvent volume was reduced in vacuo and the precipitate was dissolved in 200 mL of ethyl acetate, followed by washing of the organic layer with saturated aqueous NaCl (3 × 20 mL). The organic phase was dried over anhydrous Na2SO4 and filtered while the solvent removed in vacuo. The product was purified by flash column chromatography (Merck silica gel 60, particle size 0.040–0.063 mm; ethyl acetate/hexane 4:1) to give HoPan benzyl ester dibenzyl ether column chromatography (Merck silica gel 60, particle size 0.040–0.063 mm; ethyl acetate/hexane 3:1 to 5:1), yielding the intermediate (HoPan benzyl ester) as a clear oil (311 mg, 0.96 mmol, yield 25%). 1H NMR (300 MHz, CDCl3): δ 0.91 (s, 3H), 1.02 (s, 3H), 1.84–1.94 (m, 2H), 2.43 (t, J = 7.2 Hz, 2H), 3.30–3.38 (m, 2H), 3.50 (q, J = 16.3 Hz, 2H), 4.00 (s, 1H), 5.12 (s, 2H), and 7.36 (arom, 5H). Dibenzyloxycarbonate was subsequently prepared by stirring dibenzyl phosphite (756 mg, 2.9 mmol) and N-chlorosuccinimide (385 mg, 2.9 mmol) in dry toluene (10 mL), followed by addition of a mixture of methanol and water (10 mL), followed by the addition of an equivalent of NaOH (1 M solution) and dried by lyophilization from S2 cDNA and inserting them in the plasmid pAc 5.1 (Invitrogen) with RI and XbaI or EcoRI and KpnI. Plasmid pAc GFP was created by amplifying the GFP-encoding gene by PCR from pEGFP-C1 (Clontech) and inserting it in pAc 5.1 with Agel and BstBI.

Expression and Purification of Enzymes for Kinetic Assays. hPPCS was expressed and purified as previously reported. 26, 41 hPANK3, dPANK/FblD, and dPPCS were expressed in E. coli BL21(DE3), while hPANK1β was expressed in E. coli C41(DE3) + pLySS. A starter culture was made by adding a single colony from plate of the expression host transformed with the required plasmid and grown overnight at 37 °C. For batch expression, 500 mL of LB media was inoculated with 5 mL of starter culture and grown at 37 °C while shaking at 180 rpm. When the required density was reached (OD600 of 0.6 for dPPCS and 0.9 for the other enzymes), expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). For dPPCS, 0.1 mM IPTG was used and expression was allowed to occur overnight at 37 °C. For hPANK1β, hPANK3, and dPANK/FblD, mM IPTG was used, followed by overnight expression at 18 °C. Cultures were collected by centrifugation, and pellets were stored at −20 °C until purification. Pellets were resuspended and sonicated on ice in 25 mL of sonication buffer (25 mM Tris-HCl pH 7.9, 300 mM NaCl, 10 mM imidazole, 0.033% Brij-35, 5% glycerol) containing 2 mM PMSE, half a protease inhibitor cocktail tablet (COMplete EDTA free, Roche), and 17.5 µL of 2-mercaptoethanol. After sonication, the samples were centrifuged for 20 min at 75 600g, and the lysates were subjected to immobilized nickel affinity chromatography on an AKTAprime protein purification system. Non-specific proteins bound to the resin were washed with 75 mM imidazole in binding buffer (25 mM Tris-HCl at pH 7.9, 300 mM NaCl, and 5 mM imidazole) followed by elution of the target protein with 500 mM imidazole in binding buffer. Fractions containing the target protein were pooled and subjected to buffer exchange, replacing the elution buffer with storage buffer (20 mM Tris-HCl at pH 8.0, 5 mM MgCl2). Purified protein fractions were aliquoted, flash frozen in liquid nitrogen, and stored at −80 °C. The concentration of each enzyme was determined with the Bradford method and a BSA standard using the Rapid protein quantification kit from Bio-Rad. The purity of the enzymes was confirmed by 12% SDS-PAGE analysis.

PanK Assay and Data Analysis. PanK activity was determined using a previously described continuous PK/LDH enzyme-coupled spectrophotometric assay 37 that couples the production of ADP to the oxidation of NADH. An extinction coefficient of 6220 M−1 cm−1 was used for NADH. All assays with hPanK1β and hPANK3 were performed at 25 °C in clear, flat-bottom, 96-well plates (Greiner Bio-One), with each reaction performed in triplicate at a final volume of 300 µL. Each 300 µL reaction mixture contained 50 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 20 mM KCl, 0.5 mM NADH, 2 mM phosphoenolpyruvate, 1.5 mM ATP, 0.01 U µL−1 PK, 0.01 U µL−1 LDH, and 16.7 ng µL−1 PanK. The reactions were initiated by the addition of Pan (3.125–200 µM) and were monitored for 5 min at 6 s intervals by a Versioscan multimode plate reader (Thermo Scientific). When PanSH was tested as a substrate, 0.2 mM of tris(2-carboxyethyl)phosphine (TCEP) was added to the reaction mixture to reduce the disulfide. For dPANK/FblD, initial assays were performed under the same conditions as for the human PanKs, except at a Pan concentration range of 6.25–400 µM and at a final reaction volume of 150 µL. Due to the low expression yield of this enzyme, subsequent assays were performed in clear, flat bottom, 384-well plates (Greiner Bio-One) using a total reaction volume of 50 µL on a Versioscan Flash multimode plate reader (Thermo Scientific). For each assay, kinetic profiles were obtained by determining initial rates.
at each substrate concentration in triplicate. The kinetic parameters of interest were determined by fitting either the Michaelis–Menten equation (eq 1) or the Hill equation (eq 2; see Table 1) using SigmaPlot 14.0 (Systat Software, Inc.) to the data. The kinetic parameters reported in Table 1 are the average of those determined for the indicated number of experiments, each performed with a different batch of enzymes.

\[
\frac{v}{v_0} = \frac{V_{\text{max}} [S]}{K_{\text{m}} + [S]}
\]

(eq 1)

\[
\frac{v}{v_0} = \frac{V_{\text{max}}^b [S]^b}{K_{\text{m}}^b + [S]^b}
\]

(eq 2)

**PPCS Assay and Data Analysis.** PPCS assays were performed as previously described using a continuous enzyme coupled assay kit (pyrophosphate reagent, Merck-Millipore/Sigma-Aldrich cat. # P7275) that couples the formation of pyrophosphate by PPCS to the oxidation of NADH. An extinction coefficient of 6220 M⁻¹ cm⁻¹ was used for NADH. Assays were performed at 37 °C in clear, flat bottom, 96-well plates (Greiner Bio-One), with each reaction performed in duplicate at a final volume of 100 µL. Each 100 µL reaction contained PPCS enzyme (200 nM hPPCS or 100 nM dPPCS respectively), 1 mM ATP, 1 mM L-cysteine, 2.5 mM TCEP, 50 mM Tris-HCl (pH 7.6), 20 mM KCl, 10 mM MgCl₂, and 40 µL of pyrophosphate reagent (each vial was dissolved in 4.5 mL distilled H₂O). Reactions were initiated with 150 µM P-Pan and P-HoPan (at a range of 12.5–400 µM), and the oxidation of NADH was continuously measured with a Multiskan Sky microplate spectrophotometer (Thermo Scientific) at 340 nm at 10 s intervals over 15 min. Pyrophosphate formation was plotted as progress curves, and the fractional activities for each duplicate reaction were calculated. These fractional activities were plotted, and an IC₅₀ was calculated in SigmaPlot 14.0 (Systat Software, Inc.) using eq 3. Each IC₅₀ value was converted to a Kᵣ value, using the Cheng-Prusoff equation for competitive inhibitors (eq 4). For each enzyme, two Kᵣ values were determined from two independent experiments using two different enzyme batches; the reported value is the average Kᵣ value that was obtained.

\[
\frac{v}{v_0} = \frac{1}{1 + \left(\frac{IC_{50}}{K_{r}}\right)}
\]

(eq 3)

\[
K_{r} = \frac{IC_{50}}{1 + \left(\frac{S}{K_{s}}\right)}
\]

(eq 4)

**hPPCS Purification for Crystallization.** The hPPCS protein used for crystallization and structure determination was obtained using previously reported methods. In brief, the starter culture was made by adding a single colony from transformed plate, grown overnight at 37 °C, in 10 mL of LB media containing 50 mg mL⁻¹ of ampicillin. Five liters of LB media was inoculated with 50 mL of saturated starter culture and incubated at 37 °C. When the culture reached an OD of approximately 0.6, the temperature was lowered to 16 °C, and cells were induced with 0.1 mM IPTG for 16 h. Cells were harvested by centrifugation at 4500 rpm for 20 min, and the cell pellet was resuspended in 30 mL of ice-cold lysis buffer (50 mM Tris-HCl at pH 8.0, 500 mM NaCl, 5 mM imidazole). Cells were lysed by sonication, and cell debris was removed by centrifugation at 18 500 g for 45 min. The supernatant of the protein solution was mixed with 500 µL of a slurry of Ni-nitriotriacetic acid resin (Qiagen) pre-equilibrated in lysis buffer, followed by incubation for 2 h. The supernatant was separated by centrifugation at 250g for 25 min. Nonspecifically bound proteins were removed by extensive washing with wash buffer (50 mM Tris-HCl at pH 8.0, 500 mM NaCl, 30 mM imidazole). The His-tagged protein was eluted with the elution buffer (50 mM Tris-HCl at pH 8.0, 500 mM NaCl) using a gradient of imidazole from 60 to 300 mM. The purity of the protein in the collected fractions was verified by 15% SDS-PAGE analysis. Fractions containing pure protein were pooled, desalted in dialysis buffer (50 mM Tris-HCl at pH 8.0), and concentrated to a final concentration of 500 µM.

**Crystallization, Data Collection, and Refinement.** The initial crystallization condition for the protein was determined from the matrix screen provided by Hampton research (crystal screen 1 and crystal screen 2). 300 µM protein was preincubated with 5 mM AMP-PNP, 5 mM P-HoPan, and 10 mM MgCl₂. Crystals were grown at 18 °C by hanging drop vapor diffusion method. Diffraction quality crystals grew within 4 weeks in 0.2 M lithium sulfate monohydrate, 0.1 M Tris HCl, and 30% PEG 4000 in a drop size of 6 µL, having a 2:1 ratio of protein mixture and reservoir solution, respectively. A single wavelength data set was collected at the beamline ID23 at the European synchrotron radiation facility (ESRF), Grenoble, France. Before data collections, the crystal was incubated in the cryo-protectant by adding 20% glycerol in reservoir solution to prevent crystal damage from continuous X-ray exposure. A total of 1600 frames were collected with 0.5 s exposure time using a PILATUS detector. Frames were indexed, integrated, and scaled at 1.9 Å resolution in the C2 space group using XDS software. Phases were generated, and the structure was determined by performing molecular replacement in Phaser in the CCP4 suite against the published structure of apo hPPCS (PDB: 1PPO). Structure models were subjected to rigid body refinement and further refined using several cycles in Refmac in the CCP4 suite. Manual model building was performed with WinCoot. Crystallographic data statistics are reported in Table 1 of the Supporting Information. The structure coordinates are available from the PDB with ID 7EDZ.

**Docking of Cysteine Binding Pocket in hPPCS.** Docking studies were undertaken to model the cysteine binding site. The dimeric crystal structure of the ternary complex of hPPCS with P-HoPan and AMP-PNP was used as a template. In line with previous proposals, the cysteine enters the active pocket only after cytidylate or adenylate intermediate formation. Therefore, to mimic the intermediate state of hPPCS, the coordinates of intermediate from the P-Pan-CMP bound Cab2 crystal structure (PDB: 6AIK) were probed into the active site of hPPCS. Using this intermediate-bound hPPCS structure as a base, the cysteine binding site was docked using AutoDock 2.0 software, employing a Lamarckian genetic algorithm and free energy scoring function. AutoDock calculation includes the following steps: (1) coordinate file preparation, (2) precalculation of atomic affinities using AutoGrid, (3) docking of ligand, and (4) analysis of results. The grid box was selected in the active site formed by the two monomers, and rigid docking was carried out using 200 runs. Run 139 with the cluster rank 2 (having a maximum, i.e., 118 conformations in the cluster) and lowest binding energy of −4.19 kcal/mol was chosen as the model.

**Drosophila S2 Cell Culture, RNA Interference, and P-Pan and P-PantSH Treatment.** Drosophila Schneider’s S2 cells were maintained at 25 °C in Schneider’s Drosophila medium (Invitrogen) supplemented with heat-inactivated 10% fetal calf serum (FCS, Gibco) and antibiotics (penicillin–streptomycin; Invitrogen) under laboratory conditions. Cells in the exponential phase of growth were used for all the experiments. Synthesis of double-stranded RNA (dsRNA) and RNAi treatment were carried out as described previously. Nonrelevant (human gene; hMAZ) dsRNA was used as a control. After incubating the cells for 3 days with dsRNA to induce efficient knockdown, cells were subcultured with or without P-Pan (varying concentrations) or 100 µM P-PantSH (Syncom, >94%) and maintained for an additional 4 days until analysis for rescue efficiency of the compounds. The stock solutions of compounds were made in sterile water and stored at −20 °C until use.

**HoPan Treatment of Drosophila S2 Cells in Combination with P-Pan and P-PantSH Treatment.** Drosophila Schneider’s S2 cells were maintained under standard conditions as described above. Different concentrations of P-Pan or 100 µM P-PantSH were added to S2 cells in either the presence or the absence of 1 mM HoPan (Zhou Fang Pharm Chemical; 99%) for 48 h. As readouts, analysis of cell count and histone acetylation was performed as explained below.
orcid.org/orcid.org/

HoPan Treatment of Drosophila S2 Cells Overexpressing Fbl or PPCS. Drosophila Schneider’s S2 cells were maintained under standard conditions as described above. S2cs were seeded in Schneider’s Drosophila medium supplemented with heat-inactivated 10% dialyzed serum (Fisher Scientific), and antibiotics (penicillin-streptomycin), transfected with the indicated plasmids using Effectene (Qiagen) according to the protocol of the manufacturer. After 24 h, HoPan was added in the different concentrations and incubated for another 48 h. Cells were counted and analyzed for histone acetylation as described below.

Western Blotting Analysis and Antibodies. For Western blotting analysis, cells were collected, washed with PBS, and centrifuged. The cells were lysed and sonicated in 1× Laemml sample buffer and boiled for 5 min with 5% β-mercaptoethanol. Protein content was determined via DC protein assay (Bio-Rad). Equal amounts of protein were loaded on 10% or 12.5% mini protein TGX stain-free gels (Bio-Rad), transferred onto PVDF membranes using the Trans Blot Turbo System (Bio-Rad). Blocked with 5% fat-free milk in PBS + 0.1% Tween for 1 h at RT, after which they were incubated with the primary antibodies at 4°C overnight with primary antibodies at 4°C. The primary antibodies used were rabbit anti-dPANK/Fbl (1:4000, Eurogentec, custom-made), rabbit anti-dPPCS (1:4000, Eurogentec, custom-made), mouse antiblutlinin (1:5000, Sigma-Aldrich, T5168), and anti-acetyl-histone (1:2000, Active Motif, 39139). Appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham) were used, and incubation occurred for 1.5 h at RT. Membranes were developed using ECL reagent (Thermo Fisher Scientific), and the signal was visualized using the ChemiDoc imager (Bio-Rad), with the signal intensities was performed using Image Studio Lite (Li-Cor).

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00535.

Supplementary Figures as referenced in the manuscript, supplementary table containing the crystallographic data statistics of the structure reported in this study, and supplementary Results and Discussion detailing how the hPPCS ternary structure allows for the mapping of mutations linked to severe dilated cardiomyopathy (PDF)

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