7,8-Dihydroxyflavone is a direct inhibitor of pyridoxal phosphatase

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ABSTRACT

Vitamin B6 deficiency has been linked to cognitive impairment in human brain disorders for decades. Still, the molecular mechanisms linking vitamin B6 to these pathologies remain poorly understood, and whether vitamin B6 supplementation improves cognition is unclear as well. Pyridoxal phosphatase (PDXP), an enzyme that controls levels of pyridoxal 5'-phosphate (PLP), the co-enzymatically active form of vitamin B6, may represent an alternative therapeutic entry point into vitamin B6-associated pathologies. However, pharmacological PDXP inhibitors to test this concept are lacking. We now identify a PDXP and age-dependent decline of PLP levels in the murine hippocampus that provides a rationale for the development of PDXP inhibitors. Using a combination of small molecule screening, protein crystallography and biolayer interferometry, we discover and analyze 7,8-dihydroxyflavone (7,8-DHF) as a direct and potent PDXP inhibitor. 7,8-DHF binds and reversibly inhibits PDXP with low micromolar affinity and sub-micromolar potency. In mouse hippocampal neurons, 7,8-DHF increases PLP in a PDXP-dependent manner. These findings validate PDXP as a druggable target. Of note, 7,8-DHF is a well-studied molecule in brain disorder models, although its mechanism of action is actively debated. Our discovery of 7,8-DHF as a PDXP inhibitor offers novel mechanistic insights into the controversy surrounding 7,8-DHF-mediated effects in the brain.
INTRODUCTION

Vitamin B6 is an essential micronutrient that plays an important role in the nervous system (1, 2), with the vitamin B6 status affecting cognitive function at any age (3, 4). Population studies indicate that low vitamin B6 levels are common among older people (5), and suggest that vitamin B6 deficiency may influence memory performance and may contribute to age-related cognitive decline (6-9). Vitamin B6 deficiency is also associated with other conditions characterized by impaired learning and memory, including neuropsychiatric disorders (10-12), Alzheimer’s disease (13) and inflammation (14, 15). Nevertheless, the exact molecular mechanisms linking vitamin B6 to these pathologies are often insufficiently understood, and whether vitamin B6 supplementation improves cognition is unclear (4, 5, 16-22).

The term vitamin B6 encompasses the enzymatically interconvertible compounds pyridoxine, pyridoxamine, pyridoxal (referred to as B6 vitamers) and their phosphorylated forms. Among these, only pyridoxal 5′-phosphate (PLP) is co-enzymatically active. In humans, PLP is known to be required for 44 distinct biochemical reactions, including the biosynthesis and/or metabolism of neurotransmitters, amino acids, lipids, and glucose. In addition, B6 vitamers display antioxidant and anti-inflammatory functions (23-26).

Cellular PLP availability in the brain depends on numerous factors, including the intestinal absorption of B6 vitamers, extracellular phosphatases, inter-organ transport and intracellular enzymes and carriers/scavengers involved in PLP formation and homeostasis (2). Specifically, intracellular PLP is formed by the pyridoxal kinase (PDXK)-catalyzed phosphorylation of pyridoxal, or the pyridox(am)ine-5′-phosphate oxidase (PNPO)-catalyzed oxidation of pyridox(am)ine 5′-phosphate to PLP. PLP is highly reactive and can undergo condensation reactions with e.g., primary amino groups or thiol groups in proteins or amino
acids. Although the mechanisms of PLP delivery within the cells are still largely unknown, it
is clear that the intracellular availability of PLP for co-enzymatic functions depends on PLP
 carriers/scavengers and on the hydrolytic activity of pyridoxal 5’-phosphate phosphatase
(PDXP) (2, 27-30).

We have previously shown that the genetic knockout of PDXP (PDXP-KO) in mice increases
brain PLP levels and improves spatial memory and learning, suggesting that elevated PLP
levels can improve cognitive functions in this model (30). We therefore reasoned that a
pharmacological inhibition of PDXP may be leveraged to increase intracellular PLP levels
and conducted a high-throughput screening campaign to identify small-molecule PDXP
modulators. Here, we report the discovery and the structural and cellular validation of 7,8-
dihydroxyflavone (7,8-DHF) as a selective PDXP inhibitor. 7,8-DHF is a well-studied
molecule in brain disorder models characterized by impaired cognition, and widely regarded
as a tropomyosin receptor kinase B (TrkB) agonist with brain-derived neurotrophic factor
(BDNF)-mimetic activity (31). However, a direct TrkB agonistic activity of 7,8-DHF has
been called into question (32-36). Our serendipitous discovery of 7,8-DHF as a direct PDXP
inhibitor provides an alternative mechanistic explanation for 7,8-DHF-mediated effects. More
potent, efficacious, and selective PDXP inhibitors may be useful future tools to explore a
possible benefit of elevated PLP levels in brain disorders.
RESULTS

PDXP activity controls PLP levels in the hippocampus

The hippocampus is important for age-dependent memory consolidation and learning, and impaired memory and learning is associated with PLP deficiency (3). To study a possible contribution of PDXP and/or PDXK to age-related PLP homeostasis in the hippocampus, we performed Western blot analyses in young versus older mice. Unexpectedly, we found that both PDXP and PDXK expression levels were markedly higher in hippocampi of middle-aged than of juvenile animals (Fig. 1a). These data suggest an accelerated hippocampal PLP turnover in older mice, consistent with previous findings in senescent mice (37).

An analysis of total hippocampal PLP levels in PDXP-WT and PDXP-KO mice showed an age-dependent profile. PLP levels appeared to peak around 3 months of age (possibly reflecting PLP-dependent neurotransmitter biosynthesis and metabolism during the postnatal developmental period) and descended back to juvenile levels by 12 months of age in both genotypes. Although total hippocampal PLP levels in PDXP-KO mice also decreased with age, they consistently remained above PLP levels in control mice (Fig. 1b; two-tailed, unpaired t-test of PLP levels in PDXP-WT vs. PDXP-KO hippocampi, all ages combined: p<0.0001).

PLP is protected from hydrolysis by binding to proteins, and PDXP is expected to dephosphorylate only non-protein-bound PLP (38). To test this, we prepared protein-depleted PLP fractions from PDXP-WT and PDXP-KO hippocampal lysates using 3 kDa molecular weight cutoff centrifugal filters. The quantification of PLP in these fractions demonstrated that PDXP loss indeed only increased the pool of protein-depleted PLP, both in young (18-42 days old) and older mice (252-352 days old, corresponding to mature/middle-aged mice), whereas the levels of protein-bound PLP remained unchanged (Fig. 1c). While the hippocampal levels of non-protein-bound PLP dropped by about 60% over this time span in PDXP-WT mice, they remained elevated in PDXP-KO mice ~2-fold higher in younger, and
~5-fold higher in older PDXP-KO compared to the respective PDXP-WT; see Fig. 1 – figure supplement 1 for exact mouse ages). We conclude that hippocampi of older mice are characterized by a specific decrease in the levels of non-protein-bound PLP, and that this age-dependent PLP loss is dependent on PDXP activity. These observations establish that PDXP is a critical determinant of PLP levels in the murine hippocampus and suggest that intracellular PLP deficiency may be alleviated by PDXP inhibition.

A high-throughput screening campaign identifies 7,8-dihydroxyflavone as a PDXP inhibitor

Pharmacological small-molecule PDXP inhibitors are currently lacking. To identify PDXP inhibitor candidates, we screened the FMP small molecule repository containing 41,182 compounds for molecules able to modulate the phosphatase activity of recombinant, highly purified murine PDXP (see Fig. 2 – figure supplement 1 for a schematic of the screening campaign). Difluoro-4-methylumbelliferyl phosphate (DiFMUP) was used as a fluorogenic phosphatase substrate in a primary screen. Compounds that altered DiFMUP fluorescence by ≥50% (activator candidates) or ≤25% (inhibitor candidates) were subjected to EC$_{50}$/IC$_{50}$ value determinations. Of these, 46 inhibitor hits were selected and counter-screened against phosphoglycolate phosphatase (PGP), the closest PDXP relative (39, 40). Fourteen of the PDXP inhibitor hits (with an IC$_{50}$ PDXP <20 µM, and IC$_{50}$ PDXP < IC$_{50}$ PGP or no activity against PGP) were subsequently validated in a secondary assay, using PLP as a physiological PDXP substrate (see Fig. 2 – figure supplement 2 for all 14 inhibitor hits). Only two PDXP-selective inhibitor hits (FMP-1 and 7,8-dihydroxyflavone/7,8-DHF, a naturally occurring flavone) blocked PDXP-catalyzed PLP dephosphorylation. FMP-1 (IC$_{50}$~1 µM) was obtained from an academic donor, and its structure is undisclosed for intellectual property reasons. Further characterization was therefore only possible for 7,8-DHF (IC$_{50}$~1 µM).
In vitro activity assays using PLP as a substrate confirmed that 7,8-DHF directly blocks murine and human PDXP activity with submicromolar potency and an apparent efficacy of ~50% (Fig. 2a, b). The efficacy of 7,8-DHF under these conditions may be limited by the poor solubility of this flavone (see also Fig. 2c). This interpretation is supported by Todd et al. who established a solubility profile of 7,8-DHF and demonstrated that only ~20% of the compound remains in solution beyond a concentration of 5 µM (35).

We next examined whether commercially available 7,8-DHF analogs might be more potent PDXP inhibitors. We tested flavone, 3,7-dihydroxyflavone, 5,7-dihydroxyflavone (also known as chrysin), 3,5,7-trihydroxyflavon (galangin), 5,6,7-trihydroxyflavone (baicalein) and 3,7,8-trihydroxyflavone-4’-hydroxyphenyl. Figure 2b shows that of the tested 7,8-DHF analogs, only 3,7,8-trihydroxyflavone-4’-hydroxyphenyl was able to inhibit PDXP, albeit with an IC50 of 2.5 µM and thus slightly less potently than 7,8-DHF. These results suggest that hydroxyl groups in positions 7 and 8 of the flavone scaffold are required for PDXP inhibition.

We used a biolayer interferometry (BLI) optical biosensing technique to further characterize the binding of 7,8-DHF to PDXP (Fig. 2c). Consistent with a specific interaction, 7,8-DHF binding to PDXP was concentration-dependent and fully reversible. Steady-state analysis of a 7,8-DHF serial dilution series yielded an affinity (K_D) value of 3.1 ± 0.3 µM (data are mean values ± S.E. of n=4 measurements; see Fig. 2 – figure supplement 3 for the three other measurements) using a 1:1 dose-response model. Global analysis of the sensorgrams assuming a 1:1 binding model resulted in an affinity of 2.6 ± 0.5 µM, in line with the steady-state results (Fig. 2c). Due to the aforementioned poor solubility of 7,8-DHF (35), concentrations higher than 12.5 µM could not be evaluated. As expected, 5,7-dihydroxyflavone showed no signal in the BLI, in line with previous experiments (see Fig. 2b). With its molecular size of 254 Da and its physicochemical properties, 7,8-DHF is a typical fragment-like molecule (41). Typical association rate constants (k_on) for fragments are
limited by the rate of diffusion and are higher than $10^6 \text{M}^{-1}\text{s}^{-1}$. Interestingly, 7,8-DHF showed a slow $k_{on}$ of 1050 M$^{-1}$s$^{-1}$, which is atypical and rarely found for fragment-like molecules (42), and a $k_{off}$ rate of 0.03 s$^{-1}$. With the commonly used estimation of $\Delta G \sim pK_D$ and a heavy atom number of 19, 7,8-DHF shows a high ligand efficiency of 0.39, which makes it an interesting molecule for further medicinal chemistry optimization. Taken together, these data support a direct and reversible physical interaction between 7,8-DHF and PDXP that leads to PDXP inhibition.

**Selectivity of 7,8-DHF**

PDXP is a member of the large family of haloacid dehalogenase (HAD)-type hydrolases (43). HAD phosphatases are Mg$^{2+}$-dependent phospho-aspartate transferases that consist of a Rossman-like catalytic core linked to a cap domain. The insertion site, structure and size of the cap define the substrate selectivity of the respective enzyme. The “capless” C0-type HAD phosphatases contain either a very small or no cap, resulting in an accessible catalytic cleft that enables the dephosphorylation of macromolecular substrates. Larger C1 or C2 caps act as a roof for the entrance to the active site; most C1/C2-capped HAD phosphatases consequently dephosphorylate small molecules that can gain access to the catalytic cleft. Cap domains also contain so-called substrate specificity loops that contribute to substrate coordination. Hence, caps are distinguishing features of HAD phosphatases (38, 43-45).

To probe the selectivity of 7,8-DHF for PDXP, a C2-capped HAD phosphatase, we tested five additional mammalian HAD phosphatases, including two other C2- and two C1-capped phosphatases and one C0-type enzyme. When assayed at a (nominal) concentration of 5, 10 and 40 µM (i.e., up to ~40-fold above the IC$_{50}$ value for PDXP-catalyzed PLP-dephosphorylation), 7,8-DHF was inactive against four of the tested enzymes. 7,8-DHF inhibited PGP, the closest PDXP relative, with an IC$_{50}$ value of 4.8 µM (Fig. 2d). This result is consistent with the criteria applied during the initial counter-screen (see above). Together,
our data show that 7,8-DHF selectively inhibits PDXP, and that higher 7,8-DHF concentrations can also target the PDXP paralog PGP.

**Mode of PDXP inhibition**

To probe the mechanism of PDXP inhibition, we assayed the steady state kinetics of PLP dephosphorylation in the presence of increasing 7,8-DHF concentrations (Table 1). Analysis of the derived kinetic constants demonstrated that 7,8-DHF increased the $K_M$ up to ~2-fold, and slightly reduced $v_{max}$ values ~0.7-fold. Thus, 7,8-DHF mainly exhibits a mixed mode of PDXP inhibition, which is predominantly competitive.

**Co-crystal structure of PDXP bound to 7,8-DHF**

To investigate the mechanism of PDXP inhibition in more detail, we co-crystallized homodimeric, full-length murine PDXP in complex with this compound. 7,8-DHF-bound PDXP crystallized in the cubic space group I23 with one dimer per asymmetric unit, with protomer A containing the inhibitor and protomer B representing an apo-state (Fig. 3a). The structure was refined following molecular replacement with full-length murine PDXP (here referred to as apo-PDXP; Protein Data Bank /PDB entry 4BX3) to a resolution of 2.0 Å resulting in an R$_{work}$ of 18.4% and an R$_{free}$ of 21.1% (PDB code 8QFW). Data collection and refinement statistics are summarized in Table 2.

Like apo-PDXP, 7,8-DHF-bound PDXP homodimerizes via its cap domain. The alignment of the structures representing apo-PDXP, the inhibitor-free protomer B, and the 7,8-DHF-bound PDXP protomer A resulted in root mean square deviations (RMSDs) of 0.42 Å (A vs. B in this structure), 0.35 Å (A vs. B in 4BX3), 0.41 Å (A in this structure vs. A in 4BX3, Fig. 3b), 0.49 Å (A in this structure vs. B in 4BX3), 0.45 Å (B in this structure vs. A in 4BX3) and 0.37 Å (B in this structure vs. B in 4BX3) based on C$_\alpha$ atoms, which indicate that binding of the inhibitor did not result in significant changes in the backbone...
conformations. In particular, we did not discern distinct open and closed active site
conformations (40) or a tilting of the substrate specificity loop (46) in 7,8-DHF-bound PDXP
compared to apo-PDXP. All catalytic core residues and the Mg\(^{2+}\) cofactor are correctly
oriented in the presence of the inhibitor. Hence, 7,8-DHF binding does not appear to impact
the overall fold or the open/closed dynamics of PDXP.

7,8-DHF was observed to only bind to one subunit (the A-chain; **Fig. 3a**) with well-
defined density (**Fig. 3c**) and full occupancy since its average B-factor of 45.8 \(\text{Å}^2\) closely
matches the B-factors of the surrounding atoms. Binding to the other subunit (B-protomer) is
prevented by a salt bridge between Arg62 and Asp14 of a symmetry-related A-protomer (**Fig.
3d**). The \(\chi_1\) and \(\chi_2\) torsion angles of the Arg62 side chain observed in the B-protomer
correspond to those observed for this side chain in both protomers of the apo-structure
(4BX3). To allow binding of the inhibitor, the side chain of Arg62 needs to adopt a
completely extended conformation, which is prevented by the salt bridge.

It is currently unclear whether the PDXP crystal state with only a single inhibitor
bound per dimer reflects the state in solution. Due to the limited solubility of 7,8-DHF, we
were unable to address the stoichiometry of 7,8-DHF binding to the PDXP dimer with
isothermal calorimetry. It is conceivable that the PDXP crystal packing is very stable (indeed,
7,8-DHF-bound PDXP crystallized in the same cubic space group as apo-PDXP, see ref. (46),
including the aforementioned salt bridge between Arg62 of the B-subunit and Asp14 of a
symmetry-related molecule), and that the free energy generated by the formation of the crystal
lattice is higher than the free energy generated upon inhibitor binding. Further inspection of
7,8-DHF-bound PDXP indicated that the crystallographic neighbors of protomer A and B are
dissimilar. While the 7,8-DHF binding site is readily accessible in protomer A, the
corresponding site in protomer B is shielded by two crystallographic neighbors (**Fig. 3 –
figure supplement 1**).
In protomer A, 7,8-DHF binds directly in the PDXP active site, with its phenyl ring at a distance of 8.3 Å from the Asp25 nucleophile, and 3.8 Å from Asp27, which functions as a general acid/base in the two partial reactions of the catalytic cycle (47). The introduction of PLP from PDB code 2CFT (human PDXP in complex with PLP) for visualization purposes revealed a partial overlap between the PLP and 7,8-DHF binding sites (Fig. 3e). While PLP is deeply buried in the catalytic cleft, the bulkier 7,8-DHF molecule obstructs the active site entrance with its 1,4-benzopyrone moiety and its phenyl ring protrudes into the catalytic cleft.

7,8-DHF is embedded in a cavity of the PDXP active site that is exclusively formed by the active site of protomer A, without a contribution of the dimerization interface with protomer B. One side of this cavity is formed by the more polar residues Asp27, Asn60, Ser61 and Arg62, whereas the opposite side is established by the more hydrophobic residues Tyr146, His178, Pro179, and Leu180. Adjacent to this hydrophobic stretch, the polar residue Glu148 is located at the active site entrance, directly opposite of Arg62 on the more polar side of the 7,8-DHF binding channel (Fig. 3f). Inhibitor binding appears to be primarily stabilized by two hydrogen bonds, as well as polar and non-polar interactions. The side chain hydroxyl group of Ser61 forms a direct hydrogen bond with the ketone group of the inhibitor, which is additionally coordinated by the Ser61 backbone nitrogen atom. Furthermore, Glu148 forms a direct hydrogen bond via its carboxylic acid with the 7-hydroxyl group of 7,8-DHF. The side chains of the polar residues Asp27, Asn60, and Arg62 engage in van der Waals interactions with 7,8-DHF. The two hydroxyl groups of the 7,8-DHF benzyl ring engage in van der Waals interactions with the guanidinium group of Arg62 and the carboxylic acid function of Glu148. On the more hydrophobic side of the binding cavity, Tyr146 forms π-electron stacking interactions with the pyrone ring of 7,8-DHF. In addition, the His178 imidazole group coordinates the 7,8-DHF phenyl ring via π-π stacking. His178, located in the substrate specificity loop, and Asn60 and Arg62 are also important for PLP binding (46, 48). All PDXP
residues found to engage in 7,8-DHF interactions are identical in murine and human PDXP (Fig. 3 – figure supplement 2).

To verify the putative 7,8-DHF – PDXP interactions, we introduced single mutations into the binding interface. Asn60, Arg62, Tyr146, Glu148 and His178 were each exchanged for Ala (PDXP$^{N60A}$, PDXP$^{R62A}$, PDXP$^{Y146A}$, PDXP$^{E148A}$, or PDXP$^{H178A}$, respectively). Since the carboxamide group of Asn60 can form a hydrogen bond with the carboxylate moiety of the Asp27, and a loss of this interaction in the PDXP$^{N60A}$ variant is predicted to alter the PDXP structure, we additionally mutated Asn60 to Ser (PDXP$^{N60S}$). PDXP variants were recombinantly expressed and purified from *E. coli* (see Fig. 3 – figure supplement 3 for protein purity). Figure 3g (left panel) shows that all PDXP variants were enzymatically active. As expected, the phosphatase activities of PDXP$^{N60A}$ and of PDXP$^{H178A}$ were reduced. The somewhat elevated phosphatase activity of PDXP$^{Y146A}$ and PDXP$^{E148A}$ is currently unexplained. Importantly, all variants except PDXP$^{N60A}$ and PDXP$^{N60S}$ were resistant to 7,8-DHF, supporting the essential role of each of these residues for inhibitor binding (Fig. 3g, right panel).

Based on the inhibitor-bound structure and the predominantly competitive component of PDXP inhibition by 7,8-DHF (increased $K_M$, see Table 1), it seems likely that 7,8-DHF sterically hinders substrate access to the active site, and competes with PLP coordination. In addition, BLI measurements (see Fig. 2c) showed a relatively slow association rate and extended residence time of 7,8-DHF ($\tau=30.3$ s). This may indicate a possible reorganization of the amino acids and water molecules within the binding site as a function of slow-onset 7,8-DHF binding. The reduced rate of product formation may account for the observed non-competitive component of 7,8-DHF-mediated PDXP inhibition (reduction of $v_{max}$, see Table 1).

7,8-DHF functions as a PDXP inhibitor in hippocampal neurons
To investigate cellular target engagement of 7,8-DHF, we isolated primary hippocampal neurons from PDXP-WT and PDXP-KO embryos. PDXP deficiency increased total PLP levels 2.4-fold compared to PDXP-WT neurons (**Fig. 4a**). This finding is in good agreement with the PLP increase resulting from PDXP loss in total hippocampal extracts (see **Fig. 1**). The larger absolute PLP values in cultured neurons are likely attributable to the high concentration of the PLP precursor pyridoxal (20 µM) in the culture medium. We did not observe PDXP-dependent changes in pyridoxal kinase (PDXK) expression (**Fig. 4b**) and could not detect pyridox(am)ine-5′-phosphate oxidase (PNPO) in hippocampal neuronal cultures, suggesting that the PLP increase was primarily caused by the constitutive PDXP loss.

To assess the consequences of 7,8-DHF treatment on PLP levels in hippocampal neurons, we chose short-term incubation conditions (45 min, 20 µM) to avoid possible secondary effects of the inhibitor. As expected, the acute effect of 7,8-DHF treatment in WT cells was much more subtle (~9% increase in total PLP) than the impact of long-term PDXP-deficiency (441.3 ± 62.6 nmol PLP/g protein in DMSO solvent control-treated cells versus 482.7 ± 130.4 nmol PLP/g protein in 7,8-DHF treated cells; data are mean values ± S.E. of \( n=4 \) independent experiments). However, this effect is likely underestimated because only the PDXP-accessible pool of non-protein-bound PLP may be impacted by 7,8-DHF (see **Fig. 1c**). Due to the limited number of available hippocampal neurons, we were unfortunately unable to obtain sufficient quantities of protein-depleted PLP pools to address this question.

Acute changes in the PLP/PL ratio may be a more sensitive indicator of PDXP activity than changes in total PLP levels alone, because PDXP inhibition is expected to increase cellular levels of PLP (the PDXP substrate) and to concomitantly decrease the levels of PL (the product of PDXP phosphatase activity). The PLP/PL ratio is also independent of the exact protein concentration in a given extract of hippocampal neurons, thus optimizing comparability between samples. As shown in **Fig. 4c**, 7,8-DHF significantly increased the...
PLP/PL ratio in PDXP-WT, but not in PDXP-KO hippocampal neurons (+18% versus +1% compared to the respective DMSO controls). Together, these data indicate that 7,8-DHF can modulate cellular PLP levels in a PDXP-dependent manner and validate PDXP as a 7,8-DHF target in primary hippocampal neurons.
PLP deficiency has been associated with human brain disorders for decades (3), yet causal links remain unclear. Aside from vitamin B6 administration, pharmacological strategies to elevate intracellular PLP levels are lacking. Here, we identify 7,8-dihydroxyflavone (7,8-DHF) as a direct PDXP inhibitor that increases PLP levels in hippocampal neurons, validating PDXP as a druggable target to control intracellular PLP levels in the brain. We also present a high-resolution 7,8-DHF/PDXP co-crystal structure that could facilitate the design of more potent, efficacious, and selective PDXP inhibitors in the future. Such molecules might improve the control of intracellular PLP levels and help to elucidate a possible contribution of PLP to the pathophysiology of brain disorders. Our observation that the expression of PDXP is substantially upregulated in hippocampi of middle-aged mice suggests that a therapeutic vitamin B6 supplementation alone may not suffice to elevate intracellular PLP levels under conditions where the PLP-degrading phosphatase is hyperactive.

The discovery of 7,8-DHF as a direct PDXP inhibitor was unexpected. Interestingly, numerous in vivo studies have reported the effectiveness of 7,8-DHF in brain disorder models, including rodent models of Alzheimer’s disease (49-56), depression (57-62), schizophrenia (63-67), or epilepsy (68, 69), as well as in rodent models of autism (70-73). Although PLP deficiency is thought to contribute to the respective human conditions (3, 74, 75), PLP-dependent processes have not yet been considered in the context of 7,8-DHF-induced effects.

7,8-DHF was initially discovered as a small-molecule TrkB agonist with BDNF-mimetic activity (76). BDNF, a high-affinity TrkB ligand, is an important neuropeptide for nervous system function and pathology. Consensus is emerging that BDNF plays a key role in the treatment response to neuropsychiatric drugs (32). Therapeutics that target BDNF/TrkB-signaling are thus of interest as disease-modifying agents in several brain disorders. Since
BDNF does not cross the blood-brain barrier, attempts have been made to develop small molecule BDNF mimetics. Several candidates have been reported, including 7,8-DHF (33, 76). Nevertheless, the on-target selectivity and efficacy of these compounds is actively debated. Using quantitative and direct assays to measure TrkB dimerization and activation, TrkB downstream signaling pathways, TrkB-dependent gene expression and cytoprotection, 7,8-DHF and other reported small-molecule TrkB agonists failed to activate TrkB in cells (33-35). An electrophysiological study in acute hippocampal slice preparations demonstrated that 7,8-DHF potentiates hippocampal mossy fiber-CA3 synaptic transmission in a TrkB receptor-independent manner (77). Overall, it appears that the mechanism of action of 7,8-DHF is incompletely understood, but 7,8-DHF targets other than TrkB so far have remained elusive. The identification of 7,8-DHF as a PDXP inhibitor reported here indicates that this flavone may modulate vitamin B6-dependent processes and suggests that PDXP could be explored as a pharmacological entry point into brain disorders.
MATERIALS AND METHODS

Materials

Unless otherwise specified, all reagents were of the highest available purity and purchased from Sigma-Aldrich (Schnelldorf, Germany). 3,7,8-Trihydroxyflavone-4'-hydroxyphenyl was obtained from Ambinter (Orléans, France), all other flavones were from Sigma-Aldrich.

PDXP knockout mice

Floxed PDXP mice (Pdxp<sup>tm1Goh</sup>) were generated on a C57Bl/6J background, and whole-body Pdxp knockouts were achieved by breeding with B6.FVB-Tg(EIIa-cre)C5379Lmgd/J (EIIa-Cre) transgenic mice, as described (30). All experiments were authorized by the local veterinary authority and committee on the ethics of animal experiments (Regierung von Unterfranken). All analyses were carried out in strict accordance with all German and European Union applicable laws and regulations concerning care and use of laboratory animals.

Preparation of hippocampi and hippocampal neurons and immunocytochemistry

Mice were sacrificed by cervical dislocation, and brains were immediately placed on a pre-cooled metal plate and dissected under a Leica M80 binocular (Leica, Wetzlar, Germany). Hippocampi were weighed and flash-frozen in liquid nitrogen. The entire procedure was performed in <3 min. Hippocampal lysates were prepared by the addition of ice-cold PBS (200 µL PBS/10 mg hippocampal wet weight) and homogenized for 1 min in a TissueLyser II instrument (Qiagen, Hilden, Germany). One fourth of the obtained volume of each lysate was used for the analysis of total PLP concentrations as described below. To determine protein-depleted PLP (27), the remaining volume of each lysate was centrifuged at 14,000 × g for 15 min at 4°C. The supernatant was applied to 3 kDa MWCO filters (Amicon Ultra-0.5
Centrifugal Filter; Merck Millipore, Darmstadt, Germany), and centrifuged at 14,000 × g for 45 min at 4°C. The flow-through was collected and prepared for HPLC analysis (see below).

Primary hippocampal neuronal cultures were prepared from mouse embryos at embryonic day 17. Hippocampi were incubated with 0.5 mg/mL trypsin, 0.2 mg/mL EDTA and 10 µg/mL DNase I in PBS for 30 min at 37°C. Trypsinization was stopped by adding 10% fetal calf serum. Cells were dissociated by trituration, counted, and seeded at a density of 150,000 cells per 35 mm dish. Dissociated cells were grown in neurobasal medium supplemented with L-glutamine and B27 supplement (A3582801, Life Technologies, Dreieich, Germany) with an exchange of 50% of the medium after 6 days in culture. After 21 days of differentiation (day in vitro 21/DIV21), 7,8-DHF (20 µM) or DMSO (0.02%, v/v) was added to the hippocampal neuronal cultures for 45 min. Cells were rinsed once with PBS (37°C), lysed in 150 µL ice cold H2O, and placed at -80°C for at least 30 min.

For immunocytochemistry, DIV21 primary hippocampal neurons were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at RT. After washing twice with PBS, 50 mM NH₄Cl was added for 10 min. Cells were then permeabilized with 0.1% (v/v) Triton X-100 and blocked with 5% (v/v) goat serum in PBS for 30 min at 22°C. Cells were incubated with mouse monoclonal anti-MAP2 antibodies (1:500 dilution, clone MAB3418, Millipore, Darmstadt, Germany) for 1 h in 5% goat serum/PBS at 22°C. Alexa-488-labeled secondary goat anti-mouse antibodies (1:500 dilution; Dianova, Hamburg, Germany) were applied for 1 h. Nuclei were counter-stained with 4’,-6-diamino-2-phenylindole (DAPI), and slides were mounted with Mowiol. Images were acquired using an inverted IX81 microscope equipped with an Olympus UPLSAPO 60× oil objective (numerical aperture: 1.35) on an Olympus FV1000 confocal laser scanning system, using a FVD10 SPD spectral detector and diode lasers of 405 nm (DAPI) and 495 nm (Alexa488).
Samples were derivatized as described (78). Briefly, 100 µL of lysate were mixed with 8 µL derivatization agent (containing 250 mg/mL of both semicarbazide and glycine), and incubated on ice for 30 min. Samples were then deproteinized by addition of perchloric acid (8 µL of a 72% (w/v) stock solution), followed by centrifugation at 15,000 × g for 15 min at 4 °C. Supernatants (100 µL) were neutralized with 10 µL NaOH (25% (v/v) stock solution), and 2 µM pyridoxic acid was added as an internal standard. PLP and PL were subjected to the same derivatization protocol to establish a standard curve. Samples were analyzed on a Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, Dreieich, Germany), using 60 mM Na₂HPO₄, 1 mM EDTA, 9.5% (v/v) MeOH; pH 6.5 as mobile phase. PL, PLP and pyridoxic acid were separated on a 3 μm reverse phase column (BDS-HYPERSIL-C18, Thermo Fisher Scientific). Chromatograms were analyzed using Chromeleon 7 software (Thermo Fisher Scientific).

Western blotting

Tissue or cell homogenates (prepared as detailed above for HPLC analysis) were extracted with 4 × RIPA buffer (final concentration, 50 mM Tris, pH 7.5; 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Pefabloc), 5 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin) for 15 min at 4 °C under rotation, and lysates were clarified by centrifugation (20,000 × g, 15 min, 4 °C). Protein concentrations in the supernatants were determined using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes by semidry-blotting. Antibodies were purchased from the following providers: Merck Millipore (mouse monoclonal α-actin mAb1501, dilution 1:5000); Cell Signaling (rabbit monoclonal α-PDXP clone C85E3, dilution 1:1000; Cell Signaling, Danvers/Massachusetts, USA), Sigma Aldrich (rabbit polyclonal α-PDXK/AB1, #AV53615, dilution 1:1000), and Thermo Fisher Scientific.
Scientific (rabbit polyclonal α-PNPO, #PA5-26400, dilution 1:1000, as used in ref. (30)).

Western blots were densitometrically quantified with NIH ImageJ, version 1.45i.

**Phosphatase plasmids and cloning**

N-terminally GST-tagged, human PDXP was in pGEX-4T-1 (Amersham Biosciences, Amersham, UK). All other phosphatases were of murine origin and were subcloned into pETM11 (EMBL), as described (40). *Pdxp* point mutants (generated by nested PCR) were subcloned into the *Nco*I (*Pci*I for *Psph*) and *EcoR*I restriction sites of pETM11, using Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Frankfurt/Main, Germany). The following primers (oligonucleotide sequence 5’- 3’; fwd, forward; rev, reverse) were used:

*Pdxp*  
- fwd: TCGACCATGGCGCGCTGCGAGCGG  
- rev: AAAAGTGAATTCTCAGTCCTCCAGCCCCTC

*Pdxp-N60A*  
- fwd: TTCGTGAGCAACGCCAGCGCGCGCGCGCGG  
- rev: CGCGCGCCGGCTGGCTGTTGCTCACGAA

*Pdxp-N60S*  
- fwd: TTCGTGAGCAACAGCAGCGCGCGCGCGG  
- rev: CGCGCGCCGGCTGGCTGTTGCTCACGAA

*Pdxp-R62A*  
- fwd: AGCAACAACAGCGCGCGCGCGCGGCC  
- rev: GGGCCGCGCGCGCGCGCTGTTGTTGCT

*Pdxp-Y146A*  
- fwd: GTGCTCGTAGGCGCCGACGAGTTT  
- rev: AAACTGCTCGTCGGCGCCTACGAGCAC

*Pdxp-E148A*  
- fwd: GTAGGCTACGACGCGCAGTTTTCCT  
- rev: GAAGGAAAACTGCGTCGTAGCCTAC

*Pdxp-H178A*  
- fwd: CGCGACCCTTGGGCCCGCTACGAC  
- rev: GTCGCTGAGCGGGGCCCAAGGGTCGCG

Primers were purchased from Eurofins Genomics (Ebersberg, Germany), and all constructs were verified by sequencing (Microsynth Seqlab, Göttingen, Germany).
Expression and purification of recombinant proteins

All purification steps of murine PDXP and murine PGP were carried out exactly as described (40). N-terminally His6-tagged PDXP variants were expressed exactly as described for PDXP-WT (40), except that the His6-tag was not cleaved off. Human GST-PDXP was transformed into *E. coli* BL21(DE3) (Stratagene Europe/VWR, Darmstadt, Germany). Protein expression was induced with 0.5 mM isopropyl β-d-thiogalactopyranoside for 18 h at 20 °C. All subsequent purification steps were carried out at 4 °C. Cells were harvested by centrifugation for 10 min at 8000 × g and resuspended in lysis buffer (100 mM triethanolamine/TEA, 500 mM NaCl; pH 7.4) supplemented with protease inhibitors (EDTA-free protease inhibitor tablets; Roche, Mannheim, Germany) and 150 U/mL DNase I (Applichem, Renningen, Germany). Cells were lysed using a cell disruptor (Constant Systems, Daventry, UK), and cell debris was removed by centrifugation for 30 min at 30,000 × g. GST-PDXP was batch-purified on a glutathione sepharose 4B resin (GE Healthcare, Uppsala, Sweden). After extensive washing with 25 column volumes of wash buffer (50 mM TEA, 250 mM NaCl; pH 7.4), GST-PDXP was eluted in wash buffer supplemented with 10 mM reduced glutathione, concentrated, and further purified in buffer A (50 mM TEA, 250 mM NaCl, 5 mM MgCl2; pH 7.4) using a HiLoad 16/60 Superdex 200 pg gel filtration column operated on an ÄKTA liquid chromatography system (GE Healthcare).

High-Throughput Screen for PDXP Modulators

The screening campaign (chemical library, screening protocol, concentration-dependent assays, data analysis) was conducted exactly as described previously (40), except that the primary screen was done with PDXP, the counter-screen was done with PGP, and PDXP inhibitor hits were validated using 5’-pyridoxal phosphate (PLP) as a physiological PDXP substrate.
IC₅₀ determinations, enzyme kinetics, and compound selectivity

Buffer conditions for enzymatic assays were as previously published (40). Purified phosphatases were pre-incubated for 10 min at RT with serial dilutions of flavones. Dephosphorylation reactions were started by the addition of the indicated substrate; buffer with substrate and the respective flavone but without the enzyme served as a background control. Prior to compound testing, time courses of inorganic phosphate release from the respective phosphatase substrates were conducted to ensure assay linearity. Inorganic phosphate release was detected with malachite green solution (Biomol Green; Enzo Life Sciences, Lörrach, Germany); the absorbance at 620 nm (A₆₂₀) was measured on an Envision 2104 multilabel reader (Perkin Elmer, Rodgau, Germany). Released phosphate was determined by converting the values to nmol Pᵢ with a phosphate standard curve. Data were analyzed with GraphPad Prism version 9.5.1 (GraphPad, Boston/Massachusetts, USA). For IC₅₀ determinations, logₐinhbitor versus response was calculated (four parameter). To derive K_M and k_cat values, data were fitted by nonlinear regression to the Michaelis-Menten equation.

Biolayer interferometry (BLI)

PDXP was biotinylated using the EZ-Link NHS-PEG4-Biotin kit, as recommended by the manufacturer (Thermo Fisher Scientific), and loaded on Super Streptavidin Biosensors (SSA) (Sartorius, Göttingen, Germany) as follows. SSA sensors were equilibrated for 1 h at RT in BLI assay buffer (250 mM triethanolamine, 5 mM MgCl₂, 250 mM NaCl, 0.005% (v/v) TWEEN-20; pH 7.5), loaded with 200 µg/mL biotinylated PDXP, blocked with 2 µg/mL biocytin, and washed in BLI assay buffer. Reference SSA sensors were blocked with 2 µg/mL biocytin (79). Six point 1:1 serial dilution series of 7,8-DHF and 5,7-DHF were prepared in DMSO, and BLI assay buffer was added to the wells to obtain a 7,8-DHF starting concentration of 25 µM. The final DMSO concentration was 5% (v/v). Buffers for baseline, dissociation, and buffer correction wells were supplemented with the same amount of DMSO.
for identical buffer conditions. Four measurements were carried out per condition, using one
sensor set for two measurements. All measurements were conducted on an Octet K2 device
(Sartorius) using 96-well plates. Assay settings were as follows: baseline measurement 45 sec,
association time 90 sec, dissociation time 150 sec. The resulting data were processed using
the double reference method of the Octet analysis software for removal of drifts and well-to-
well artefacts. Kinetic analyses were performed using the Octet analysis software. The steady
state analysis was carried out with OriginPro 2021b (OriginLab, Northampton/Massachusetts,
USA), using a dose-response model for regression.

**PDXP crystallization and data collection**

For co-crystallization with 7,8-DHF, full-length murine PDXP (10 mg/mL in 50 mM
triethanolamine; 250 mM NaCl; 5 mM MgCl₂; pH 7.4) was supplemented with a three-fold
molar excess of the flavone. Prism-shaped crystals of 7,8-DHF-bound PDXP were grown at
20°C in 0.1 M phosphate citrate (pH 4.2) and 40% (v/v) PEG 300 using the sitting-drop vapor
diffusion method. Crystals were cryoprotected for flash-cooling in liquid nitrogen by soaking
in mother liquor 25% (v/v) glycerol. Diffraction data were collected from flash-cooled
crystals after addition of 25% glycerol at a temperature of 100 K on beamline BL 14.1 at the
BESSY (Helmholtz Zentrum Berlin, Germany) synchrotron. The structure of 7,8-DHF-PDXP
was solved by molecular replacement with the program Phaser (80) with the structure of the
murine PDXP (PDB entry 4BX3) as search model.
ACKNOWLEDGMENTS

We thank Carola Seyffarth and Nicole Bader for excellent technical assistance, Dr. Jochen Kuper for collecting the diffraction data and the staff at beamline BL14.1 of the BESSY synchrotron.

DATA AVAILABILITY STATEMENT

The previously published PDB entry 4BX3 [http://doi.org/10.2210/pdb4BX3/pdb] of murine apo-PDXP and 2CFT [http://doi.org/10.2210/pdb2CFT/pdb] of PLP-bound human PDXP are used in this manuscript. X-ray crystallographic data of 7,8-DHF-bound PDXP generated in this study have been deposited in the PDB and can be accessed under the PDB entry 8QFW [http://doi.org/10.2210/pdb8QFW/pdb].

AUTHOR CONTRIBUTIONS


COMPETING INTERESTS

The authors declare no competing interests.
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Figure 1. Role of PDXP in hippocampal PLP homeostasis.

(a) Age-dependent expression of PDXK and PDXP in murine hippocampi. **Left panels**, representative Western blots of three hippocampi for each genotype. The same blots were reprobed with α-actin antibodies as a loading control. The age of the investigated mice is indicated above the blots. **Right panel**, densitometric quantification of hippocampal PDXP.
and PDXK Western blot signals, corrected by the corresponding actin signals. Young mice were 18-42 days old, older mice were 252-351 days old; n=7 hippocampi were analyzed per group. Data are mean values ± S.D. Statistical analysis was performed with unpaired, two-sided t-tests; p-values are indicated. (b) Age-dependent, total PLP-concentrations in isolated hippocampi of PDXP-WT and PDXP-KO mice. PLP was derivatized with semicarbazide and analyzed by HPLC. Each symbol represents the result of the PLP determination in an individual hippocampus. Data were fitted by Gaussian least-squares analyses. (c) Determination of protein-bound and protein-depleted PLP in PDXP-WT and PDXP-KO hippocampal lysates of young (18-42 days old) and older mice (252-352 days old). The number of analyzed hippocampi is indicated in the bars. Data are mean values ± S.D. Statistical analysis was performed with two-way ANOVA and Tukey's multiple comparisons test. Significant differences (adjusted P-values) in protein-depleted PLP levels are indicated. The exact age of analyzed mice is listed in Figure 1 – supplementary figure 1. Source data are available for this Figure.
Figure 2: Characterization of the 7,8-DHF/PDXP interaction.
(a) Determination of half-maximal inhibitory constants (IC$_{50}$) of 7,8-DHF (2D-structure shown on top) for purified murine or human PDXP, using pyridoxal 5'-phosphate (PLP) as a substrate. Phosphatase activities in the presence of 7,8-DHF were normalized to the respective enzyme activities measured in the presence of the DMSO solvent control. Data are mean values ± S.D. of $n=3$ (human PDXP) and $n=4$ (murine PDXP) independent experiments. (b) IC$_{50}$ values of different flavones for purified murine PDXP with PLP as a substrate. Phosphatase activities in the presence of flavones were normalized to the respective enzyme activities in the presence of the DMSO solvent control. All data are mean values ± S.D. The inhibition of PDXP by 3,7,8-trihydroxyflavone-4'-hydroxyphenyl (2D-structure shown on top) was assessed in $n=6$ independent experiments. All other data are from $n=3$ biologically independent experiments. Apparently missing error bars are hidden by the symbols. (c) Biolayer interferometry (BLI) measurements of the interaction of 7,8-DHF with purified murine PDXP. Left panel, example sensorgram overlayed with the global 1:1 binding model (red) and the negative control (gray). The dashed line indicates the start of the dissociation phase. Right panel, steady-state dose-response analysis for 7,8-DHF based on $n=4$ measurements. (d) Sensitivity of the indicated HAD phosphatases to 7,8-DHF. Phosphatase activities in the presence of 7,8-DHF were normalized to the respective enzyme activities measured in the presence of the DMSO solvent control. Data are mean values ± S.D. of $n=4$ (PGP) or $n=3$ independent experiments (all other phosphatases). Phosphatase substrates and cap types are indicated in the legend. PGP, phosphoglycolate phosphatase; LHPP, phospholysine phosphohistidine inorganic pyrophosphate phosphatase; PHOP2, phosphatase orphan 2; PSPH, phosphoserine phosphatase; MDP1, magnesium-dependent phosphatase-1.

Source data are available for this Figure.
Figure 3. X-ray crystal structure of murine PDXP in complex with 7,8-DHF.

(a) [Image of X-ray crystal structure] (b) [Image of X-ray crystal structure] (c) [Image of X-ray crystal structure] (d) [Image of X-ray crystal structure] (e) [Image of X-ray crystal structure] (f) [Image of X-ray crystal structure] (g) [Graph showing PDXP activity vs. 7,8 DHF concentration]
(a) The model was refined to a resolution of 2.0 Å (PDB code 8QFW). Protomer A of full-length, homodimeric PDXP is shown in pink and protomer B in green. 7,8-DHF is shown in sphere representation with its C-atoms in pink and the catalytically essential Mg$^{2+}$ ions are shown as cyan spheres. (b) Structural overlay of apo-PDXP (PDB code 4BX3, chain A, in brown) and the 7,8-DHF-bound PDXP protomer A (in pink). 7,8-DHF is shown in stick representation with its C-atoms in pink. The catalytic Asp25 residues are shown in red or purple sticks, respectively, and the Mg$^{2+}$ ion is depicted as a cyan sphere. The substrate specificity loops are highlighted in yellow. (c) $2F_o-F_c$ electron density map contoured at an RMSD of 1 in blue, overlaid with the refined model. The $F_o-F_c$ map of 7,8-DHF contoured at an RMSD of 3 is shown with positive densities in green mesh. (d) Arg62 blocks 7,8-DHF binding in the B-protomer. A salt bridge between Arg62 (C-atoms shown as green spheres) in the B-protomer (in green) and Asp14 (C-atoms shown as pink spheres) of a symmetry-related A-protomer (in pink) blocks the 7,8-DHF binding site. 7,8-DHF (in stick representation with gray C-atoms) is modeled based on the A-protomer. (e) Comparison of the 7,8-DHF and PLP binding sites. The active site of 7,8-DHF-bound PDXP is in gray. 7,8-DHF is shown in stick representation (pink C-atoms), and the Mg$^{2+}$ ion as a cyan sphere. PLP (in stick representation with blue C-atoms) was modelled based on a superposition of the human PDXP-PLP complex (PDB code 2CFT). (f) Structural details of bound 7,8-DHF and adjacent residues of the active site. (g) Verification of 7,8-DHF - PDXP interactions. Left panel, phosphatase activity of purified PDXP or the indicated PDXP variants. Data are mean values ± S.D. of $n=3$ independent experiments. Right panel, determination of the IC$_{50}$ values of 7,8-DHF for purified PDXP or the indicated PDXP variants. Data are mean values ± S.D. of $n=3$ independent experiments. Apparently missing error bars are hidden by the symbols. Source data are available for this Figure.
Figure 4. Effect of 7,8-DHF on the PLP/PL ratio in cultured hippocampal neurons from WT or PDXP-KO mice.

(a) Effect of long-term PDXP deficiency on total PLP levels in hippocampal neurons. Data are mean values ± S.E. of n=4 independent experiments. Statistical significance was assessed with a two-tailed, unpaired t-test. A representative image of primary hippocampal neurons stained for the neuronal marker protein MAP2 is shown in the insert (pixel intensities were color-inverted for better visualization). Scale bar, 100 µm. (b) Western blot analysis of PDXP and PDXK expression in hippocampal neuron samples shown in (a). The same blots were reprobed with α-actin antibodies as a loading control. The densitometric quantification of PDXK signals is shown on the right; data are mean values ± S.E. of n=4 independent experiments. (c) Effect of 7,8-DHF (20 µM, 45 min) or the DMSO solvent control (0.02%
v/v, 45 min) on the PLP/PL ratio in hippocampal neurons of PDXP-WT or PDXP-KO mice.

Source data are available for this Figure.
### Table 1. Kinetic constants of PDXP-catalyzed PLP hydrolysis in the presence of 7,8-DHF.

<table>
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<th>7,8-DHF [µM]</th>
<th>0</th>
<th>1.0</th>
<th>1.5</th>
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<th>3.0</th>
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<th>10.0</th>
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<tr>
<td>$K_M$ [µM]</td>
<td>14.98 ± 1.28</td>
<td>18.54 ± 6.24</td>
<td>20.20 ± 6.19</td>
<td>18.97 ± 5.15</td>
<td>24.83 ± 2.61</td>
<td>32.96 ± 2.13</td>
<td>30.61 ± 2.57</td>
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<tr>
<td>$v_{max}$ [µmol/min/mg]</td>
<td>1.08 ± 0.04</td>
<td>0.95 ± 0.01</td>
<td>0.89 ± 0.04</td>
<td>0.85 ± 0.02</td>
<td>0.80 ± 0.04</td>
<td>0.81 ± 0.05</td>
<td>0.74 ± 0.06</td>
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<td>$k_{cat}$ [s$^{-1}$]</td>
<td>0.57 ± 0.02</td>
<td>0.5 ± 0.01</td>
<td>0.47 ± 0.02</td>
<td>0.45 ± 0.01</td>
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<tr>
<td>$k_{cat}/K_M$ [s$^{-1}$·M$^{-1}$]</td>
<td>3.93 ± 0.29</td>
<td>3.27 ± 0.84</td>
<td>2.75 ± 0.67</td>
<td>2.72 ± 0.66</td>
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The data are mean values ± S.E.M. of $n=3$ independent experiments, except for the solvent control samples ($n=6$). Curves were fitted and parameters $K_M$ (Michaelis–Menten constant); $v_{max}$, (maximum enzyme velocity); $k_{cat}$ (turnover number) were derived using the Michaelis-Menten model in GraphPad Prism 9.5.1. The $k_{cat}$ values were calculated from the maximum enzyme velocities using a molecular mass of 31,828 Da for PDXP. DMSO concentrations were kept constant (0.1% DMSO under all conditions, including the solvent control samples).
### Table 2. Data Collection and Refinement Statistics.

<table>
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<td>Ramachandran statistics (%)</td>
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$aR_{sym}= \sum_{hkl} |I_i| - <I>| / \sum_{hkl} \Sigma_i I_i$ where $I_i$ is the $i^{th}$ measurement and $<I>$ is the weighted mean of all measurements of $I$.

$bR_{pim} = \sum_{hkl} 1/(N-1)^{1/2} \Sigma_i I_i(hkl) - \bar{I}(hkl) | / \sum_{hkl} \Sigma_i I_i(hkl)$, where $N$ is the redundancy of the data and $\bar{I}(hkl)$ the average intensity.

$c<|I/\sigma I|>$ indicates the average of the intensity divided by its standard deviation.

$dR_{work} = \sum_{hkl} ||F_o|| - |F_c|| / \sum_{hkl}||F_o||$ where $F_o$ and $F_c$ are the observed and calculated structure factor amplitudes.

$eR_{free}$ same as $R$ for 5% of the data randomly omitted from the refinement. The number of reflections includes the $R_{free}$ subset.

Numbers in parentheses refer to the highest resolution data shell.

Ramachandran statistics reflect the percentage of residues in favored/allowed/outlier regions.
**SUPPLEMENTARY INFORMATION**

**Figure 1 – figure supplement 1. Analysis of total hippocampal PLP levels in PDXP-WT and PDXP-KO mice.**

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Each value represents the result of the PLP determination in an individual hippocampus. Analysis for a statistically significant difference between PLP levels in PDXP-WT and

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PDXP-KO hippocampi (all ages combined; two-tailed, unpaired t-test) $p<0.0001$. Bold table entries indicate those hippocampal extracts that were further separated for an analysis of protein-depleted and protein-bound PLP (see Fig. 1c).
A primary screen was conducted using 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) as an artificial substrate. Out of 41,182 screened compounds, 256 compounds were discarded that showed very high autofluorescence (as recognized by elevated fluorescence at the start of the kinetic curve); 26 compounds showed statistically significant PDXP activation, and 255 compounds showed PDXP inhibition (as recognized by an elevated or decreased slope of the kinetic curve, respectively). The average Z' factor of the screen was 0.75 ± 0.112. These 281 compounds were selected for DiFMUP-based concentration-dependent validation, and the 46 most potent compounds were selected. A counter-screening
was conducted in parallel, also in a concentration-dependent fashion, against the PDXP paralog and closest relative phosphoglycolate phosphatase (PGP). The 14 compounds that were inactive against PGP were validated in a secondary assay, using the PDXP substrate pyridoxal 5′-phosphate (PLP). Two PDXP inhibitor hits blocked PLP dephosphorylation by ≥50%. Source data are available for this Figure.
**Figure 2 – figure supplement 2. PDXP inhibitor hits.**

<table>
<thead>
<tr>
<th>InChI Key</th>
<th>IC$_{50}$ [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>7,8-DHF COCYGNDCWFKTMF-UHFFFAOYSA-N</td>
<td>0.8 *</td>
</tr>
<tr>
<td>FMP-1</td>
<td>~1</td>
</tr>
<tr>
<td>RDMYXZLESVAHOO-UHFFFAOYSA-N</td>
<td>&gt;40</td>
</tr>
<tr>
<td>FMP-2</td>
<td>&gt;40</td>
</tr>
<tr>
<td>RBZAGLIUHTVMFL-UHFFFAOYSA-N</td>
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<tr>
<td>FMP-3</td>
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<tr>
<td>HSJXOMZEPTVVQC-UHFFFAOYSA-N</td>
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<td>OPTDAYWBFRIGB-UHFFFAOYSA-N</td>
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</tr>
<tr>
<td>GCUCIFQCGJIRNT-UHFFFAOYSA-N</td>
<td>&gt;40</td>
</tr>
</tbody>
</table>

Determination of half-maximal inhibitory constants (IC$_{50}$) of 14 PDXP inhibitory compounds (see InChI Key for chemical substance identification) using purified murine PDXP and pyridoxal 5’-phosphate (PLP) as a substrate. Data marked with an asterisk (*) are results of $n=3$ independent experiments. Because of the limited quantity of most compounds available for these assays, all other data are results of $n=1$ determinations. 7,8-DHF, 7,8-dihydroxyflavone; ~, approximate IC$_{50}$ value. FMP-1, FMP-2 and FMP-3 are compounds obtained from academic donors whose structure is undisclosed for intellectual property reasons.
Figure 2 – figure supplement 3. BLI measurements of the interaction of 7,8-DHF with purified murine PDXP.

Sensorgrams of three additional experiments overlayed with the global 1:1 binding model (red) and the negative control (gray). The dashed line indicates the start of the dissociation phase.
Figure 3 – figure supplement 1. Unit cell environment of the 7,8-DHF ∙ PDXP crystal.
The 7,8-DHF-bound protomer A is depicted in pink, the inhibitor-free protomer B is colored in light green. 7,8-DHF is shown in stick representation (C-atoms in pink), and its position is marked with a white asterisk. Arg62 and Glu148 at the active site entrances of protomers A and B are highlighted in blue. Surrounding symmetry mates within 4 Å of the centrally located 7,8-DHF-PDXP/apo-PDXP dimer are shown in surface representation in magenta or dark green. Whereas the active site in 7,8-DHF-bound protomer A is accessible, the corresponding site in protomer B is shielded by two crystallographic neighbors (white arrow). The marked area is enlarged in the bottom panel. Source data are available for this Figure.
Figure 3 – figure supplement 2. Alignment of human and murine PDXP.

Protein sequences of human PDXP (UniProtKB Q96GD0) and murine PDXP (UniProtKB P60487) were aligned with the EMBL-EBI multiple sequence alignment tool Clustal Omega version 1.2.4. PDXP residues found to engage in 7,8-DHF interactions (highlighted in red color) are identical in human and murine PDXP.

Figure 3 – figure supplement 3. Purity of the employed PDXP and PDXP variants.

A Coomassie Blue-stained gel is shown.