Novel pharmacological chaperones that correct phenylketonuria in mice

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Phenylketonuria (PKU) is caused by inherited phenylalanine-hydroxylase (PAH) deficiency and, in many genotypes, it is associated with protein misfolding. The natural cofactor of PAH, tetrahydrobiopterin (BH₄), can act as a pharmacological chaperone (PC) that rescues enzyme function. However, BH₄ shows limited efficacy in some PKU genotypes and its chemical synthesis is very costly. Taking an integrated drug discovery approach which has not been applied to this target before, we identified alternative PCs for the treatment of PKU. Shape-focused virtual screening of the National Cancer Institute’s chemical library identified 84 candidate molecules with potential to bind to the active site of PAH. An in vitro evaluation of these yielded six compounds that restored the enzymatic activity of the unstable PAH/V106A variant and increased its stability in cell-based assays against proteolytic degradation. During a 3-day treatment study, two compounds (benzylhydantoin and 6-amino-5-(benzylamino)-uracil) substantially improved the in vivo Phe oxidation and blood Phe concentrations of PKU mice (Pahenu1). Notably, benzylhydantoin was twice as effective as tetrahydrobiopterin. In conclusion, we identified two PCs with high in vivo efficacy that may be further developed into a more effective drug treatment of PKU.

INTRODUCTION

Phenylketonuria (PKU) is an autosomal recessive inborn error of metabolism caused by deficiency of hepatic phenylalanine-4-hydroxylase (PAH; EC 1.14.16.1). The clinical phenotypes of hyperphenylalaninemia due to PAH deficiency range from classic PKU (blood Phe concentrations >1200 μM) to mild PKU (600–1200 μM) and mild hyperphenylalaninemia (120–600 μM). Untreated patients with classic and mild PKU can develop severe mental retardation (1). For the better part of the past 60 years, the only treatment available was dietary Phe restriction which is a burdensome therapy often leading to malnutrition and to psychosocial complications (2,3). Therefore, there is an urgent need for alternative therapies (4). Recent strategies to replace the dietary treatment comprise: tetrahydrobiopterin (BH₄), large neutral amino acids (LNAA), enzyme replacement therapy with PEGylated phenylalanine ammonia lyase (PEG-PAL) and gene therapy. LNAA compete with phenylalanine for transport across the blood brain barrier via L-type amino acid transporter and thus can reduce the intracerebral Phe concentration. But to which extent it allows for diet liberalization still needs to be clarified in large clinical trials (5–7). Bacteria-derived PEG-PAL therapy substantially decreased blood Phe in both murine and clinical studies. Immunogenicity in the subcutaneous application and instability...
A decade ago, the observation that pharmacological doses of BH4 can restore enzyme function in a significant share of PKU patients (14–18) ushered in a new era. Finally, 5 years ago sapropterin dihydrochloride, which is the synthetic form of BH4, was marketed approved for the treatment of PKU by the Food and Drug Administration and the European Medicines Agency (19,20). However, its underlying mechanism is not fully clarified. In BH4-responsive PKU genotypes, BH4 can act as a pharmacological chaperone (PC) that upon specific binding to the active site restores PAH function by structural stabilization of the misfolded PAH enzyme (17,21,22). Wild-type PAH, however, is shifted from an activated labile conformation towards an inactive but stable state by BH4 (23). Thus, the BH4-induced gain of function of misfolded PAH is the outcome of a trade-off between a gain in protein stability and enzyme inhibition. Structural and biochemical studies have demonstrated that the inhibitory effects of BH4 are induced by its interaction with the regulatory domain of PAH. The inhibitory effect is lower or missing in some BH4 analogues, such as BH2 or other ligands of the PAH active site (24–27). We therefore hypothesized that potent PC could be found by screening putative ligands of the PAH active site. This search was further motivated by the low efficacy of BH4 in some PKU genotypes and its unfavourable pharmaceutical and pharmacological characteristics (low bioavailability, instability, costly synthesis). Pey et al. showed that putative PC for the treatment of PKU can be identified by screening 1000 commercially available molecules with an in vitro HTS (high throughput put screening) stability assay. However, the proof of concept for in vivo efficacy of alternative chaperones in PKU is missing so far.

Virtual screening is established as a major pillar of rational drug discovery and has led to the successful identification of numerous therapeutically relevant agents (19,28,29). Therefore, we applied a shape-focused virtual screening method to identify compounds with potential to be active on PAH. This pre-selection step of molecules resulted in high odds of positive compound selection. The virtual hits were evaluated and characterized in several functional in vitro assays, and their in vivo efficacies were proved in Pahenu1 mice. This mouse model was generated by germline mutagenesis and carries the missense mutation V106A in the regulatory domain of PAH which leads to a mild hyperphenylalaninemia phenotype (30–33). Recently, it was shown that the amino acid change V106A leads to protein misfolding, instability and accelerated degradation of PAH in Pahenu1 and that BH4 corrects this pathology (22,34). Thus, Pahenu1 is a mouse model of misfolding-induced PAH deficiency which is responsive to BH4 (22).

In conclusion, we identified two molecules chemically different from the previously reported candidates and with high potential for further drug development, one of which was twice as effective as BH4. This confirms our initial hypothesis and illustrates the value of our approach.

RESULTS

Shape-focused virtual screening of the National Cancer Institute compound database

The National Cancer Institute’s (NCI) chemical library was screened for potential PC of hPAH. More than 115 000 compounds were screened and ranked using a shape-focused virtual screening engine, selected by visual inspection and ordered from the NCI for experimental evaluation.

BH4 was selected as query structure for shape-focused virtual screening of the NCI structural database using ROCS (see Materials and Methods). Eighty-four candidate molecules were screened and ranked using a shape-focused virtual screening engine, selected by visual inspection and ordered from the NCI for experimental evaluation.

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and their potential interaction pattern (Fig. 1). According to X-ray experimental structures of PAH (in this study, PDB 1j8u), BH4 causes the formation of a well-ordered complex H-bonding network. Direct interactions are formed with G247, L249 and S251, while indirect interactions via water molecules and the catalytic iron are formed with H264, H285, E286, H290, A322, Y325 and E330.

**Screening for binding partners of mPAH with the use of surface plasmon resonance (SPR): compound selection**

Surface plasmon resonance (SPR) allows for rapid label-free characterization of interactions between the immobilized target protein and the screened small molecules in real time. This method has been used successfully in drug screening (35). Thus, we screened the 84 in silico selected compounds in binding to mPAH using SPR. Thirteen of the aforementioned 84 tested compounds bound to the immobilized mPAH with a fast dissociation characteristic of reversible binding. Compounds showing higher affinity, saturability and reversible binding were classified as positive hits and they were included in further analysis. In order to determine the equilibrium constants, the interaction of the hits with mPAH was analysed in a concentration-dependent way until a saturation response was achieved as depicted in Figure 2 for compound 2 (2; numbers in **bold** will be used to indicate the compounds assigned number). Higher concentrations of the compounds that showed ambiguous binding behaviour in the initial screening were subsequently tested to discard false negatives. We selected six compounds that bound to mPAH in a reversible and specific way with affinities in the micromolar range and that belong to three different chemical clusters (Table 1). Three of the compounds (1, 5, 6) are based on a (thio)hydantoin scaffold with a short linker to a phenyl or furan moiety. Other compounds are based on uracil (2) and guanine (3, 4) scaffolds. Hydantoin derivatives can be found in phenylketonuric urine (36) and literature relates hydantoin-based compounds such as 1 to inhibitory activity on PAH (37). Compounds related to 2 such as pyrimidines have been identified and investigated with regard to their function as cofactors of PAH (38–41).

**Compound effect on mPAH intrinsic tryptophan fluorescence**

SPR analyses were performed with a surface immobilized protein which may be slightly different from dissolved PAH in its binding capabilities due to the non-directional attachment of the protein to the matrix after the amino-coupling step (42). Thus, we used fluorescence spectroscopy as a second independent method to validate the SPR results in solution. Equal to human and rat homologs, the mouse PAH has three tryptophan residues partially buried in the protein structure that produce—after excitation at 295 nm—an emission maximum at ~342 nm (26,43). The fluorescence spectra of mPAH with the test compounds were recorded. A typical compound titration curve is shown in Supplementary Material, Figure S1. 1, 4, 5 and 6 increased the quantum yield at 342 nm in a concentration dependent and saturable way reminiscent of the effect caused by the substrate L-Phe. The increase in fluorescence intensity that accompanies activation of PAH by L-Phe mainly originates from changes in the emission of W120 (43). It has been shown that this residue becomes more solvent-exposed in the substrate-activated enzyme due to domain movements around the hinge region R111–T117 (44). The effect of 2 and 3 on the conformation of PAH could not be determined, as these substances show a fluorescence emission spectrum overlapping with that of PAH. Mapping the data of the fluorescence corrected curves against the compound concentration (expressed as total ligand present) and assuming a 1:1 binding, we obtained the apparent dissociation constants (KdF; Table 1). The observed increase in fluorescence intensity at 342 nm was competed and quenched by adding increasing concentrations of BH4 (Supplementary Material, Fig. S1). These results confirmed the binding of 1, 4, 5 and 6 to PAH with affinity in the order of the BH4 interaction (KdF 0.49 ± 0.04). The KdF were one to two orders of magnitude lower than those observed in SPR experiments. This result is not surprising as proteins in solution present a higher degree of conformational flexibility than immobilized proteins.

**PAH stabilization against endogenous proteolysis**

The V106A amino acid change in PAH results in a reduction of PAH function caused by aberrant protein folding and consecutive accelerated protease degradation as shown for the mouse model Pahenu1 (34,45). BH4 corrects this pathology by stabilizing the protein conformation (22). We evaluated whether the identified ligands have the potential to stabilize PAHV106A. TsA201 cells were transiently transfected with plasmids encoding for PAH wild-type or PAHV106A and the cells were incubated with the compounds or control vehicle. Next, protein translation was blocked by addition of puromycin, and the compounds’ effect on protein stability was assessed by immunoblotting with the corresponding antibodies. After 6 h of protein translation inhibition, PAH wild-type remained stable to proteolysis and the compounds had no significant effect on protein stability. On the contrary, 1–
stabilized PAHV106A against endogenous protease degradation between 1.3 ± 0.04 (1) and 1.8 ± 0.2 (4) fold (Fig. 3A). These results indicate that the compounds assist in stabilizing the protein conformation against intracellular proteolysis and therefore can be regarded as potential PCs.

Recovery of the enzymatic activity of PAHV106A

As aforementioned, PAHV106A is a partially misfolded protein susceptible to proteolysis. Still maltose-binding protein (MBP) -tagged PAHV106A is a stable protein with a specific activity in the order of wild-type PAH (22). To explore the effect of the compounds on PAHV106A, we compared the enzymatic activity of the tagged protein before and after enterokinase processing of MBP. The digestion reaction was performed after incubation of the protein with the compounds (5–100 μM) or vehicle control (Fig. 3B). The catalytic activity of MBP-PAHV106A was decreased almost 2-fold after cleavage with enterokinase for 1 h. sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analyses and Coomassie staining of the enterokinase digestion reaction showed that the protease cuts specifically after the recognition sequence D-D-D-D-K located between MBP and PAHV106A and additionally leads to the appearance of degradation products. Pre-incubation of MBP-PAHV106A led to retention of enzymatic activity to levels reached by the stable tagged protein in a dose-dependent manner. Only 5 increased the activity to 100% at the lowest concentration tested. These results support the notion that incubation with compounds 1–6 adjusts the enzyme conformation of PAHV106A, protecting it against non-specific proteolysis and enhances its catalytic activity as a result of it.

Cell viability

The discovery of the selected compounds was intended for their in vivo use in the Pahnull mouse model of PKU. For this reason, we next studied their effect on the viability of HepG2 cells. These cells were incubated with increasing concentrations of compounds as shown in Figure 3C. In all cases, low concentration of the compounds (100 μM) was well tolerated by the cells; 5 and 6 reduced cell viability at the maximum concentration tested (1 mM) by more than 50%. The data indicate that these compounds show cell toxicity at high concentrations. In contrast to the above, 1, 2 and 4 did not show any or just a low-level toxic effect (3).

In vivo efficacy on 13C-Phe oxidation and blood Phe concentrations

The 13C-Phe oxidation rate assessed as cumulative tracer recovery within a 13C-Phe breath test reflects the PAH activity in vivo (15,22,46,47). We used this endpoint to evaluate the in vivo efficacy of compounds 1–6. First, Pahlnull animals received single doses of 10 μg/g i.p. Only 1 and 2 which induced significant increases in Phe oxidation after a single dose were further tested in a 48h multiple dose trial (3 × 10 μg/g i.p.). We evaluated the effect of 1 and 2 in comparison to the vehicle used [distilled water for 1 and dimethyl sulfoxide (DMSO) for 2] as well as BH4 10 μg/g (positive control) (Fig. 4). The cumulative recovery was significantly increased

### Table 1. Six out of 84 selected compounds bind to mPAH

<table>
<thead>
<tr>
<th>ID</th>
<th>Structure</th>
<th>CAS</th>
<th>NCI</th>
<th>Name</th>
<th>KdEQ (μM)</th>
<th>KdF (μM)</th>
<th>Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH4</td>
<td><img src="17528-72-2.png" alt="Image" /></td>
<td>17528-72-2</td>
<td>5,6,7,8-tetrahydrobiopterin</td>
<td>26</td>
<td>0.49 ± 0.04</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><img src="3530-82-3.png" alt="Image" /></td>
<td>3530-82-3</td>
<td>30459</td>
<td>5-benzylhydantoin</td>
<td>4.6</td>
<td>0.75 ± 0.18</td>
<td>c. III</td>
</tr>
<tr>
<td>2</td>
<td><img src="25507-29-3.png" alt="Image" /></td>
<td>25507-29-3</td>
<td>100192</td>
<td>6-amino-5-benzylaminouracil</td>
<td>135.0</td>
<td>a.f.</td>
<td>c. I</td>
</tr>
<tr>
<td>3</td>
<td><img src="7780-10-1.png" alt="Image" /></td>
<td>7780-10-1</td>
<td>211854</td>
<td>8-phenylguanine</td>
<td>14.7</td>
<td>a.f.</td>
<td>c. II</td>
</tr>
<tr>
<td>4</td>
<td><img src="171813-05-1.png" alt="Image" /></td>
<td>171813-05-1</td>
<td>310778</td>
<td>3,5,6,7-tetrahydro-6,7-dihydroxy-6-methyl-9H-imidazo[1,2-a]purin-9-one</td>
<td>18.9</td>
<td>0.48 ± 0.3</td>
<td>c. II</td>
</tr>
<tr>
<td>5</td>
<td><img src="583-46-0.png" alt="Image" /></td>
<td>583-46-0</td>
<td>7937</td>
<td>5-benzylidene-2-thiohydantoin</td>
<td>67.5</td>
<td>0.52 ± 0.23</td>
<td>c. I</td>
</tr>
<tr>
<td>6</td>
<td><img src="4349-14-8.png" alt="Image" /></td>
<td>4349-14-8</td>
<td>16601</td>
<td>5-furfurylhydantoin</td>
<td>72.7</td>
<td>4.72 ± 1.88</td>
<td>c. III</td>
</tr>
</tbody>
</table>

The identified ligands belong to three different clusters. Their registry (CAS) and NCI numbers are indicated. The dissociation constants obtained by SPR and tryptophan intrinsic fluorescence spectroscopy with mPAH were obtained as indicated in Materials and Methods. CAS, Chemical Abstract Service; Eq, equilibrium; F, fluorescence spectroscopy; A.f., autofluorescence at the excitation wavelength used.
by 1 (median 29.5 versus 7.9%; \( P < 0.001 \)) and 2 (median 41.1 versus 22.7%; \( P < 0.01 \)) versus the vehicle. Correspondingly, blood Phe concentrations were decreased by 1 (median 371–139 \( \mu M \); \( P < 0.001 \)) and 2 (median 271–142 \( \mu M \); \( P < 0.05 \)) as a function of the increased PAH activity. Compound 1 showed significantly stronger effects on Phe oxidation (median 29.5 versus 14.9%; \( P < 0.001 \)) and blood Phe concentrations (median 236 versus 371 \( \mu M \); \( P < 0.05 \)) when compared with \( BH_4 \). DMSO increased the tracer recovery but decreased the blood Phe concentration only slightly. This is in line with the well-known unspecific induction and activation of liver enzymes (48,49).

Secondary effects of compound exposure on the animal’s health
No secondary effects of compound exposure such as weight loss, automutilation, tumours, abnormal movements, dyspnoea, tremors, ascites or signs of centralized circulation have been observed.

Compounds 1 and 2 bind at the \( BH_4 \) pocket and stabilize PAH
To verify that the effects of 1 and 2 observed in transfected cells and in vivo are due to a direct molecular interaction with PAH, we pre-incubated purified MBP-PAH106A with Phe and 1, 2, \( BH_4 \) or a control vehicle followed by ProteinaseK digestion. Within 30–90 s, ProteinaseK cleaves MBP-PAH106A at multiple sites rendering the peptide pattern shown in Figure 5A. The degradation pattern of the protein was reduced by \( BH_4 \) pre-incubation, and 1 and 2 improved upon this effect. Moreover, 1 and 2 did not inhibit the protease activity per se as demonstrated in a control experiment with bovine serum albumin (BSA) (Fig. 5A, right panel).

Hereafter, we performed SPR competition-binding experiments to verify our initial assumption that the new PC

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**Figure 3.** Compound effects on stability and activity of PAH106A and cell viability. (A) PAH stability in cell culture. TsA201 cells were transfected with PAH or PAH106A and incubated with the indicated compounds (200 \( \mu M \)). Protein translation was stopped with 10 \( \mu g/ml \) of puromycin and PAH stability was quantified in relation to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression by immunoblotting with anti-PAH and anti-GAPDH. The bar chart depicts the quantification for PAH106A and PAH in three independent experiments except for \( BH_4 \) (two experiments). A discontinuous line separates two samples that were run in the same gel. (B) PAH106A enzymatic activity is improved in the presence of the compounds. Left panel: MBP-PAH106A was pre-incubated with the different compounds for 30 min. After digestion with enterokinase for 1 h, Phe was added and the reaction was started by addition of 50 \( \mu M \) BH4. Tyr production was quantified by the nitrosonaphthol method. Right panel: Coomassie stained gel of the time course digestion by enterokinase. The values are the mean of three reactions ± SD. Indicated one experiment out of two. (C) Viability of the HepG2 cell-line in the presence of the compounds. Cells were pre-incubated in the presence of vehicle (1% DMSO) or increasing concentrations of the compounds for 6 h. The amount of viable cells was determined with the CSK-8 assay and it is expressed as a percentage of the control values. Values are means ± SD of three independent experiments with duplicate samples.
should bind to the BH₄-binding pocket and not to a different
patch on the PAH surface. Binding of BH₄ to immobilized
PAH at a saturating concentration (500 µM) was competed
with increasing concentrations of 1 (0–300 µM) as indicated
by the non-additive intensity of the observed sensorgrams
with increasing compound concentration (Fig. 5B).

We investigated the potential orientation of 1 and 2 when
aligned to the protein-bound ligand conformation of BH₄
(Fig. 5C and D; binding mode of 3 and 6 in Supplementary
Material, Fig. S2). Analogous to BH₄, both compounds may
maintain the water network mediating interactions with the
iron and surrounding protein residues as well as the hydrophobic
interactions seen for BH₄ by its acyclic substituent. The
hydrogen bonding network is highly susceptible to the tauto-
meric form of the ligand. In these depictions, the tautomeric
forms are adapted for a best-possible agreement of interaction
patterns with the one observed for BH₄.

Therefore, we can conclude that the selected compounds,
similar to BH₄, most probably bind to the cofactor-binding
pocket of PAH and act as PC.

**DISCUSSION**

The market authorization of sapropterin dihydrochloride,
which is the synthetic form of BH₄, represents a substantial
progress in PKU management as it allows for liberalization
or replacement of burdensome lifelong treatment for many
patients (15,16). However, the high costs (~37 000–
166 000 USD per adult; [50,51]) of this prototype PC and
the low efficacy in some patients (52,53) justify a search for
alternative molecules. Pey et al. (54) showed that this is a real-
istic aim by screening 1000 compounds with a HTS
fluorescence-based stability assay. Their elegant study identi-
fied two putative PC for PKU treatment that increased stability
and activity of misfolded PAH in vitro. Oral application to
wild-type mice enhanced PAH activity and the level of the
PAH protein in the liver. Nonetheless, a final confirmation
of their efficacy in a mouse model of PKU remained elusive. Here, we report two novel PC that correct
misfolding-induced loss of PAH function in the Pah_emul
PKU mouse model. Remarkably, one compound was twice
as effective in increasing PAH activity and reducing blood
Phe concentrations when compared with BH₄. These results
confirm the high potential of the identified molecules for
further drug development to a more potent therapeutic agent
than BH₄ and they also support the validity of our PC screening
approach.

The discovery of PC has so far been based on the primary
screening of compound libraries (1000–50 000 molecules)
for mild enzyme inhibitors (55,56) or protein stabilizers
(54,57). These approaches are plausible as PC act by stabiliza-
tion against protein degradation and additionally most PC are
mild inhibitors of the target enzyme. Furthermore, this does
not limit the focus to compounds interacting with a particular
site of the target. Yet most potent PCs including BH₄ are
active-site ligands (55–58). We therefore used a structure-
based ‘directed’ in silico search to identify putative ligands
of the PAH active site. The protein-bound BH₄ conformation
was extracted from the PDB entry 1j8u and used as a query
for shape-focused screening of the NCI’s database using
ROCS. Out of more than 115 000 screened compounds, 84 poten-
tially active molecules were selected from the top-ranked
positions of the in silico hit list by visual inspection and
then tested for PAH binding.

The physical interaction of the selected compounds with
PAH was screened using SPR. Earlier, Flatmark et al. (59)
described the binding of Phe and BH₄ to immobilized
hPAH. We found equivalent patterns in the murine homolog.
Thus, we used SPR to identify compounds with favourable
binding characteristics. This led to the selection of 6 com-
 pounds out of 84 in silico hits, and the binding was confirmed
in solution using intrinsic tryptophan fluorescence spectro-
copy for 4 of the substances (2 were not eligible due to
autofluorescence). Pah_emul has previously been established as a model of
BH₄-responsive PAH deficiency (22,46). Its V106A exchange
is located in the regulatory domain of PAH and induces mis-
folding, namely a conformational pre-activation, which is inde-
dependent from substrate exposure (60). This pre-activated
enzyme is instable and prone to accelerated proteolytic de-
gradation. Even though the specific activity of PAHV106A is
unimpaired, the loss of functional PAH in Pah_emul causes a mild
hyperphenylalaninemic phenotype. In combination with a
severe mutation (compound heterozygosity), V106A leads
to an intermediate phenotype in patients (61) and mice
(34,46) equivalent to BH₄-responsive mild PKU (46,61). The

**Figure 4. In vivo efficacy of ¹³C-Phe oxidation and blood Phe concentration.**
The effects of multiple doses of the compounds on in vivo Phe oxidation and
blood Phe levels have been assessed in a 48 h trial. (A) Phe oxidation
expressed as cumulative ¹³C recovery. Left: compound 1 (3 × 10 µg/g bw)
is compared with BH₄ (same dose) and solvent (water, ascorbic acid 1%); right:
compound 2 is compared with solvent (DMSO). (B) Left: differences
of cumulative recovery at 86 min after ¹³C-Phe injection are statistically evalu-
ated by ANOVA with Dunnet’s multiple comparison test. Blood Phe concen-
trations 120 min after the last dose of compounds or controls respectively
(right). All results are expressed as means and standard errors of the mean;
the significance is indicated (*P < 0.05, **P < 0.01, ***P < 0.001); n = 6 in
all groups but 2 (n = 4).
misfolding-induced loss-of-function pathology in \( \text{Pah}^{\text{V106A}} \) is disrupted by BH4 (24). Correspondingly, our results indicate that the PC candidates identified follow the same mechanism: the six candidate compounds led to the restoration of the enzymatic activity of PAHV106A (Fig. 3B), increased protein stability of PAHV106A against intracellular proteolysis (Fig. 3A) and reduced degradation of purified PAHV106A by ProteinaseK as a result of improved conformation (Fig. 5A) with \( \text{1 and 2} \).

In vivo toxicity data were not available for all compounds. Thus, we examined the viability of HepG2 cells after incubation with our hits in order to exclude highly toxic compounds from in vivo testing. \( \text{5 and 6} \) showed toxic effects but only at rather high concentrations (\( >0.5 \text{ mM} \)). This is in good agreement with the published LD50 of \( \text{5} \) in mice (960 mg/kg; available from http://chem.sis.nlm.nih.gov/chemidplus/direct.jsp?regno=583-46-0; access date: 28th of June 2011) which is almost 100-fold above the dose applied in our in vivo tests. Consequently, all six compounds have been tested in vivo.

In single-dose experiments, two of the six compounds increased the PAH activity as quantified by cumulative \( ^{13}\text{C-Phe} \) recovery in breath tests and lowered blood Phe concentrations. In a 3-day multiple dose treatment, the response to \( \text{2} \) was in the range of that achieved by BH4 and \( \text{1} \) was twice as effective. Notably, those compounds showing a higher in vitro activity and protein stabilization effect (\( \text{3–6} \)) appeared to be ineffective in our in vivo model. Several pharmacokinetic features such as low uptake by hepatocytes and rapid metabolism or excretion may account for a low exposure of the target PAH to the active form of these compounds.

In conclusion, our innovative integrated in silico—in vitro—in vivo approach allowed for the successful identification of novel PCs for the treatment of BH4-responsive PKU. The selection of molecules that lead to the stabilization of PAHV106A without relevant inhibitory effects resulted in the identification of very potent candidates. However, ligand-binding affinity can be mutation specific (17,54,62). Thus, further studies will have to evaluate whether our compounds act similarly in the presence of other mutations rather than V106A. The necessary information will be provided by the future application of our test system and the identified compounds to other PAH variants; this may lead to the identification of other mutation-specific PCs that can be further developed in the sense of an individualized PKU therapy. Moreover, our approach can be applied to >25 other ‘misfolding diseases’ qualifying for PC treatment (22).

Figure 5. Mode of action of compounds \( \text{1 and 2} \). (A) Stabilization of PAH against proteolysis. MBP-PAHV106A was pre-incubated with \( \text{1, 2 or BH4} \) and digested with ProteinaseK (left). Control digestion of BSA treated in the same way as MBP-PAHV106A (right). (B) SPR competition of the BH4 binding by \( \text{1} \). The binding of BH4 at saturating concentration (green) was competed by increasing concentrations of \( \text{1 (0–300 \text{ mM})} \). Mixed injections of BH4 plus \( \text{1} \) showed no increase in the sensogram-response in comparison to BH4 alone. (C) Proposed binding mode of \( \text{1} \). Compound \( \text{1} \) is depicted placed in the ligand interaction site based on the alignment derived from ROCS in a tautomeric form optimized with regard to the protein-ligand hydrogen bond interaction network. The interactions observed for BH4 in Figure 1A can be largely preserved. (D) Proposed binding mode of \( \text{2} \). All major interactions between the ligand and the protein are preserved. For (C) and (D), the ligand colour code is similar to BH4 in Figure 1A.

MATERIALS AND METHODS

Compounds

Stock solutions were either prepared in DMSO (Sigma Aldrich) or in water at a concentration of 10 mM or 50 mM depending on the solubility of the compound. All molecules tested were ordered from the NCI database, USA (63). Compounds 1 and 2 were additionally ordered from Chemos GmbH and ABI Chem for animal experiments.

Hardware setup

Shape-focused virtual screening and protein-ligand docking were processed on an Intel Core 2 Quad Q6600 workstation with 4 × 2.4 GHz, 2 × 4 MB L2-Cache and 4 GB RAM using openSUSE 11.2 as operating system.

Shape-focused virtual screening

The NCI database was downloaded from the NCI’s website (2009-08-24) and their data were edited using Pipeline Pilot Student Edition (version 6.1.5, Accelrys, San Diego, CA, USA). The library was filtered using the Organic Filter and Bad Valence Filter nodes and ionized at pH 7.0. The following property filtering was applied: number of carbon atoms \( \leq 15 \); 140 < molecular weight < 600, number of H-bond donors \( \leq 8 \) and AlogP (16) \( \leq 6 \). Conformational ensembles were calculated for the refined database using OMEGA (version 2.3.2, OpenEye, Santa Fe, NM, USA) and stored in the OpenEye binary format .oeb.gz for virtual screening.

Candidate molecules for experimental testing were selected using a shape-focused virtual screening method. The basic idea of such computational approaches is to generate a complementary image of the binding site by considering the shape of a ligand (template or query). Molecules complying with this mapping are expected to be potentially active on the particular target addressed by the template. The software package ‘ROCS’ (‘Rapid Overlay of Chemical Structures’, version 3.0.0, OpenEye) was employed for similarity-based screening of the NCI database. ROCS is one of the most established programs available and provides sound performance regarding virtual 3D similarity screening (64,65).

The program assesses the volume overlap between two molecules by Gaussians that are parameterized according to the volume of heavy atoms. For activity of small organic molecules on a particular target, complementary properties in both shape and chemical functionalities are required. ROCS considers both aspects featuring dedicated scoring functions, the latter employing a so-called ‘colour force field’. This force field is based on SMARTS patterns encoding six different chemical feature types. In this way, ROCS is able to maximize both molecular shape overlay and chemical functionality overlap. ROCS’s ‘ComboScore’ function weight both components equally (functions ‘Shape Tanimoto’ and ‘Scaled Color’); they are normalized to a range from 0 to 1 and are subsequently summed up for the ComboScore. Hence, ComboScore’s values range from 0 to 2 whereby 2 stands for ‘best overlap possible’ and 0 for ‘no similarity’.

BH₄ was used as template for shape-focused virtual screening. A representative query conformation was determined using OMEGA (using OMEGA’s parameter ‘-maxconf’ and set to 1). ROCS screening was performed using default settings. The hits were ranked using ComboScore.

LigandScout (version 3.0, Inte:Ligand, Vienna, Austria) was used for visualization (66,67).

Recombinant mouse PAH purification

Recombinant mouse PAH was expressed as a fusion protein using the pMAL system (NEB) in E. coli DH5α and purified as previously described (62). Shortly, after a first amylose affinity chromatography step, the fusion protein was cleaved overnight with bovine enterokinase (NEB) and the tetrameric PAH protein was isolated by size-exclusion chromatography.

Surface plasmon resonance

A BiacoreX device (GE Healthcare) was used to screen the binding of small molecules to PAH under assay conditions similar to the procedure previously outlined (59). Per assay, 15 μg of purified PAH were immobilized by the amine coupling reaction at pH 4.5 in a CM5 matrix to achieve an immobilization level of ≈20000 RU. The reference surface was pre-treated equally to the test surface, however, without immobilized protein. The assays were carried out at 20°C with PBS running buffer at a flow of 10 μl/min. A DMSO correction was performed where necessary. Sequential 30 μl injections of each compound (25 μm) were run onto 15 μg of mPAH immobilized in a CM5-chip surface. The selection threshold for positive binding molecules was set to three times the binding signal in comparison to the noise signal typical of negative-binding compounds (68). The integrity of the enzyme was regularly checked by comparison of the response units of BH₄-binding before and after a set of injections. Compounds increasing the baseline signal irreversibly and those decreasing by more than 10% the control response of BH₄ (250 μm; RU 30) were discarded.

Tryptophan intrinsic fluorescence

Fluorescence measurements were performed with an LS50B spectrofluorimeter (PerkinElmer) at 25°C. Tryptophan fluorescence emission spectra (300–500 nm) were recorded with the excitation wavelength at 295 nm (slit width 7 nm). The compounds were added at the indicated concentrations to a quartz cuvette containing 590 μl of size exclusion chromatography (SEC) buffer (20 mM HEPEs, pH 7.0, 200 mM NaCl). The background spectra of the compounds were recorded and subtracted from the spectra obtained at the same concentrations in the presence of 0.6 μg of purified tetrameric PAH.

Assay of PAH activity

In a 96-well plate format, 75 μg of purified recombinant MBP-PAHV106A per well in Tris–HCl buffer (pH 7.4) with 40 μM ammonium iron sulphate (II) and CaCl₂ 2 mM were pre-incubated for 30 min with the compounds. Then 4 units of enterokinase were added and incubated for 1 h. PAH was
activated by the addition of 1 mM Phe for 5 min and the reaction was started by the addition of 50 μM BH₄. The produced Tyr by 1 mg of protein for 1 min (nmol Tyr min⁻¹ g⁻¹) was quantified by the nitrosonaphtol method (69) using fluorimetric detection (485/535 nm excitation and emission wavelengths) in a Victor2 plate reader (PerkinElmer).

**Compound toxicity in cell culture**

HepG2 cells were grown in Dulbecco’s modified Eagle medium media (PAA) with 4.5 g/l glucose, 10% fetal calf serum, stable glutamine and 1% antibiotics at 37°C in 5% CO₂. In a 96-well plate, 10⁴ cells per well were seeded and grown overnight. After a 6 h lasting incubation with the compounds, the Cell Counting Kit-8 (CCK-8; Sigma Aldrich) was added to the media and incubated at 37°C. The colour development indicative of cell viability was measured by absorbance at 450 nm in a plate reader (Bio-rad).

**PAH stability**

In cell culture, TsA201 cells were kept as indicated for the HepG2 cell line. 1.5 × 10⁵ cells per well were seeded in 24 well plates, grown overnight and transfected with 0.5 μg of pDEST-PAH or pDEST-PAHV106A plasmid DNA per well and the transfection reagent Primefect (Polyplus). Six hours after transfection 1 mM Phe was added to the culture media in order to simulate hyperphenylalaninemic conditions. After 18 h, the cells were incubated with the compounds or the media control during 1 h and the protein translation was stopped by addition of 10 μg/ml of puromycin. After 6 h, the cells were collected and lysed in SEC buffer with 1% Triton X and proteinase inhibitors (Roche). The cell debris were removed by centrifugation at 4°C (10⁴ g) and 100 μg of the cell lysates were analysed by SDS-PAGE on 10% polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Peqlab). Immunoblotting was done with goat anti-PAH, donkey anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (Sta. Cruz Biotechnology) and anti-PAH, donkey anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (Sta. Cruz Biotechnology) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-HRP (Sigma Aldrich). Finally, chemiluminescence detection was carried out by Immobilon western reagent (Millipore) and the heterologous protein expression was quantified relative to the protein expression controls using the freeware ‘ImageJ’. Proteinase digestion of recombinant PAH: per reaction, 15 μg of purified recombinant MBP-PAHV106A were pre-incubated with the compounds or control solvent at 4°C for 30 min. Next 25 ng of ProteinaseK (Roth) were added. Following incubation for 90 s at 37°C, the proteolysis was stopped by addition of SDS buffer and by boiling at 95°C for 5 min. The different reactions were run onto 12% SDS-PAGE gels; silver stained and the degradation products were quantified with ImageJ.

**Animals**

Pal⁺em1 (BTBR.Cg-Pah⁺em1/J) mice (30) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The animals were housed under controlled temperature conditions and kept on the basis of a cycle of 12 h light/dark period. Between experiments, water and food were available ad libitum. All blood samples were taken from the tail and collected on filter cards. Animal experiments were approved by the Austrian Ministry of Science. Tests were carried out in adult animals at age between 3 and 6 months.

**L-[1-13]C-Phe breath test and blood sampling**

The L-[1-13]C-Phe breath tests were performed as described (22,46). Sampling time was shortened to 86 min in order to reduce the mice’s stress. The cumulative recovery of ¹³C was calculated based on the ratio of ¹³CO₂ to ¹²CO₂ as previously described (47), assuming a total CO₂ production rate of 94 ml per minute per g body weight × m² body surface area (70). For the quantification of Phe, 40 μl of blood was taken from the tail vein and collected on filter cards. Blood Phe was quantified by electron spray ionization-tandem mass spectrometry.

**Single dose and 3-day treatment**

The compounds were dissolved in DMSO or water to a concentration of 2 μg/μl and injected as either a single dose or as doses of 10 μg/g body weight on three consecutive days. Two hours after the last injection, the breath test was performed and blood was taken thereafter. As a control, the solvent (DMSO or water 5 μl/g body weight) was injected.

During and after the test, the animals were observed for any signs of toxicity, with special attention paid to weight loss, autumutilation, tumours, abnormal movements or breathing, tremors, signs of ascites or centralized circulation.

**Statistics**

Statistical analyses were performed using GraphPadPrism (GraphPad Software, San Diego, CA, USA). Group mean values were compared by one-way analysis of variance (ANOVA) applying the Dunnet’s multi-comparison test or the unpaired Student’s t-test in cases of analysing two groups only.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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