Pyridoxamine and pyridoxal are more effective than pyridoxine in rescuing folding-defective variants of human alanine:glyoxylate aminotransferase causing primary hyperoxaluria type I

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Abstract

Vitamin B6 in the form of pyridoxine (PN) is one of the most widespread pharmacological therapies for inherited diseases involving pyridoxal phosphate (PLP)-dependent enzymes, including primary hyperoxaluria type I (PH1). PH1 is caused by a deficiency of liver-peroxisomal alanine:glyoxylate aminotransferase (AGT), which allows glyoxylate oxidation to oxalate leading to the deposition of insoluble calcium oxalate in the kidney. Only a minority of PH1 patients, mostly bearing the F152I and G170R mutations, respond to PN, the only pharmacological treatment currently available. Moreover, excessive doses of PN reduce the specific activity of AGT in a PH1 cellular model. Nevertheless, the possible effect(s) of other B6 vitamers has not been investigated previously. Here, we compared the ability of PN in rescuing the effects of the F152I and G170R mutations with that of pyridoxamine (PM) and PL. We found that supplementation with PN raises the intracellular concentration of PN phosphate (PNP), which competes with PLP for apoenzyme binding leading to the formation of an inactive AGT–PNP complex. In contrast, PNP does not accumulate in the cell upon PM or PL supplementation, but higher levels of PLP and PM phosphate (PMP), the two active forms of the AGT coenzyme, are found. This leads to an increased ability of PM and PL to rescue the effects of the F152I and G170R mutations compared with PN. A similar effect was also observed for other folding-defective AGT variants. Thus, PM and PL should be investigated as matter of importance as therapeutics for PH1 patients bearing folding mutations.

Introduction

Vitamin B6 refers to a group of six water-soluble pyridine compounds (B6 vitamers) comprising pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL) and their respective phosphorylated forms (PNP, PMP and PLP) (Fig. 1). The biologically active form of vitamin B6 is PLP, which serves as coenzyme for more than 160 enzymatic reactions including transaminations, racemizations, decarboxylations and aldol cleavage (1). In mammals, diet is the only source of B6 vitamers, being mainly present in meat, cereals and vegetables (2). Phosphorylated B6 vitamers must be hydrolyzed to PM, PL and PN in order to be absorbed by the intestine. They are then internalized by the liver (2), where they are interconverted.
by a pathway that relies on the action of PN (PM) 5'-phosphate oxidase (PNPOx), a flavin mononucleotide-dependent enzyme that catalyzes the oxidation of PNP or PMP to PLP, and PL kinase (PLK), an adenosine triphosphate-dependent enzyme that catalyzes the phosphorylation of PN, PM and PL to PNP, PMP and PLP, respectively (Fig. 1) (3).

Besides the protective role against cardiovascular diseases and its reported antioxidative properties (4), the beneficial effects of vitamin B6 for human health are mainly related to its effectiveness as a drug for rare diseases involving PLP-dependent enzymes (5). Vitamin B6 is usually administered as PN, which is known to be converted to PLP inside the cell and has proven to be effective in treating the symptoms of several disorders, even if the percentage of responsive patients is variable (2). Furthermore, biochemical and cell biology studies have shown that the coenzyme can exert a chaperone role, by improving the folding efficiency of variants bearing conformational defects (5).

Inherited mutations in the AGXT gene encoding human liver PLP-dependent alanine:glyoxylate aminotransferase (AGT) lead to the rare disorder primary hyperoxaluria type I (PH1) (OMIM 259900) (6). AGT catalyzes a transamination reaction that, by converting L-alanine and glyoxylate to pyruvate and glycine, plays a pivotal role in glyoxylate detoxification (7). The AGT deficiency allows glyoxylate accumulation and its oxidation to oxalate. An increased plasma and urinary oxalate concentration causes the formation of calcium oxalate (CaOx) stones in the urinary tract, which are the first disease symptoms, followed, when renal failure occurs, by systemic oxalosis with CaOx deposition in other tissues (8). The administration of vitamin B6 in the form of PN has proven to be effective in 25–35% of PH1 patients. The responsiveness seems to be associated with two of the most common missense mutations, G170R (9) and F152I (10), which cause the aberrant targeting of AGT to mitochondria where the enzyme cannot detoxify peroxisomal glyoxylate (11–13). Both mutations are pathogenic only when they co-segregate with a polymorphic form of the AGXT gene, named the ‘minor allele’ (14). The protein encoded by the minor allele (AGT-Mi) mainly differs from that encoded by the most common ‘major allele’ (AGT-Ma) due to the presence of a potential mitochondrial targeting sequence (MTS) generated by the P11L mutation. The MTS however is functionally weak, probably because AGT-Mi rapidly dimerizes and becomes incompatible with mitochondrial import (15). The G170R and the F152I amino acid substitutions have been found to destabilize the dimeric structure of purified recombinant AGT in the apo-form (16–18). This could increase the population of monomeric folding intermediates able to interact with the mitochondrial import machinery, thus explaining why the G170R-Mi and F152I-Mi variants are imported predominantly into mitochondria (12,19). Recently, Fargue et al. (20) investigated the mechanism of action of PN in a cellular model of PH1 involving Chinese Hamster Ovary (CHO) cells stably expressing glyoxylate oxidase (GO) (21). They found that PN administration increases the expression level and promotes the peroxisomal targeting of F152I-Mi and G170R-Mi. However, an unexpected reduction in the specific activity of AGT-Ma and AGT-Mi was observed, which suggested that excessive doses of PN could be detrimental for PH1 patients (20). Interestingly, it has been reported (22) that PM administration to a rat model of PH1 was able to reduce kidney crystals, thus suggesting that it could be a therapeutic agent in PH1. Although the authors rationalized the effect of PM on its ability to react non-enzymatically with the carbonyl group of glyoxylate, it cannot be excluded that PM administration could also lead to the intracellular accumulation of B6 vitamers able to improve AGT folding and/or activity. However, neither the effect of PM nor that of PL, the direct precursor of PLP, has ever been investigated in PH1.

Based on these results and considerations, we compared the effect of PM and PL with that of PN on the two PN-responsive variants G170R-Mi and F152I-Mi stably expressed in CHO-GO cells. We found that (i) the administration of PN leads to the intracellular accumulation of PNP, which competes with PLP for apoenzyme binding leading to the formation of an ‘inactive holo-enzyme’ and (ii) the administration of PM or PL increases the glyoxylate detoxification ability of cells expressing the F152I-Mi and G170R-Mi variants more efficiently than PN. This can be ascribed partly to the increased intracellular levels of PLP and PMP, which reduce the aggregation propensity and increase the intracellular stability of the variants, and partly to the absence of
intracellular PNP, which would reduce the specific activity. Transient transfection experiments also suggest that PM and PL are more efficient than PN in counteracting other PH1-causing mutations including the G41R and G161R on the major allele and the I244T on the minor allele. The possible therapeutic implications of the results obtained for PH1 patients are discussed.

Results

In order to study the effects of different B6 vitamers on the behavior of AGT-Ma, AGT-Mi and the pathogenic variants F152I-Mi and G170R-Mi, we used CHO-GO cells, a well-characterized cellular model of PH1 (21). We compared cells adapted to grow in the absence of B6 vitamers (zero-B6) with cells grown in the presence of known amounts of PN, PM or PL, the unphosphorylated B6 vitamers taken up by the cell. Cells were grown for 3 weeks, a period long enough to guarantee that all the AGT present in the cell is synthesized in the presence of the selected B6-vitamer. We also included in the study cells grown in a low-B6 medium (PN) \( \approx 0.3 \mu M \), a condition that mirrors the physiological B6 plasma concentration (20,23–25).

Effect of B6 vitamers on AGT functionality

We first determined how each vitamer affects the functionality of AGT-Ma, AGT-Mi, F152I-Mi and G170R-Mi in CHO-GO cells. Cell viability was measured upon addition to the culture medium of known amounts of PN, PM or PL, the unphosphorylated B6 vitamers taken up by the cell. Cells were grown for 3 weeks, a period long enough to guarantee that all the AGT present in the cell is synthesized in the presence of the selected B6-vitamer. We also included in the study cells grown in a low-B6 medium (PN) \( \approx 0.3 \mu M \), a condition that mirrors the physiological B6 plasma concentration (20,23–25).

Effect of B6 vitamers on AGT functionality

Figure 2. Effect of B6 vitamers on AGT functionality. The histogram shows the viability of CHO-GO cells expressing AGT-Ma, AGT-Mi, F152I-Mi and G170R-Mi after 24 h treatment with 500 \( \mu M \) glycolate. Data are expressed as percentage of the untreated control. Bars are coded as follows: cells grown in zero-B6 (black), cells grown in low-B6 (white), cells grown in the presence of 10 \( \mu M \) PN (black and white squares), 10 \( \mu M \) PM (black and white dots) and 10 \( \mu M \) PL (black and white lines). Bar graphs represent the mean + SEM. **P < 0.005 and ****P < 0.0005.

As shown in Figure 3, the expression levels of AGT-Mi, F152I-Mi and G170R-Mi in the absence of B6 vitamers relative to AGT-Ma were 72, 9 and 20%, respectively, in agreement with previous results (20). When cells were cultured in the presence of B6 vitamers (10 \( \mu M \)), we observed that, relative to culture in zero-B6 and low-B6 media: (i) the expression level of AGT-Ma or AGT-Mi did not change, (ii) the expression level of F152I-Mi increased \( \sim 7 \)- and 3-fold, respectively, and (iii) the expression level of G170R-Mi increased \( \sim 5 \)- and 2-fold in the presence of PN and \( \sim 7 \)- and 3-fold in the presence of PM or PL, respectively. No differences were observed upon increasing vitamer concentrations to 100 \( \mu M \) (data not shown). However, although the effect of PM or PL on the expression level was similar to PN in the case of F152I-Mi, it was significantly higher than PN in the case of G170R-Mi. To better clarify the effect of B6 vitamers on the expression level of the variants, we investigated whether the presence of 10 \( \mu M \) PN, PM or PL in the culture medium could change the aggregation propensity and the half-life of the two proteins (Supplementary Material, Fig. S1). Although in zero-B6 medium F152I-Mi and G170R-Mi were equally distributed between the soluble and insoluble fractions of the lysate, in the presence of B6 vitamers, the variants were almost exclusively present in the soluble fraction (Supplementary Material, Fig. S1A and B). Moreover, we found that the half-life of G170R-Mi in zero-B6 medium was 46 \( \pm \) 6 h, whereas in the presence of B6 vitamers, ~70% of the protein was still present after 72 h. Similar observations can be made for F152I-Mi,
even if, in this case, we could not determine the protein half-life in zero-B6, due to the very low expression level (Supplementary Material, Fig. S1C and D). These data suggest that all B6 vitamers prevent the aggregation and increase the intracellular stability of the two pathogenic variants.

To test whether the increased AGT expression level was paralleled by an increased specific activity, we measured the transaminase activity on the soluble lysate of cells expressing AGT-Ma, AGT-Mi, F152I-Mi or G170R-Mi grown either in the absence or in the presence of PN, PM or PL at 10 or 100 µM concentration. In agreement with previous results (20), the specific activity in lysates of CHO-GO-AGT-Mi, CHO-GO-F152I-Mi and CHO-GO-G170R-Mi cells grown in zero-B6 was about 29, 1 and 4%, respectively, relative to CHO-GO-AGT-Ma cells (Fig. 3). The presence of PN (10 µM) in the culture medium strongly decreased the specific activity of CHO-GO-AGT-Ma and CHO-GO-AGT-Mi cells to 40–50% relative to cells grown in zero-B6 or low-B6 medium. The residual activity decreased to 20% at 100 µM PN concentration (data not shown). This effect has been previously hypothesized as being due to the presence of some inactive B6 vitamers inside the cell (discussed subsequently). PN supplementation increased the specific activity of cells expressing F152I-Mi and G170R-Mi by ∼6- and 4-fold, respectively, relative to cells grown in zero-B6, and by ∼3- and 2-fold, respectively, relative to cells grown in low-B6. In contrast, the presence of PM or PL (10 µM) did not alter, or even increase, the specific activity of cells expressing AGT-Ma and AGT-Mi, whereas it increased the specific activity of cells expressing the two pathogenic variants ∼9–15-fold when compared with cells grown in zero-B6 or low-B6 medium. Similar results were obtained by treating with 100 µM PM or PL (data not shown).

Overall, the results obtained indicate that PM and PL (i) do not cause the decrease of the specific activity of cells expressing AGT-Ma and AGT-Mi observed upon PN supplementation and (ii) are significantly more effective than PN in rescuing the expression and/or the specific activity of the pathogenic variants F152I-Mi and G170R-Mi in CHO-GO cells.

Effect of treatment with B6 vitamers on AGT subcellular localization

Studies on liver biopsies and eukaryotic cells (12,13) have shown that the F152I and G170R pathogenic mutations in the background of the minor allele cause the mislocalization of AGT to mitochondria and that PN is able to partly redirect the variants to peroxisomes (20). Thus, we performed immunofluorescence microscopy experiments to investigate whether PM or PL were also able to restore the proper targeting of F152I-Mi and G170R-Mi. The results obtained were analyzed both qualitatively and quantitatively (Fig. 5 and Supplementary Material, Fig. S2). As expected, in both zero-B6 and low-B6 media, the localization of AGT-Ma and AGT-Mi was entirely peroxisomal, and this did not change upon addition of B6 vitamers (Fig. 5C and Supplementary Material, Fig. S2C). On the contrary, F152I-Mi and G170R-Mi were found predominantly in mitochondria, and the addition of 10 µM PN, PM or PL to the culture medium induced a partial redirecting of the variants to peroxisomes (Fig. 5 and Supplementary Material, Fig. S2). As expected, in both zero-B6 and low-B6 media, the localization of AGT-Ma and AGT-Mi was entirely peroxisomal, and this did not change upon addition of B6 vitamers (Fig. 5C and Supplementary Material, Fig. S2C). On the contrary, F152I-Mi and G170R-Mi were found predominantly in mitochondria, and the addition of 10 µM PN, PM or PL to the culture medium induced a partial redirecting of the variants to peroxisomes (Fig. 5A and B and Supplementary Material, Fig. S2A and B). Indeed, in the presence of PN, PM or PL, the Pearson’s coefficient for the colocalization AGT/peroxisomes in cells expressing F152I-Mi and G170R-Mi reaches values higher than 0.5, thus indicating that there is a significant amount of protein inside peroxisomes. Interestingly, relative to PN, the Pearson’s coefficient for the
colocalization AGT/peroxisomes of F152I-Mi and G170R-Mi is about 1.4- and 1.3-fold higher in the presence of PM and PL, respectively (Fig. 5C). Similar results can be observed after treatment with 100 µM vitamers (data not shown). Thus, PM and PL appear to be more efficient than PN in promoting the correct peroxisomal targeting of the two pathogenic variants. However, a significant colocalization of F152I-Mi and G170R-Mi with mitochondria was still detected in the presence of B6 vitamers (Supplementary Material, Fig. S2C), thus indicating that they do not completely prevent the mitochondrial targeting.

**Effect of treatment with PN, PM or PL on the intracellular concentration of B6 vitamers**

In order to rationalize the different effects of B6 vitamers on the functionality, expression level, specific activity and subcellular localization of the analyzed enzymatic species, we determined how the intracellular concentration of all B6 vitamers in CHO-GO cells changed upon addition of PN, PM or PL at 10 or 100 µM concentration (Fig. 6 and Supplementary Material, Fig. S3). When we supplemented cells with PN, we observed a significant increase in the intracellular concentration of all B6 vitamers (data not shown). Thus, PN and PL appear to be more efficient than PM in promoting the correct peroxisomal targeting of the two pathogenic variants. However, a significant colocalization of F152I-Mi and G170R-Mi with mitochondria was still detected in the presence of B6 vitamers (Supplementary Material, Fig. S2C), thus indicating that they do not completely prevent the mitochondrial targeting.

Analyses of the interaction of PN and PNP with AGT-Ma, AGT-Mi, F152I-Mi and G170R-Mi in the purified form

The data indicate that (i) the specific activity of both AGT-Ma and AGT-Mi expressed in CHO-GO cells is reduced only in the presence of PN, but not in the presence of PL and PM, and (ii) only upon PN supplementation, high levels of intracellular PN and PNP are present. Thus, we investigated the interaction of PN and PNP with all the analyzed species in the recombinant purified form. We compared the catalytic activity of AGT-Ma, AGT-Mi, F152I-Mi and G170R-Mi in the apo-form (0.2 µM) after 30 min incubation with either PLP alone (100 µM) or mixtures of PN/PLP or PNP/PLP in molar ratios similar to those observed inside the cells (100 µM/200 µM) (Fig. 6 and Supplementary Material, Fig. S3). The catalytic activity of AGT-Ma, AGT-Mi, F152I-Mi and G170R-Mi, although unaltered in the presence of the PN/PLP mixture when compared with PLP alone, dropped to 58, 48, 65 and 42%, respectively, in the presence of the PNP/PLP mixture. This could suggest that PNP, but not PN, is able to compete with PLP for apoenzyme binding, leading to the formation of an inactive AGT–PNP complex. In agreement with these data, the addition of PNP, but not PN, changed the CD spectrum of all the analyzed enzymatic species, even if to a variable extent, giving rise to the formation of (i) a negative dichroic signal at 340 nm, attributable to PNP bound to the active site, and (ii) a positive dichroic signal at 260 nm, likely related to subtle changes of the aromatic residues in the vicinity of the active site occurring upon PNP binding (Supplementary Material, Fig. S4). We determined the equilibrium dissociation constant for PNP of each species [Kd(PNP)] by measuring the intensity of the CD signal at 340 nm as a function of PNP concentration and found it in the micromolar range in all cases (Table 1). As expected, neither PM nor PL up to 200 µM concentration were able to interact with each of the analyzed enzymatic species in the apo-form. All these data, along with the finding that the amount of intracellular PNP and PLP is comparable in CHO-GO cells grown in the presence of PN, suggest that the two phosphorylated vitamers could compete for apoAGT binding inside the cell. Unfortunately, as the rate constants for coenzyme binding to the apo-form of each species (=0.3 min⁻¹) are lower than those of the other steps of the enzymatic reaction, we were not able to investigate the inhibition mechanism of PNP as well as to determine the inhibition constants. However, it
has been shown previously that PNP behaves as a competitive inhibitor toward PLP in rat tyrosine aminotransferase (27).

Effect of treatment with B6 vitamers on other folding-defective AGT variants

We investigated whether the action of PM and PL could extend to other folding-defective variants of AGT including G41R-Ma, G161R-Ma and I244T-Mi, which are susceptible to variable degrees of aggregation and/or proteolytic degradation (12,28–30). The variants were transiently expressed in CHO-GO cells grown in zero-B6 medium either in the absence or in the presence of PN, PM or PL at 10 μM concentration (Fig. 7). In the case of G41R-Ma, all B6 vitamers increased the protein expression level by ~1.5-fold. However, although the specific activity of this variant decreased slightly in the presence of PN, in the presence of PM it increased by 1.3-fold. G161R-Ma displayed a similar behavior, with the expression level increasing by 2.2-fold in the

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Figure 5. Effect of B6 vitamers on the peroxisomal localization of AGT pathogenic variants. CHO-GO cells expressing F152I-Mi (A) and G170R-Mi (B) grown in media at different B6 vitamer concentrations were fixed and colored as follows: anti-AGT (red) and anti-peroxisomal GO (green). Nuclei were stained with Dapi (blue). Merge and single-channel images come from a single z-plane. Scale bar: 10 μm. (C) Quantitative analysis of the effect of B6 vitamers. The histogram bars represent the Pearson’s coefficient calculated for the colocalization AGT/peroxisomes and are coded as follows: cells grown in zero-B6 (black), cells grown in low-B6 (white) and cells grown in the presence of 10 μM PN (black and white squares), 10 μM PM (black and white dots) and 10 μM PL (black and white lines). The results are given as means (+SEM); at least, 30 individual cells were analyzed for each sample.
presence of B6 vitamers and the specific activity increasing by 1.5-fold in the presence of PN and by 2.7- and 2.4-fold in the presence of PM or PL, respectively. In the case of I244T-Mi, although PN did not affect the expression of the variant, PM and PL increased the protein levels by ∼2.5-fold. Moreover, the specific activity decreased by 1.7-fold in the presence of PN, whereas it increased by 1.6- and 1.4-fold in the presence of PM or PL, respectively. These data suggest that PM and PL could better counteract the effects of other folding mutations associated with PH1, by more efficiently improving their specific activity and/or their expression level.

**Discussion**

PN administration is the only effective pharmacological treatment currently available for PH1 patients (31). Although the administration of PN has been used routinely in the management of PH1 patients during the last 40 years, it has proved effective in only 25–35% of the cases. The responsiveness appears to be associated with the presence of the F152I (9) and G170R (10) mutations, which primarily affect AGT folding and subcellular localization (11–13). However, the majority of the patients respond to PN doses that are lower than recommended (10 mg/kg/day) (9,32), and urinary oxalate levels are not correlated with serum B6 levels (31). These findings suggest that the molecular and cellular effects of PN administration could be more complex than expected.

In agreement with previously published results (20), we found that addition of PN to the culture medium raised the intracellular concentrations of PLP, PN, PL and PNP. PLP does not only work as a coenzyme, but also exerts a stabilizing effect on the dimeric structure of AGT (16). It has therefore been suggested that it may act as a pharmacological chaperone by promoting the attainment of the native conformation and the correct targeting of the F152I-Mi and G170R-Mi variants (20). Our results show that PN and PL do not bind AGT-Ma and AGT-Mi or the two pathogenic variants in the recombinant purified form when used in concentrations up to 200 µM. This is expected giving that these vitamers lack the phosphate group, which establishes several important interactions with active site residues of the apoprotein (33). All of the variants were however able to interact with PNP (Supplementary Material, Fig. S4). The $K_{D(PNP)}$ value of each enzyme is about 10-fold higher than the corresponding $K_{D(PLP)}$ value (Table 1), probably because PNP does not form the Shiff base linkage with the ε-amino group of Lys209 (33). Nevertheless, when both vitamers are present in vitro at comparable concentrations, they compete for apoAGT binding, as demonstrated by the significantly reduced catalytic activity of the apoenzyme of AGT-Ma, AGT-Mi, F152I-Mi and G170R-Mi upon incubation with a PNP/PLP mixture when compared with PLP alone. Given that PNP and PLP intracellular levels are similar upon PN supplementation, it is then reasonable to hypothesize that PNP could also compete with PLP for AGT binding in our cellular model, leading to the formation of an inactive AGT–PNP complex. This would explain the reduced AGT specific activity and functionality of CHO-GO-AGT-Ma.

**Table 1.** Equilibrium dissociation constants for PNP [$K_{D(PNP)}$] and PLP [$K_{D(PLP)}$] of AGT-Ma, AGT-Mi, F152I-Mi and G170R-Mi

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_{D(PNP)}$ (µM)</th>
<th>$K_{D(PLP)}$ (µM)</th>
</tr>
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<tbody>
<tr>
<td>AGT-Ma</td>
<td>2.23 ± 0.42</td>
<td>0.27 ± 0.03a</td>
</tr>
<tr>
<td>AGT-Mi</td>
<td>2.91 ± 0.46</td>
<td>0.26 ± 0.02b</td>
</tr>
<tr>
<td>F152I-Mi</td>
<td>2.61 ± 0.72</td>
<td>0.09 ± 0.001b</td>
</tr>
<tr>
<td>G170R-Mi</td>
<td>1.21 ± 0.41</td>
<td>0.4 ± 0.1c</td>
</tr>
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$^a$From (7).
$^b$From (18).
$^c$From (16).
and CHO-GO-AGT-Mi cells in the presence of PN in the culture medium. Although F1521-Mi and G170R-Mi are similar to AGT-Ma and AGT-Mi with regard to their binding affinities for PNP and PLP, no inhibitory effects of PN supplementation were observed in CHO-GO-F1521-Mi and CHO-GO-G170R-Mi cells. It is likely that the global improvement of the specific activity and glyoxylate detoxification ability observed in the presence of PN is a balance between the inhibitory effect exerted by PNP and the chaperone effect exerted by PLP. If this were the case, the administration of other B6 vitamers, which do not increase the intracellular PNP levels, could enhance the chaperone effects of the coenzyme by preventing the formation of inactive holoenzyme complexes.

On the basis of these suggestions, we investigated the effect of supplementing CHO-GO cells expressing AGT-Ma, AGT-Mi, F1521-Mi and G170R-Mi with PM or PL. We found that the presence of PM or PL increased the intracellular concentration of PL, PLP, PM and PMP, but not that of PN and PNP (Fig. 6). The absence of PNP correlates with the finding that the specific activity and functionality of CHO-GO-AGT-Ma and CHO-GO-AGT-Mi cells did not decrease upon PM or PL supplementation with respect to cells grown in zero-B6. Interestingly, when compared with PN, the supplementation of cells expressing F1521-Mi and G170R-Mi with PM or PL resulted in a significantly higher increase in their ability to detoxify glyoxylate, as measured by increased cell survival (Fig. 2). This is consistent with the higher effects exerted by these two vitamers on the specific activity, the peroxisomal localization and, in the case of G170R-Mi, the expression level of the variants (Figs 3–5). These beneficial effects cannot be ascribed to PM or PL accumulation, because the non-phosphorylated vitamers do not bind the apoenzyme. Rather, they are probably due to (i) the slightly increased amount of intracellular PLP (particularly in the case of PL supplementation) and (ii) the accumulation of PMP (particularly in the case of PM supplementation), which is able to form an enzymatically active AGT–PMP complex (7) and could exert a chaperone role similar to that of PLP. Further evidence supporting the chaperone effect of B6 vitamers is the finding that supplementation with PN, PM or PL reduced the aggregation and increased the intracellular stability of both F1521-Mi and G170R-Mi, thus indicating that coenzyme binding favors the correct folding and prevents the misfolding and the consequent degradation of the two variants, in agreement with previous reports on other PH1-causing variants (17,20,28,30). It should also be noted that the chaperone effect of PM and PL is comparable with that of PN in the case of F1521-Mi, whereas it is higher than that of PN in the case of G170R-Mi. This could be explained by the very low affinity that F1521-Mi has for PMP and by the finding that it is mostly present as apoenzyme in the cell (12,18). Thus, this variant cannot benefit from the chaperone effect of PMP that accumulates in the cell upon PM or PL supplementation. Nevertheless, when compared with PN supplementation, the administration of PM and PL had a greater impact on the specific activity than the expression level of the variants. This implies that the increased specific activity is not solely due to the chaperone effect of PLP and possibly PMP, but also to the absence of the inhibitory effect exerted by PNP. Thus, we can conclude that while the net effect of PN supplementation is a balance between the positive chaperone effect of PLP and the negative inhibitory effect of PNP, supplementation of cells with PM or PL is more successful at correcting the enzyme deficiency by only eliciting the positive effects of PLP and PMP. On the basis of our data, we can also suggest that while for F1521-Mi the greater effect of PM and PL relative to PN occurs mainly due to the absence of PNP inhibition, in the case of

Figure 7. Effect of B6 vitamers on expression level and specific activity of CHO-GO cells transiently expressing G41R-Ma (A), G161R-Ma (B) or I244T-Mi (C). CHO-GO cells previously adapted to grow in the absence or presence of 10 µM cellular lysate were subjected to SDS–PAGE, immunoblotted with anti-AGT from rabbit (1:6000) and then detected by chemiluminescence. Immunoblot lanes are colored as follows: cells grown in zero-B6 (Z) and cells grown in the presence of 10 µM PN, 10 µM PM and 10 µM PL. For the determination of the AGT-specific activity, 100 µg of lysate was incubated with 0.5 m M l-alanine, 10 mM glyoxylate and 200 µM PLP at 25°C in 100 mM KP, pH 7.4, and the pyruvate produced was determined by a spectrophotometric assay. Bars are colored as follows: cells grown in zero-B6 (black) and cells grown in the presence of 10 µM PN (black and white squares), 10 µM PM (black and white dots) and 10 µM PL (black and white lines). Data are representative of two independent experiments. Bar graphs represent the mean ± SEM.
G170R-Mi, the effects seen are also due to the chaperone effect of PLP and/or PMP.

Notably, we did not observe any significant difference between the effects of PM or PL on F152I-Mi and G170R-Mi at 10 or 100 µM concentration, although some differences in the intracellular content of B6 vitamers were present. It can be speculated that the intracellular levels of PLP and PMP at 10 µM concentration are sufficient to give the maximum chaperone effect of the coenzyme for each variant.

Overall, our data demonstrate that PM and PL are more effective than PN in correcting the effect of the F152I and G170R mutations in a cellular model of PH1. Although we are aware that CHO-GO cells may not reflect in full the physiological hepatocyte environment, our data suggest that PM or PL could be used in the treatment of patients bearing the mutations described. Moreover, as most pathogenic mutations cause folding defects without significantly altering the AGT active site (34), it could be argued that PN could exert a more general inhibitory effect on many AGT variants. As such, variants that appear to be non-responsive to PN could respond to PM or PL administration. In this regard, preliminary data on the folding-defective variants G41R-Ma, G161R-Ma and I244T-Mi transiently expressed in CHO-GO cells indicate that PM or PL performs better than PN in rescuing the expression level and/or the activity (Fig. 7). We can also speculate that although the performance of PM and PL is likely due to an increased chaperone effect in the case of I244T-Mi, it is mainly driven by the absence of PNP inhibition in the case of G41R-Ma and G161R-Ma. Whatever the mechanism is, these data would suggest that the action of PM and PL could be more general, thus supporting their possible clinical use.

All B6 vitamers are characterized by a high bioavailability and can be found in different types of foods including meat, cereals and vegetables (2). In addition, the administration of PM and PL to mice does not result in the subtle neurological symptoms that can appear after PN administration (2). All these considerations suggest that PM and PL should be considered as possible therapeutic approaches for PH1 patients bearing folding mutations, with a rapid bench-to-bedside transition. In this regard, preliminary data on the folding-defective variants G41R-Ma, G161R-Ma and I244T-Mi transiently expressed in CHO-GO cells indicate that PM or PL performs better than PN in rescuing the expression level and/or the activity (Fig. 7).

Materials and Methods

Materials

L-alanine, sodium glyoxylate, rabbit muscle l-lactate dehydrogenase, glycolate, PN, PM, PL, PLP and PMP were purchased from Sigma-Aldrich (MO, USA). Modified Ham’s F12 Glutamax medium without PN, fetal bovine serum (FBS), dialyzed FBS (DFBS), zeocin and geneticine (G-418) were purchased from Life Technologies (CA, USA). The anti-rabbit HRP antibody was purchased from GE Healthcare (MA, USA). PL methyl D3 hydrochloride (>98% atom % D) was purchased from Isotec, PM methyl D3 hydrochloride (>98% atom %D) was purchased from Sigma, PN D2 hydrochloride (5-hydroxymethyl-D2) (>98% atom %D) from CDN Isotopes and D2 PL 5'-phosphate was kindly supplied as a gift by Professor Coburn, Department of Chemistry, Indiana University, Purdue University, Forte Wayne. All other chemicals were of the highest purity available.

Cell culture, transfection and lysis

CHO-GO cell lines stably expressing AGT-Ma, AGT-Mi, F152I-Mi or G170R-Mi were adapted for growing in customized Ham’s F12 Glutamax medium without PN hydrochloride supplemented either with 10% (v/v) DFBS, referred as ‘zero-B6’, or with 10% (v/v) FBS, referred as ‘low-B6’ ([PN] ≪ 0.3 µM) (20). To analyze the effect of B6 vitamers, PN, PM or PL at 10 or 100 µM concentration was added to the zero-B6 medium, cells were allowed to grow for 3 weeks and medium was replaced every 48 h. In all cases, media were supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml), and cells were cultured in an incubator at 37°C under O2/CO2 (19:1) atmosphere. The expression of AGT and GO was maintained by adding G-418 (0.8 mg/ml) and zeocin (0.4 mg/ml) to the culture medium, respectively. For transient transfection, 6 × 10⁵ CHO-GO cells adapted to grow in zero-B6 medium either in the absence or in the presence of 10 µM PN, PM or PL were transfected with Turbofect™ Transfection Reagent (Fermentas), according to the manufacturer’s instructions.

Cells were harvested and lysed by freeze/thawing (five cycles) in phosphate-buffered saline (PBS), pH 7.2, containing protease inhibitor cocktail (Complete Mini, Roche). The lysate was then treated with DNAse (10 U) at room temperature for 45 min (29).

The whole cell extract was subjected to centrifugation (2900g, 10 min, 4°C) to separate the soluble and insoluble fractions. The protein concentration in the soluble fraction was measured using the Bradford assay (29).

Glycolate toxicity assay

Cells grown in the absence or in the presence of 10 or 100 µM PN, PM or PL were harvested and seeded at a density of 8000 cells/well in a 96-well plate. After 24 h, glyoxylate production was induced by the addition of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid-buffered glycolate, pH 7.0, at a final concentration of 0.5 mM. Cell viability was evaluated after 24 h by crystal violet staining (Sigma), as reported previously (39). Seven replicates were performed for each assay condition and for each cell line. Data were expressed as percentage of live cells relative to their untreated control.

Western blot analysis and half-life determination

About 2 µg of cell lysate was loaded per lane on a Mini Protein TGX™ (10%) pre-cast gel (Bio-Rad), along with the Precision plus Kaleidoscope™ (Bio-Rad, Hercules, CA, USA) molecular mass markers. Following transfer to a nitrocellulose membrane by the iBlot device (Life Technologies), the membrane was blocked with 5% milk solution in TBST (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h at 37°C. For AGT detection, the membrane was incubated with polyclonal rabbit anti-AGT serum (1:6000), washed three times in TBST and then incubated with peroxidase-conjugated anti-rabbit IgG (1:10 000). Blotted proteins were detected and quantified with ECL™ (Millipore), using the ChemiDoc XRS Imaging System (Bio-Rad). The band volume (intensity × mm²) was determined using the software Quantity One 4.6.3.

To determine the half-life of F152I-Mi and G170R-Mi, the stable clones of CHO-GO cells were treated with cycloheximide.
Immunofluorescence microscopy

Cells grown in the absence or in the presence of 10 or 100 µM PN, PM or PL were harvested and seeded at a density of 3 x 10^6 cells/well into a 13-mm glass cover slip on a 24-well plate. After 24 h, cells were fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.3% Triton X-100 in PBS and blocked in 3% bovine serum albumin. For the immunolabeling, guinea-pig or rabbit polyclonal anti-human AGT and anti-peroxisomal G0 from rabbit were used as primary antibodies, and Alexa Fluor-conjugated antibodies (AF488 and AF555, Life Technologies) were used as secondary antibodies. Mitochondria were stained with Mitotracker Red (CMXRos version, Molecular Probes, Invitrogen) and nuclei with DAPI. The cover slips were mounted over slides in AF1 medium (Dako). Images were captured using a confocal laser-scanning fluorescence microscope Leica SPS (Leica Microsystems, Mannheim, Germany) at 63× magnification and processed using Adobe Photoshop and Image software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA [http://rsb.info.nih.gov/ij/, 1997–2008]). Pearson’s coefficients were calculated using the ImageJ JACoP plugin (http://rsb.info.nih.gov/ij/).

Protein expression and purification

His-tagged AGT-Ma, AGT-Mi, F152I-Mi and G170R-Mi variants were expressed in Escherichia coli and purified with the procedure already described, and the apo-form of each variant was prepared as described previously (7). The protein concentration in the AGT samples was determined by absorbance spectroscopy using an extinction coefficient of 9.54 x 10^4 M/cm at 280 nm (7).

Preparation of PNP

An aliquot of 200 µl of NaBH₄ (1 M) was added to a freshly prepared solution of PLP (10 mM) in 100 mM potassium phosphate buffer (KP), pH 7.4, as reported previously (40). The complete PLP reduction was spectroscopically verified. The reaction was then acidified with trichloroacetic acid (TCA) 10% (v/v) final concentration and loaded onto a Supelcosyl C18 HPLC column previously equilibrated and run with 50 mM KP, pH 2.35. The absorbance at 295 nm was monitored, and the peak corresponding to PNP was collected, lyophilized and resuspended in water. The PNP concentration was determined by absorbance spectroscopy using an extinction coefficient of 7.4 x 10^3 M/cm at 325 nm (40).

Enzymatic activity assays

The AGT enzymatic activity of CHO-G0 cells expressing AGT-Ma, AGT-Mi, F152I-Mi or G170R-Mi was determined by incubating 100 µg of soluble lysate with 0.5 mM L-alanine and 10 mM glyoxylate for 30 or 60 min in the presence of 200 µM PLP. The reactions were stopped by adding TCA 10% (v/v). Pyruvate formation was measured by the spectrophotometric assay described previously (7). The enzymatic activity of the purified enzymes was assayed by incubating the apo-form (0.2 µM) of all the analyzed AGT variants with PLP (100 µM) or with a mixture of PLP and PNP (100 and 200 µM, respectively) or PLP and PN (100 and 200 µM, respectively) for 30 min at 25°C in 100 mM KP, pH 7.4. Upon addition of 0.5 M L-alanine and 10 mM glyoxylate, the reaction was stopped after 10 min at 25°C in TCA 10% (v/v). Pyruvate formation was measured by using a spectrophotometric assay coupled with lactate dehydrogenase, as described previously (7).

Spectroscopic studies

Absorption and circular dichroism (CD) spectra were registered with a Jasco V-550 spectrophotometer and Jasco J-710 spectropolarimeter, respectively, both equipped with 1 cm path length quartz cuvettes at a protein concentration ranging from 1 to 10 µM in 100 mM KP buffer, pH 7.4 at 25°C. The equilibrium dissociation constants for PNP (K_{D(PNP)}) of AGT-Ma, AGT-Mi, F152I-Mi and G170R-Mi were determined by measuring the CD signal at 340 nm of the apo-form (9.5 µM) in the presence of PNP at concentrations ranging from 0.5 to 100 µM. All the experiments were carried out in 100 mM KP buffer, pH 7.4. The K_{D(PNP)} values for the AGT–PNP complexes were obtained by using the equation:

\[ Y = \frac{[P_{\text{NP}}]}{[E]_{\text{t}} + [P_{\text{NP}}] + K_{D(PNP)}} - \left(1 - \frac{4[E][P_{\text{NP}}]}{2[E]_{\text{t}}[P_{\text{NP}}]}\right)^{1/2} \]

where [E] and [P_{\text{NP}}] represent the total concentrations of the enzyme and PNP, respectively, Y refers to the 340 nm dichroic signal changes at a PNP concentration, [PNP], and Y_{\text{max}} refers to the aforementioned spectral change when all the enzyme molecules are complexed with PNP.

Measurement of the concentration of the B6 vitamers

After 3 weeks of culture in the presence of controlled concentration of B6 vitamers, cells were harvested, resuspended in 50 µl of water and vortexed prior to freeze-thawing five times in a methanol-dry ice mix and a 37°C water bath to gently lyse cells. The lysate was centrifuged at 4°C for 10 min (29,200:g) to remove any cellular debris. An aliquot of 10 µl of the resulting supernatant was precipitated with 0.15 N TCA (D3-PM and D2-PN, 10 nmol/l; D3-PL, 50 nmol/l) were added to the TCA to enable quantification of the individual vitamers. Each sample was then vortexed for 30 s, left on ice in the dark for 60 min and centrifuged at 4°C for 10 min. The resulting supernatant was transferred to a HPLC vial and placed in an autosampler, protected from light and kept at 4°C until sample injection (23). LC-MS/MS was performed using an Acquity Ultra Performance LC system linked to a triple quadrupole Xevo TQ-S instrument (Waters, Manchester, UK). An aliquot of 15 µl of the supernatant was injected, and the B6 vitamers were separated on an Acquity UPLC HSS T3 column (1.8 µm x 2.1 mm x 50 mm) fitted with a HSS T3 VanGuard guard column (Waters). A gradient mobile phase consisting of A (3.7% acetic acid with 0.01% HFBA) and B (100% methanol) at a flow rate of 0.4 ml/min was used. The initial mobile phase consisted of 97.5% A for 0.4 min with 2.5% B then increasing linearily to 50% B over 3.35 min prior to re-equilibration of the column. Analytes were detected in positive ion mode using multiple reaction monitoring (MRM). All B6 vitamers could be uniquely identified on the basis of their retention time and the m/z ratios of their corresponding parent and daughter ions (23). Data were acquired and analyzed using MassLynx software (Waters). B6 vitamers were quantified by the addition of a known concentration of deuterated internal standard. The same concentration of internal standard was used to construct calibration curves with reference vitamers (except PNP which was not available). The amount of PLP and PMP present...
was calculated from the ratio of the signal area for the vitamer to the signal area for D2 PLP. Similarly, D3 PL was used to calculate the amount of PL and PM present, D2 PN for PN and D2 PA for PA. PNp was quantitated by determining the ratio of the signal from PNp to the signal from deuterated PLP. This was converted to a concentration by using the calibration curve for PLP. If the calibration for PNp was identical to that for PLP, this would be the concentration of PNp in nmol/l; however, we cannot make that assumption and so PNp concentrations are expressed in the results as ‘concentration units’.

Statistical analysis

Experiments were performed at least in triplicate. Statistical analysis was performed with GraphPad Prism Version 5.0 (GraphPad software, San Diego, CA, USA).

Supplementary Material

Supplementary Material is available at HMG online.

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Conflicts of Interest statement. The authors have no conflict of interest to declare.

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