Rescue of lethal molybdenum cofactor deficiency by a biosynthetic precursor from \textit{Escherichia coli}

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Substitution therapies for orphan genetic diseases, including enzyme replacement methods, are frequently hampered by the limited availability of the required therapeutic substance. We describe the isolation of a pterin intermediate from bacteria that was successfully used for the therapy of a hitherto incurable and lethal disease. Molybdenum cofactor (Moco) deficiency is a pleiotropic genetic disorder characterized by the loss of the molybdenum-dependent enzymes sulphite oxidase, xanthine oxidoreductase and aldehyde oxidase due to mutations in Moco biosynthesis genes. An intermediate of this pathway—‘precursor Z’—is more stable than the cofactor itself and has an identical structure in all phyla. Thus, it was overproduced in the bacterium \textit{Escherichia coli}, purified and used to inject precursor Z-deficient knockout mice that display a phenotype which resembles that of the human deficiency state. Precursor Z-substituted mice reach adulthood and fertility. Biochemical analyses further suggest that the described treatment can lead to the alleviation of most symptoms associated with human Moco deficiency.

**INTRODUCTION**

All molybdenum (Mo)-containing enzymes in man, animals, plants, archaea and bacteria—with the sole exception of the prokaryotic nitrogenase—require a cofactor consisting of an organic moiety, called molybdopterin (MPT), and Mo (1). This unique and ‘universal’ molybdenum cofactor (Moco) has the same core structure in all Mo-enzymes from all phylogenetic groups and is very unstable in a free form, especially under aerobic conditions, when it is not bound to an apoprotein (2). The multistep biosynthetic pathway that converts a guanosine derivative to active Moco is evolutionarily conserved (1) and the corresponding proteins from different organisms involved in Moco synthesis are extremely homologous (3–7). A mutational block in Moco biosynthesis leads to the combined loss of activities of all Mo-enzymes including sulphite oxidase (8,9). Human Moco-deficiency is a severe autosomal-recessive genetic disorder that is clinically indistinguishable from the less frequently isolated form of sulphite oxidase deficiency (10–12). Although a few mild cases have been reported (13), most affected patients exhibit neurological abnormalities such as untreated seizures and brain dysmorphologies, which result from sulphite toxicity, sulphate deficiency or both. Since until now no effective therapy has been available such patients usually die in early childhood (14).

The first eukaryotic genes involved in Moco biosynthesis to be isolated were from the plant \textit{Arabidopsis thaliana} (15). Search for homologous sequences led to the identification of the first human gene required for Moco biosynthesis, \textit{MOCS1} (3). The gene products MOCS1A and MOCS1B, whose expression involves a complex pattern of alternative splicing (16–18), convert a guanosine derivative to the sulphur-free precursor Z that already contains the unique four-carbon side chain of MPT (19). Mutations in the \textit{MOCS1} gene are found in two-thirds of Moco-deficient patients, which represent...
Results

Purification and stability of precursor Z

Precursor Z was purified to homogeneity from E. coli by a three-step HPLC chromatographic procedure (Fig. 1) as evidenced by UV–visible spectroscopy (Fig. 2B) as well as mass spectrometry (data not shown). To obtain large quantities, we used a bacterial strain that accumulates precursor Z due to a defect in its conversion to MPT. A further increase in yield was achieved by the over-expression of MoaA and MoaC proteins catalyzing the first step of Moco synthesis. This resulted in a ~5000-fold accumulation of precursor Z when compared with wild-type cells with an average yield of 40 μg/l E. coli culture and a final concentration of 30–140 μg/ml. Purified precursor Z exhibits the same absorption spectrum as noted before (19) and it could be quantitatively oxidized to compound Z (23,24) while its biosynthesis is conserved among all organisms (15–17).

We have recently described the construction of an animal model for human Moco deficiency type A (25). These MOCS1 knockout mice display a severe phenotype reflecting the biochemical characteristics of human Moco-deficient patients. They fail to thrive and die within the first 12 days after birth, with an average life span of 7.5 days. Here we show that this lethal phenotype can be efficiently neutralized by a biosynthetic intermediate, precursor Z, purified from Escherichia coli.

In vitro restoration of MPT biosynthesis

The biological activity of purified precursor Z was proven by quantitative in vitro conversion to MPT using purified E. coli MPT synthase (26). With a 2-fold excess of MPT synthase we were able to convert precursor Z almost completely into MPT as determined by HPLC formA analysis (data not shown). In order to determine the amount of precursor Z needed for a successful restoration of Moco biosynthesis in MOCS1-deficient mice, we performed an in vitro reconstitution in liver crude protein extract derived from a 5-day-old MOCS1-deficient mouse with different amounts of precursor Z (Fig. 3). Almost complete restoration of Moco biosynthesis—as quantified by the nit1-reconstitution assay (see Materials and Methods)—was achieved by using 134 ng precursor Z/mg protein, whereas 27 ng precursor Z resulted in ~50% reconstitution. Total protein content of the liver extract used in the experiment was 6 mg (derived from 50 mg total liver tissue) resulting in a total requirement of 0.8 μg precursor Z per animal for complete restoration of MPT synthesis under in vitro conditions. Accordingly, for an adult animal with a body weight of 20 g and an approximate liver mass of 1 g, 16 μg precursor Z would be needed for complete restoration, but only 3 μg for 50% activation. Since the production of precursor Z was limited, we made no attempt to use saturating amounts of precursor Z in the following experiments, but rather used the indicated dosages. Although the in vitro titration experiment did not address how much of the Moco will be incorporated into apo-sulphite oxidase, the determined amount of precursor Z reflects the maximal capacity of MPT production in the liver of the MOCS1-deficient mice.

Rescue of the lethal phenotype in Moco-deficient mice

It has been firmly established that the loss of sulphite oxidase activity is exclusively responsible for the severe neurological...
damage seen in Moco deficiency (11). The half-life of sulphite oxidase in rat liver was determined to be 3–4 days (27). Based on this and the earlier calculations, we performed intra-hepatic injections of precursor Z as summarized in Table 1. Whilst untreated homozygous mutant animals showed a significantly slower gain of weight in comparison with their littermates and died very early (25), it was virtually impossible to identify the mutant homozygotes in litters subjected to the earlier-mentioned treatment before genotyping (Fig. 4). No weight, behavioural or other phenotypic differences between precursor Z-treated MOCS1-deficient mice and their wild-type and heterozygous littermates were observed until weaning.

As can be seen in Table 1, there is a strong correlation between the total amount of precursor Z injected per week and the average lifespan of MOCS1-deficient mice. The lowest dosage was 0.2 μg precursor Z injected every second day. Under this treatment, 11 MOCS1−/− mice from three litters developed normally and gained weight comparably to their littermates. Although no behavioural differences were observed, all 11 homozygous animals were selectively killed by the parents between days 14 and 19. With 1–2 μg every third day the mice grew older and weaning was performed between days 20 and 30, after which the MOCS1−/− animals appeared to be slower in terms of their escape reactions and exploratory behaviour than the corresponding control animals. This difference disappeared around day 40, after which all MOCS1−/− animals were as agile and lively as the controls. Approximately half of the substituted animals were lost between days 20 and 40. The most successful treatment involved injections of 2–4 μg precursor Z every third day. Under this regimen, no animal died between days 40 and 70, a time in which male and female mice reached adulthood and fertility. Ten matings of these substituted mice amongst each other and another four with MOCS1 heterozygotes (two matings with homozygous males and two with homozygous females) resulted in timely delivered healthy offspring, which in the case of a MOCS1−/− genotype had to be substituted with precursor Z again for normal development.

**Deficiency phenotype appearance after drug withdrawal**

To determine whether the external supply with precursor Z could be withdrawn after certain developmental stages, we stopped the injection of precursor Z in homozygous mutant animals at days 1 (n = 3), 12 (n = 4), 26–28 (n = 3) and 64–68 (n = 2) after birth. A single precursor Z injection at day 1 resulted in death after 15–18 days. Withdrawal of precursor Z at day 12 resulted in diminished exploratory behaviour starting on day 18 (i.e. 6 days after the last injection) and death 1–3 days later (i.e. 7–9 days after the last injection). At the age of 26–28 days, which falls in the above-described critical period, withdrawal of precursor Z led to death within 4–7 days. Withdrawal at days 64–68 led to reduced exploratory behaviour after 7 days and death

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**Figure 2.** Purified precursor Z is stable under physiological conditions. (A) Oxidation of precursor Z to compound Z. (B) UV–VIS absorption spectra of 22 μM precursor Z incubated for 17 h at room temperature in 2× PBS buffer (pH 6.9). Spectra were recorded in time intervals after dilution in buffer as indicated. (C) Kinetic analysis of the oxidation of precursor Z recording the absorption at 350 nm of the spectra shown in (B).

**Figure 3.** In vitro reconstitution of MOCS1-deficient liver extracts with precursor Z. Crude protein extract of a 5-day-old MOCS1−/− mouse was incubated (1 h) with the indicated amounts of purified precursor Z per mg protein. Reconstitution of MPT synthesis was determined by the nit1 reconstitution assay.

**Table 1.** Survival of MOCS1−/− mice under different treatment schemes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Average lifespan (days)</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>28</td>
<td>7.5</td>
</tr>
<tr>
<td>0.2 μg every second day</td>
<td>11</td>
<td>17.7</td>
</tr>
<tr>
<td>1 μg every third day</td>
<td>5</td>
<td>25.2</td>
</tr>
<tr>
<td>1 μg (before weaning) and 2 μg</td>
<td>30</td>
<td>32.9</td>
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<tr>
<td>(after weaning) every third day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 μg precursor Z every second day</td>
<td>15</td>
<td>47.4</td>
</tr>
<tr>
<td>2 μg (before weaning) and 4 μg</td>
<td>18</td>
<td>91.8</td>
</tr>
<tr>
<td>(after weaning) every third day</td>
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*Precursor Z was injected transcutaneously into the liver.
*Data from (25).
*Weaning was done between days 20 and 30 p.p.
after 9–10 days. For a successful reversal of the phenotype, the external supply with precursor Z had to start within the first five days p.p. Onset of therapy at days 7 and 10 resulted in no significant improvement of either the phenotype or the longevity (data not shown).

**In vivo restoration of Moco synthesis and Moco-dependent enzyme activities**

For homozygous $MOCS1^{-/-}$ animals, which had regularly received precursor Z injections and appeared normal in weight, appearance and behaviour, treatment was discontinued and the animals were subsequently sacrificed on days 0, 1, 4 and 7 after their last precursor Z injection to determine MPT content (using the nit1-reconstitution assay) and Mo-enzyme activities in the liver (Fig. 5A–C). On the day of drug injection the highest MPT level (16 ± 1% of wild-type) was observed because of a rapid conversion of injected precursor Z into MPT (Fig. 5A). Within the following 4 days the MPT level dropped continuously to <4% of the wild-type level.

Sulphite oxidase activity (Fig. 5B) showed an increase from day 0 to day 1 after the last injection. Both sulphite oxidase and xanthine dehydrogenase activities (Fig. 5C) reach a relative level (expressed in percent of wild-type) that is higher than the reconstituted MPT level. After day 1, xanthine dehydrogenase activity diminishes rapidly. Morphological analysis of precursor Z-substituted knockout mice revealed bilateral abnormal kidneys observable from day 20 p.p. onward. This corresponds to very high levels of xanthine in the urine ($2803 \pm 355 \mu mol/l$). Besides the obstructed kidneys, no other morphological anomalies were found.

**DISCUSSION**

*In vivo and in vitro* restoration of murine Moco biosynthesis by bacterial precursor Z corroborates the genetic evidence for a unique and highly conserved pathway resulting in Moco and Moco-dependent enzyme activities (12). Recently, the chemical structure of precursor Z has been determined by mass spectrometry and $^1$H NMR spectroscopy (28). This structure confirmed previous assumptions that precursor Z is, similar to MPT, also a pyranopterin. A novel feature, the formation of a unique geminal diol in the C2$^2$, has been observed which, together with the pyranoring, might contribute to the relatively high stability of precursor Z. We observed a strong correlation between the amount of regularly injected precursor Z and the longevity of $MOCS1$-deficient mice (Table 1). At the lowest dosage applied here, the selective killing of affected offspring by the parents might indicate either a different smell—possibly caused by elevated concentrations of sulphur compounds—or some other subtle social disadvantage. The high morbidity between days 20 and 40 p.p., observed after the injection of medium amounts of precursor Z (1–2 mg) and after drug withdrawal, might reflect a critical period in brain development, in which the animals are very sensitive to elevated sulphite or diminished sulphate concentrations. At a high dosage, the activity of sulphite oxidase activity increases within the first day of intrahepatic injection of precursor Z, indicating that available and newly synthesized apo-enzyme molecules are quickly saturated with Moco. When compared with wild-type levels, the activity of both sulphite oxidase and xanthine dehydrogenase exceeds the percentage of restored MPT (Fig. 3). This finding suggests a very efficient incorporation of available Moco into the apo-enzymes as well as a pool of Moco that is not bound to Mo-
enzymes in the wild-type but might be essential for cellular Mo-enzyme turnover. Furthermore, this finding is in agreement with previous observations in gephyrin-deficient cell lines, where minimal amounts of reconstituted Moco resulted in over-proportionally high sulphite oxidase activity (22).

To our knowledge, the in vivo stability of xanthine dehydrogenase has not yet been determined, but the half-life of the corresponding mRNA was estimated to be 12–16 h (29), which is in the same range as the half-life of the enzyme activity as estimated from the difference between days 1 and 4 after precursor Z injection (Fig. 5C). Sulphite oxidase activity follows, after attaining its maximum at day 1, the published half-life of 3–4 days (27) (Fig. 5B). Apparently, due to the strong fluctuations, the mean xanthine dehydrogenase activity is lower than the mean sulphite oxidase activity. This is the primary reason for the development of xanthine stones as in human classical xanthinuria (type I) and finally renal malfunction, which seems to be the cause of death in the older substituted animals after 70 days. Human xanthinuria type I, however, is asymptomatic in the majority of cases (30) despite the total absence of xanthine dehydrogenase activity, and even in symptomatic cases, rarely results in life-threatening situations. Therefore an additional influence of the sulphite oxidase deficiency on renal function must be considered here (31). Clearly, treatment of human Moco deficiency should focus primarily on the restoration of sulphite oxidase activity. To date, no effective therapy for Moco-deficient patients has been described (11). For the rare type C deficiency caused by gephyrin mutations, we have demonstrated rescue in patient-derived fibroblasts with molybdate, which already indicated that small amounts of active Moco are sufficient for restoration of a normal phenotype (22). Since two-thirds of the known Moco-deficient patients belong to deficiency group A (10,12), and all components necessary for precursor Z conversion into MPT are present in those patients (20,24), purified precursor Z represents the first potentially effective drug for the majority of Moco patients.

Since the MOCS\textsuperscript{−/−} mice match the phenotype of human patients with respect to biochemical parameters, the correction of the human deficiency by precursor Z delivery appears very promising at the level of enzymatic restoration and metabolic correction. It remains to be seen whether delayed onset of the described therapy (expected in the majority of cases due to the time required for clinical manifestation and diagnosis) will still allow reversal of neurological damage. The described precursor Z therapy provides the basis to study the progression or reversal of this damage in detail, as we are now able to induce Moco deficiency in the animal model at any time by altering the dose of the drug.

Once sufficient amounts of purified precursor Z can be produced, the results of our study strongly suggest its clinical trials for the treatment of Moco-deficient patients. We have shown here that a Moco-deficient mouse with a maximum body weight of 40 g can reach adulthood and fertility with a dose of 4 μg precursor Z every third day. This would correspond to 100 μg precursor Z per kg body weight for a human patient in the same interval. A child with a body weight of 10 kg thus would need 1 mg of purified precursor Z approximately twice a week or 100 times a year resulting in a minimal annual requirement of 100 mg.

MATERIALS AND METHODS

Animals

The generation of MOCS\textsuperscript{−/−} mice used in this study, their phenotype and MOCS\textsuperscript{1} genotyping by polymerase chain reaction (PCR) has been described previously (25). All experiments have been approved by the Bezirksregierung Braunschweig, Germany.

Construction of plasmids for co-expression of moaA and moaC

E. coli moaA and moaC (32) were cloned by PCR from pJR11 (33) into the NdeI and XhoI sites of the pET15b expression vector (Novagen) yielding pPH15moaA and pPH15moaC. The complete moaC expression unit, including the isopropyl-β-D-thiogalactoside (IPTG)-inducible T7 promoter/operator element and the synthetic ribosomal binding site of pPH15moaC, was then sub-cloned between the SphI and HindIII sites of pLysS (Novagen) resulting in pPHLysmoaC.

Isolation and purification of precursor Z

Precursor Z was purified from E. coli based on the method described by Wuebbens and Rajagopalan (19). MJ7 chlI (DE3) (34) cells containing plasmids pPH15moaA and pPHLysmoaC were grown anaerobically at 20°C in Luria–Bertani medium supplemented with 120 μg/ml ampicillin, 30 μg/ml chloramphenicol and 50 μM IPTG and harvested by centrifugation (5 min, 12,000g, 4°C). Prior to purification, cells were re-suspended in two volumes of 0.4 M HCl, sonicated and centrifuged. The clear supernatant was injected onto a semi-preparative reversed phase column (C8, 5 μm, 250 x 10 mm, Kromasil, EKA Chemicals) equilibrated in 5 mM ammonium acetate pH 5.0. Precursor Z was eluted in

**Figure 5.** Biochemical analysis of precursor Z-substituted MOCS1-deficient animals. Mice were injected with increasing amounts of precursor Z twice a week starting with 2 μg after birth up to 8 μg after weaning. On days 25–55 p.p. treatment was stopped. On days 0 (4 h after the last injection), 1, 4 and 7 two mice each were sacrificed. Two liver samples of each animal were taken and processed separately. Each value in the graph thus was determined 4-fold. Total MPT including Moco (A), sulphite oxidase activity (B) and xanthine dehydrogenase (C) are depicted as percentage of the corresponding wild-type values (n = 4).
the first absorbing peak and immediately frozen in liquid nitrogen. In the second purification step, precursor Z-containing samples were loaded onto a semi-preparative strong anion exchange column (15 μm, 250 × 10 mm, Adsorbosphere, Alltech) equilibrated with 10 mM citrate buffer pH 3.0. Precursor Z was eluted with starting buffer around 30 ml and frozen in liquid nitrogen. Final purification was achieved by loading the precursor Z onto an analytical reversed phase column (basic spacer C18, 5 μm, 250 × 4 mm) equilibrated in 10 mM formic acid. Pure precursor Z was shock frozen in liquid nitrogen and stored at −80°C until used for injections. The concentration of precursor Z was calculated using a molar extinction coefficient at 267 nm of 8960 mol cm⁻¹ at pH 3.0 (19).

**In vitro synthesis of MPT**

Purified precursor Z was quantitatively converted into MPT using either recombinant *E. coli* MPT synthase or mouse MPT synthase present in liver protein crude extracts from 5-day-old *MOCS1*-deficient animals and detected as described earlier (26) or by the nit1-reconstitution assay.

**In vivo experiments**

After thawing, precursor Z was immediately brought to pH 6.95 by adding 10× PBS and H₂O to adjust a concentration of 50–100 ng/μl in 2× PBS with an injection volume of 20–80 μl unless otherwise indicated. Transabdominal intrahepatic injections were performed within 10 min of thawing precursor Z. Sterile endogen-free plastic ware (Eppendorf ‘Biopure’) was used throughout.

**Determination of MPT by nit1 reconstitution and HPLC form A analysis**

*Neurospora crassa* nit1 extract was prepared as described (35) and desalted prior to use by gel filtration using Nick columns (Amersham-Bioscience). Crude liver protein extracts were prepared using two volumes of nit1 buffer [50 mM sodium phosphate, 200 mM NaCl, 5 mM ethylene diamine tetra-acetic acid (EDTA), pH 7.2] following sonication and centrifugation. In *vitro* MPT synthesis was performed in a total volume of 30 μl with 5 mM sodium molybdate and 2 mM reduced glutathione containing 20 μl nit1 extract, 1–3 μl liver extract diluted according to the linear range of the reconstitution and 1 μl of different precursor Z dilutions (0–672 ng/mg liver protein). The reaction was incubated for 1 h under anaerobic conditions at room temperature followed by the reconstitution reaction carried out overnight at 4°C. After addition of 20 mM reduced nicotiamide adenine dinucleotide phosphate (NADPH) for 10 min, reconstituted NADPH–nitrate reductase activity was determined. One unit MPT activity is defined as reconstituted nit1 nitrate reductase sufficient to produce an increase at 540 nm of 1.0 absorbance units per 20 min reaction time. Activity is expressed as units per mg crude extract protein.

Crude liver protein extracts from precursor Z treated and untreated mice were prepared using two volumes of 50 mM sodium phosphate, 200 mM NaCl, 2 mM reduced glutathione, pH 7.2 following sonication and clarification. Total MPT (which includes Moco) was determined by triplicate HPLC formA analysis as described previously (36) using 250 μg total protein in each experiment.

**Mo-enzyme assays**

Sulphite oxidase activity was determined according to Johnson *et al.* (37). Crude protein extracts from mouse liver were prepared using one volume of 0.1 M Tris–HCl, 0.1 mM EDTA, pH 8.5 by sonication. Enzyme activity was assayed in a reaction volume of 300 μl containing 0.2 mM Tris–HCl, pH 8.5, 0.17 mM sodium deoxycholic acid, 50 mM potassium cyanide, 0.2 mg cytochrome c and 160 μM sulphate monitoring the reduction of cytochrome c at 550 nm. One unit sulphite oxidase activity is defined as enzyme activity needed to produce an increase of 1.0 absorbance at 550 nm per min at 25°C. Activity was expressed in percentage of wild-type activity. Xanthine dehydrogenase activity was determined combining two methods described previously (38,39). About 200–750 μg of liver crude protein extract (prepared as described for MPT analysis) was loaded in triplicates on each lane of a 9% native polyacrylamide gel. Xanthine dehydrogenase activity was detected by in-gel activity staining overnight using 300 μM hypoxanthine as substrate, 1 mM 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide and 0.1 mM phenazine methosulphate in 250 mM Tris–HCl, pH 8.5. Duplicates of stained xanthine dehydrogenase bands were quantified densitometrically (HEROLAB EasyWin32, version 3.99.199) and expressed as percentage intensity of wild-type.

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**NOTE ADDED IN PROOF**

In the meantime, we injected precursor Z in adult *MOCS1*-deficient mice also intraperitoneally or intravenously (tail vein) and observed results comparable to the intrahepatic injections of the same dosage.

**REFERENCES**


