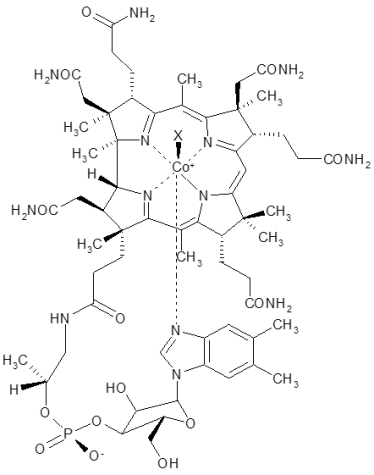
Self-reliant bioactive forms of vitamin B12

Technology



Vitamin B12 deficiency can originate due to poor nutrition or due to genetic mutations in genes that are essential for the utilization of this micronutrient in cells. Current chemical forms of vitamin B12 are unable to halt disease progression, i.e. neurological and hematological symptoms, in conditions that block this micronutrient utilization in humans. One case is the cblC genetic disease as well as other pathologies leading to B12-dependent homocystinuria and methylmalonic aciduria where B12 reaches the cells but it cannot be utilized. Herein, a new generation of B12 forms is presented, with the intrinsic property of bypassing intracellular enzymatic processing, undergoing self-activation in cells, and thus promising a superior therapeutic avenue for patients with the cblC disease and other forms of vitamin B12 deficiency. This new generation of B12 forms is expected to alleviate or correct vitamin B12 deficiencies associated with aging or with neurological impairments such as Alzheimer and Parkinson's disease, where brain atrophy and cognitive decline are hallmarks.

Innovation

- Self-reliant bioactive forms of vitamin B12 that bypass intracellular enzymatic processing of the micronutrient

Application

- Newborns with a gene defect in the cblC (MMACHC) gene and other vitamin B12-dependent disorders leading to:
  - homocystinuria
  - methylmalonic aciduria
- Elderly people with latent, subclinical vitamin B12 deficiency
- Supportive treatment of Alzheimer's and dementia patients
- Vegetarians and vegans

Developmental Status

-This new generation of self-reliant forms of vitamin B12 has been synthesized and isolated in pure form.
-Chemical reactivity with physiological reductants exhibited self-reliant activation
-The enzymatic activity of pathogenic variants of the cblC enzyme causing early and late disease onset in humans was restored to near normal levels
-Homocystinuria and methylmalonic aciduria where corrected in cblC patient cells

Responsible Scientist

Dr. Luciana Hannibal

University Medical Center, Freiburg,
Center for Pediatrics

Branch

Pharma / Dietary supplements

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Contact
Dr. Claudia Skamel
Campus Technologies Freiburg GmbH
Stefan-Meier-Str. 8 | D-79104 Freiburg
Email: Claudia.Skamel@campus-technologies.de
Tel: +49 (0)761 203-4987
Fax:+49 (0)761 203-5021

Self-reliant Bioactive Forms of Vitamin B12

Luciana Hannibal, Victoria Wingert and Ute Spiekerkötter

Laboratory of Clinical Biochemistry and Metabolism, Center of Pediatrics, Medical Center, University of Freiburg, Freiburg, Germany

Cobalamin Metabolism in Humans

Vitamin B12 deficiency can originate due to poor nutrition or due to genetic mutations in genes that are essential for the utilization of this micronutrient in cells. Current chemical forms of vitamin B12 are unable to halt disease progression, i.e. neurological and hematological symptoms, in conditions that block this micronutrient utilization in humans. One case is the cblC genetic disease as well as other pathologies leading to B12-dependent homocystinuria and methylmalonic aciduria where B12 reaches the cells but it cannot be utilized. Herein, a new generation of B12 forms is presented, with the intrinsic property of bypassing intracellular enzymatic processing, undergoing self-activation in cells, and thus promising a superior therapeutic avenue for patients with the cblC disease and other forms of *genetic vitamin B12 deficiency*. This new generation of B12 forms is expected to alleviate or correct *vitamin B12 deficiencies associated with aging or with neurological impairments* such as Alzheimer and Parkinson's disease, where brain atrophy and cognitive decline are hallmarks.

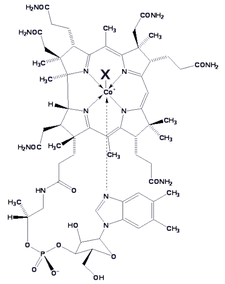

Figure 1. A. Structure of cobalamins. Cobalamins are tetrapyrroles assembled in a corrin macrocycle with cobalt as the metal center. Cobalamins possess seven side chains, namely, acetamides and propionamides. The fifth axial coordinating position (alpha-ligand) is occupied by a nitrogen atom from the dimethylbenzimidazole moeity. The sixth coordination position (herein shown as 'X'), known as the beta-ligand, can be occupied by different types of ligands. Naturally occurring ligands include methyl (methylcobalamin), 5-deoxyadenosine (adenosylcobalamin), hydroxo or aqua (hydroxocobalamin, aquacobalamin), cyano (cyanocobalamin), glutathionyl (glutathionylcobalamin) and so forth. New ligands in this invention were attached to position X, such to preserve the structure of the cobalamin for optimal binding and transport by transport protein transcobalamin. The cobalamin derivatives of the present invention feature Co-S and Co-C axial coordination and were designed to meet three properties for their enhanced bioactivity:

- a higher pKa of the base-on to base-off transition;
- a higher redox potential for the reduction of the cobalt centre and
- more facile removal of the β-axial ligand

These properties render the new cobalamins self-reliant, by bypassing the intracellular B12-processing machinery for direct furnishing of the B12-dependent reactions in humans.

Results

1. High-scale synthesis and crystallization of pure CyaCbl and MPGCbl.

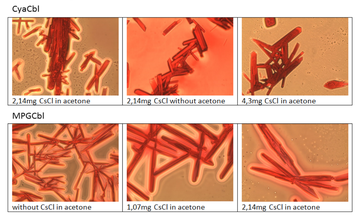

Figure 2. Crystals of CyaCbl and MPGCbl. Crystals were grown successfully via vapor-diffusion in acetone, at 4 oC, in the dark**.** Addition of CsCbl favored formation of crystals with size suitable for x-ray analysis. Crystals of CyaCbl formed both with and without acetone vapor diffusion; however, crystals of MPGCbl only grew under acetone vapor diffusion. Mass spectrometry analysis confirmed the appropriate molecular mass, as follows: [M/2+1], m/z= 765 for MPGCbl and m/z=722 for CyaCbl.

2. UV-visible spectroscopy of CyaCbl and MPGCbl: two novel bioactive Cbl derivatives

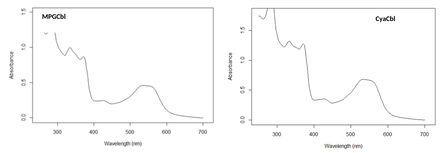

Figure 3. UV-visible spectra and absorption coefficients of two model thiolatocobalamins, namely, CyaCbl and MPGCbl. The newly synthesized thiolatocobalamins exhibit spectral properties identical to that of previously synthesized Co-S ligated species. Absorption maxima for CyaCbl and MPGCbl appear at 370 nm and 531 nm, in accord with the properties of previously described for other Co-S cobalamins.

3. Correction of elevated Hcy and MMA with new generation cobalamin derivatives in cblC patient fibroblasts: short term and long-term treatment effects after wash-off.

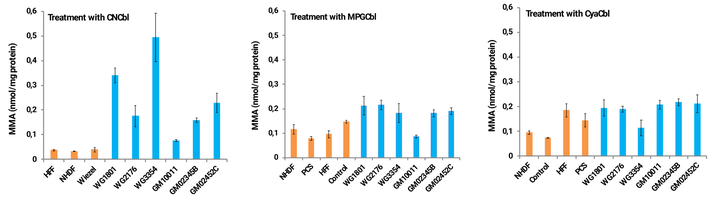

Figure 4. Cellular response to treatment with new derivatives CyaCbl and MPGCbl. The ability of the new cobalamin derivatives CyaCbl and MPGCbl to achieve metabolic control, i.e. reduction of MMA production by cblC fibroblasts in culture was examined. CyaCbl or MPGCbl were given to cultured fibroblasts from healthy subjects (HFF, NHDF, PCS, Wiesel, Control) or from cblC patients (WG1801, WG2176, WG3354, GM10011, GM02345B, GM02452C) for 7 days. As can be seen from these results, CyaCbl and MPGCbl are far superior compared to CNCbl (Figure 4) in reducing MMA to the levels observed in fibroblasts from healthy subjects. This result shows proof of the following events:
(a) CyaCbl and MPGCbl were transported successfully into the cells using the canonical TC transporter and its cellular receptor CD320.
(b) CyaCbl and MPGCbl were not cytotoxic under our experimental conditions.
(c) CyaCbl and MPGCbl could furnish the reaction of mitochondrial MCM to a greater extent than CNCbl, one of the current therapeutic forms.
(d) The fact that mitochondrial metabolism of vitamin B12 could be restored by treatment with CyaCbl or MPGCbl suggest that the new derivatives are bioactive both in the cytosol (where Cbl processing occurs) as well as in the mitochondrion, where the micronutrient is essential for the reaction of MCM.

Table 1: Metabolic control achieved with new Cbl by monitoring of MMA

	MMA (nmol/mg protein)				
Concentration of MMA in conditioned medium after 7 days:					
Subject	MeCbl	Me-S-MeCbl	GSCbl	MPGCbl	CyaCbl
HFF	0.106	0.132	0.105	0.116	0.095
NHDF	0.152	0.103	0.078	0.096	0.184
Wiezel	0.095	0.132	0.135	0.148	0.144
WG1801	0.429	0.254	0.198	0.212	0.194
WG2176	0.459	0.083	0.316	0.216	0.189
WG3354	0.891	0.148	0.269	0.183	0.114
GM10011	0.066	0.041	0.052	0.087	0.208
GM02345B	0.250	0.150	0.223	0.182	0.217
GM02452C	0.243	0.150	0.167	0.190	0.369
Controls	0.118	0.122	0.106	0.120	0.141
cblC patients	0.390	0.138	0.204	0.178	0.215
Concentration of MMA in conditioned medium after 7 days wash-off period (*)					
Controls	0.131	0.145	0.134	0.121	0.154
cblC patients	0.298	0.177	0.275	0.185	0.190

*** Cells were first cultured in the presence of the given cobalamins for a total period of time of 7 days without replacement of the culture medium. After this, the cultured medium was removed and replaced with fresh medium lacking supplemental cobalamin (wash-off experiment). The cells were grown for another 7 days (wash-off experiment), and the levels of MMA were determined for comparative purposes.**
Control healthy fibroblasts used herein are: HFF, NHDF and Wiezel. Cell lines labeled as WG and GM are fibroblasts isolated from patients with cblC disease.

4. Binding of CyaCbl and MPGCbl to human recombinant cblC

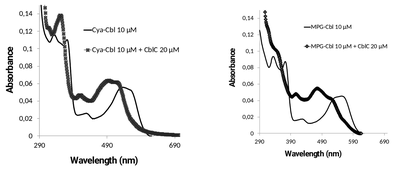

Figure 5. Binding of human recombinant cblC to Cya-Cbl and MPG-Cbl. Incubation of human recombinant cblC with CyaCbl and MPGCbl leads to conversion of the cobalamins to their base-off configuration.

5. Dethiolation of MPGCbl by human recombinant cblC

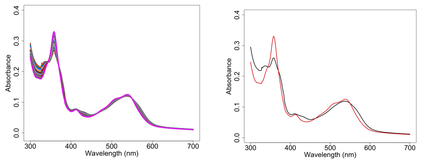

Figure 6. Dethiolation of MPG-Cbl by human cblC. Incubation of human cblC with co-substrates MPGCbl and GSH results in rapid formation (less than 5 min, left panel) of aquacobalamin as the product (right panel, red trace).

6. Dethiolation and dealkylation of new cobalamin derivatives by human recombinant cblC

Table 2: Catalytic activity of human recombinant cblC in the presence of new Cbl derivatives as the substrate

	Half-life of Beta-axial ligand removal (minutes)*		Product under ambient conditions	
Cobalamin	Wild type CblC	Mutant Arg161Gly	Wild type CblC	Mutant Arg161Gly
MeCbl	10	60	H_2OCbl	H_2OCbl
PrCbl	318	0	H_2OCbl	No reaction
BuCbl	1155	0	H_2OCbl	No reaction
Me-S-MeCbl	8.5	12	H_2OCbl/Cob(II)alamin	Cob(II)alamin
Me-S-EtCbl	7.5	11.5	H_2OCbl/Cob(II)alamin	Cob(II)alamin
GSCbl	2.5	9.8	H_2OCbl	H_2OCbl
MPGCbl	< 0.5	1.5	H_2OCbl	H_2OCbl
CyaCbl	< 0.5	2.1	H_2OCbl	H_2OCbl

Abbreviations: PrCbl: propylcobalamin (analogue for Me-S-MeCbl); BuCbl: butylcobalamin (analogue for Me-S-Et-Cbl); GSCbl: glutathionylcobalamin (analogue for thiolatocobalamins)
*** Dealkylation of Co-C cobalamins was carried out with CblC-alkylCbl complexes (20 µM:10 µM) in EPPS buffer (40 mM, pH 7.6) supplemented with 150 mM NaCl and 10% glycerol at 25 oC. The reactions were started by addition of 5 mM GSH and monitored for 60 minutes.**
Dethiolation of Co-S cobalamins was carried out with CblC-alkylCbl complexes (20 µM:10 µM) in EPPS buffer (40 mM, pH 7.6) supplemented with 150 mM NaCl and 10% glycerol at 25 oC. The reactions were started by addition of 50 µM GSH and monitored for 60 minutes. The reactions were monitored by UV-visible spectrophotometry (one full scan (300 to 700 nm) was collected every 2 minutes).

Concluding Remarks

- A new generation of bioactive cobalamin derivatives featuring Co-S and Co-C axial coordination has been designed, synthesized and isolated in pure form.
- The new Cbl derivatives bind to human recombinant cblC, both wild type and pathogenic variants, and undergo conversion to the base-off configuration, the primed form required for downstream catalysis
- Observed rates of axial ligand removal by wild type and pathogenic variants of cblC Arg161Gly show superior activity compared to the corresponding structural analogues and natural substrates MeCbl and GSCbl.
- The new Cbl derivatives reduced the production of toxic metabolites Hcy and MMA by cblC patient fibroblasts, compared to CNCbl and HOCbl, the currently available therapeutic forms.
- The new cobalamin derivatives exhibit superior capacity in sustaining low levels of Hcy and MMA after a Cbl wash-off period, compared to currently available therapeutic forms.

Acknowledgments

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