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A dodecylamine derivative of cyanocobalamin potently inhibits the activities of cobalamin-dependent methylmalonyl-CoA mutase and methionine synthase of *Caenorhabditis elegans*

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ABSTRACT

In this study, we showed that cyanocobalamin dodecylamine, a ribose 5′-carbamate derivative of cyanocobalamin, was absorbed and accumulated to significant levels by *Caenorhabditis elegans* and was not further metabolized. The levels of methylmalonic acid and homocysteine, which serve as indicators of cobalamin deficiency, were significantly increased in *C. elegans* treated with the dodecylamine derivative, indicating severe cobalamin deficiency. Kinetic studies show that the affinity of the cyanocobalamin dodecylamine derivative was greater for two cobalamin-dependent enzymes, methylmalonyl-CoA mutase and methionine synthase, compared with their respective coenzymes, suggesting that the dodecylamine derivative inactivated these enzymes. The dodecylamine derivative did not affect the levels of mRNAs encoding these enzymes or those of other proteins involved in intercellular cobalamin metabolism, including methylmalonyl-CoA mutase (*mmcm-1*), methylmalonic acidemia cobalamin A complementation group (*mmaa-1*), methylmalonic aciduria cblC type (*cblc-1*), and methionine synthase reductase (*mtrr-1*). In contrast, the level of the mRNAs encoding cob(I)alamin adenosyltransferase (*mmab-1*) was increased significantly and identical to that of cobalamin-deficient *C. elegans*. These results indicate that the cyanocobalamin-dodecylamine derivative acts as a potent inhibitor of cobalamin-dependent enzymes and induces severe cobalamin deficiency in *C. elegans*.

1. Introduction

Cobalamin (vitamin B_{12}, Cbl), an essential nutrient for humans, undergoes a complex process of gastrointestinal absorption when provided in the diet [1,2]. After uptake by its target cells, Cbl is converted into 5′-deoxyadenosylcobalamin (AdoCbl) and methylcobalamin (CH_3-Cbl), which function as coenzymes for methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) [3] and methionine synthase (MS; EC 2.1.1.13) [4], respectively. The major symptoms of Cbl deficiency are megaloblastic anemia and neuropathy [5], and the underlying cause(s) of the associated developmental disorders, metabolic abnormalities, and neuropathy are poorly understood [6].

Animal models of Cbl deficiency are required for investigating the molecular mechanisms of these metabolic disorders; however, they are difficult to establish, because animals (e.g., rats) must be fed a Cbl-deficient diet for long periods to achieve Cbl deficiency [7]. Our recent study indicates that *Caenorhabditis elegans* grown under conditions of Cbl deficiency for five generations (approximately 15 days) develops severe Cbl deficiency associated with various phenotypes that include decreased egg-laying capacity (infertility), prolonged life cycle (growth retardation), and reduced lifespan [8]. These phenotypes resemble those of Cbl-deficient mammals.

McEwan et al. [9] synthesized ribose 5′-carbamate derivatives of cyanocobalamin (CN-Cbl) and demonstrated high-affinity binding of intrinsic factor (IF, the gastric Cbl-binding protein) to certain alkylamine derivatives. Our preliminary experiments indicate that

these alkylamine derivatives lack detectable biological activity in certain microorganisms that require Cbl for growth, such as *Escherichia coli* 215, *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830, and *Euglena gracilis* Z. Further, CN-Cbl dodecylamine derivative significantly decreases the levels of Cbl-dependent enzymes in mammalian cells cultured in vitro. Therefore, development of animal models of Cbl deficiency may be facilitated if the dodecylamine derivative acts as a potent inhibitor of Cbl-dependent enzymes. In the present study, we show that CN-Cbl dodecylamine derivative potently inhibited the Cbl-dependent enzymes MCM and MS of *C. elegans*.

2. Results and discussion

2.1. Effects of the CN-Cbl dodecylamine derivative on Cbl-related biomarkers of C. elegans

Although McEwan et al. [9] demonstrated high affinity binding of the gastric Cbl-binding protein IF to the CN-Cbl dodecylamine derivative (Fig. 1), our preliminary experiments indicated that this derivative was inactive in Cbl-dependent microorganisms typically employed in Cbl the bioassay. The dodecylamine derivative significantly inhibited the activities of Cbl-dependent enzymes (MCM and MS) in mammalian cells cultured in vivo (our unpublished data). Therefore, we evaluated effects of the CN-Cbl dodecylamine derivative on Cbl-related phenotypes using *C. elegans* as a model. Table 1 shows the concentrations of Cbl and its dodecylamine derivative in homogenate prepared from adult worms grown in the presence of the CN-Cbl derivative for 3 days. Remarkably, the Cbl concentration of worms grown in the presence of the CN-Cbl derivative was only 35% compared with that of control worms and was similar to that of worms grown for two generations (6 days) in the absence of Cbl [8]. In contrast, the CN-Cbl dodecylamine derivative was absorbed and accumulated by worms grown in the presence of the CN-Cbl derivative (approximately 110 ng/g wet weight). These results suggest that the CN-Cbl dodecylamine derivative did not inhibit the uptake of Cbl in the intestine, but it was readily accumulated in worms and significantly decreased their Cbl concentrations.

Table 1
Contents of CN-Cbl and the CN-Cbl dodecylamine derivative in worms.

|	CN-Cbl (ng/g wet weight)	CN-Cbl dodecylamine derivative
Control worms	132.2 ± 26.7	–
Treated worms	46.5 ± 7.8	110.0 ± 17.2

Control and treated worms were grown on plates containing CN-Cbl- and CN-Cbl dodecylamine derivative-supplemented (each at 100 µg/L) M9 media for 3 days, respectively. Corrinoids were extracted from the treated worms by boiling with KCN at acidic pH. CN-Cbl and the CN-Cbl dodecylamine derivative were separated each other using a Sep-Pak Plus C18 cartridge and their levels were determined using the microbiological assay and HPLC, respectively. Data represent the mean ± SD of five independent experiments.

To determine whether the dodecylamine derivative detected in the treated worms was converted into other forms of Cbl, corrinoid compounds were extracted using 80% (v/v) ethanol from worms grown in the presence of the dodecylamine derivative and analyzed using reversed-phase HPLC. The retention times of authentic OH-Cbl, CN-Cbl, AdoCbl, CH₃-Cbl, OH-Cbl dodecylamine, CN-Cbl dodecylamine, and AdoCbl dodecylamine were 3.5, 8.1, 9.5, 12.8, 22.0, 30.6, and 36.2 min, respectively (Fig. 2A–D). The compounds extracted from worms exposed to the derivative eluted with retention times of 3.8–30.3 min (Fig. 2E). A major peak with the retention time of 30.3 min was identical to that of authentic CN-Cbl dodecylamine, and peaks were not detected with retention times of CN-Cbl (8.1 min), OH-Cbl dodecylamine (22.0 min), or AdoCbl dodecylamine (36.2 min). These results indicate that the CN-Cbl dodecylamine derivative accumulated by worms was not converted to any other Cbl-related compound, including its coenzyme forms.

The levels of MMA and Hcy were assayed in *C. elegans* grown for 3 days in the presence of the CN-Cbl dodecylamine derivative (Fig. 3A and B). The levels of both indicators were significantly increased in worms exposed to the dodecylamine derivative compared with those of the control worms. The increased MMA and Hcy levels were identical to those of Cbl-deficient worms. These results show that the worms developed severe Cbl deficiency when they were treated with the CN-Cbl dodecylamine derivative for only 3 days.
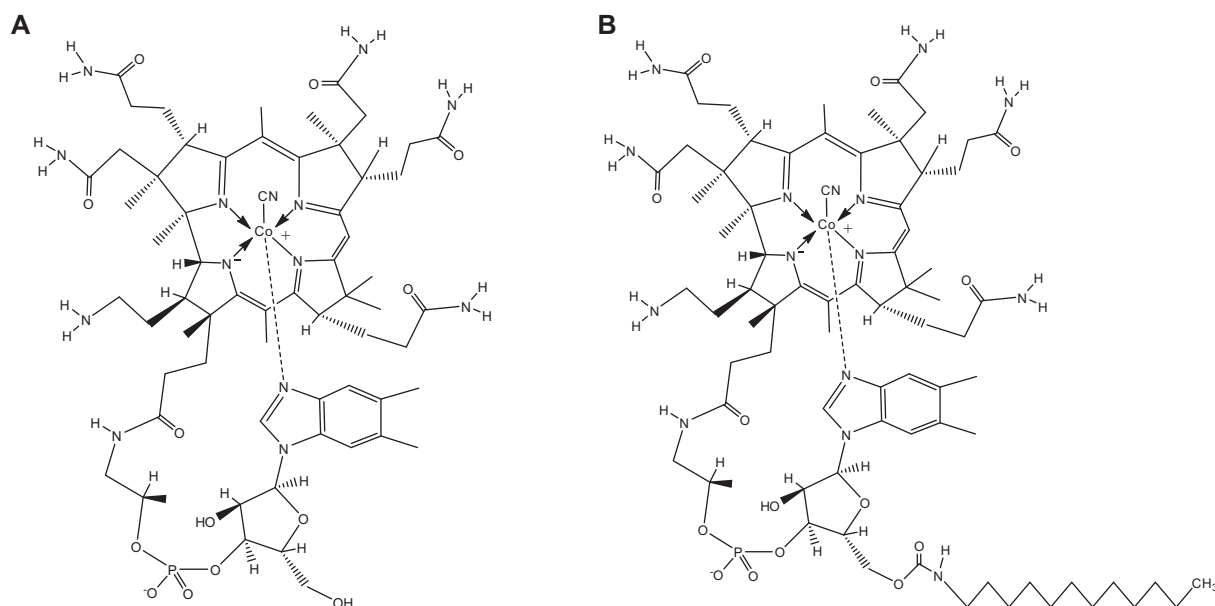
Fig. 1. Structures of cyanocobalamin and its dodecylamine derivative. (A) CN-Cbl; (B) CN-Cbl dodecylamine derivative.
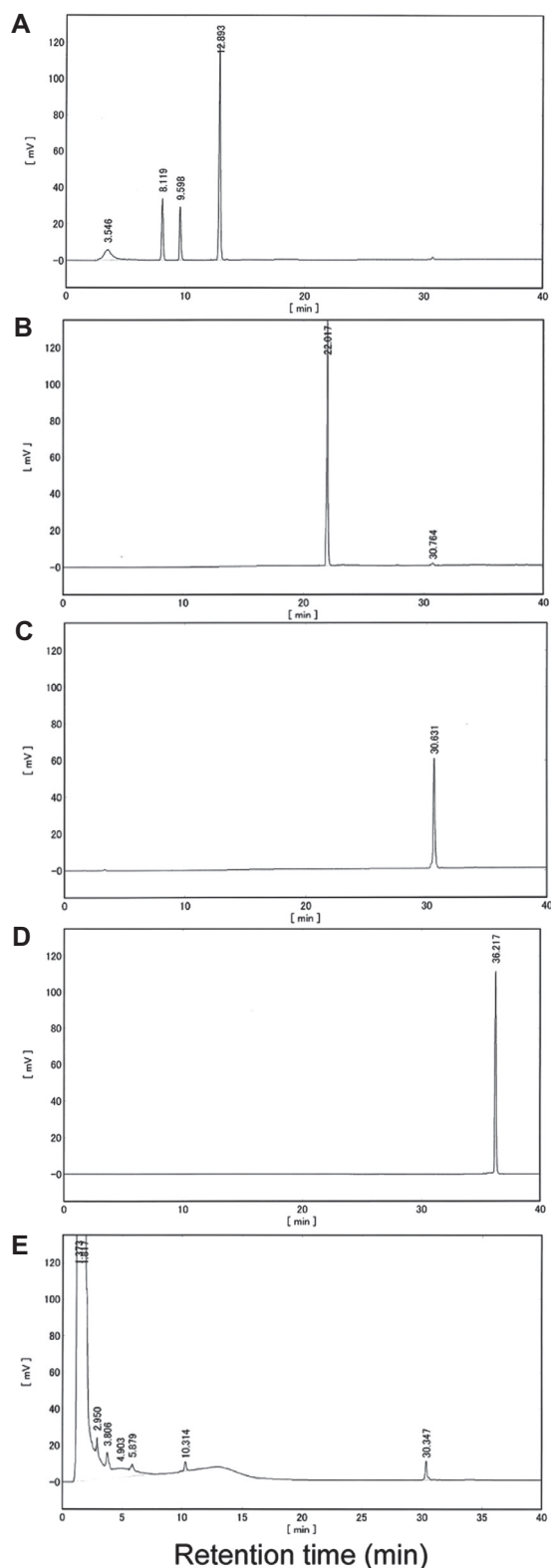
Fig. 2. HPLC analysis of Cbl compounds detected in worms treated with the CN-Cbl dodecylamine derivative. Cbl analogs were analyzed using HPLC as described in Section 3. (A) Authentic Cbl compounds. Retention times of OH-Cbl, CN-Cbl, AdoCbl, and CH₃-Cbl were 3.5, 8.1, 9.5, 12.8 min, respectively. (B) OH-Cbl dodecylamine derivative. The retention time of the OH-Cbl derivative was 22.0 min. (C) CN-Cbl dodecylamine derivative. The retention time of the CN-Cbl dodecylamine derivative was 30.6 min. (D) AdoCbl dodecylamine derivative. The retention time of the AdoCbl derivative was 36.2 min. (E) Corrinoid compounds detected in the worms grown in the presence of the CN-Cbl dodecylamine derivative.
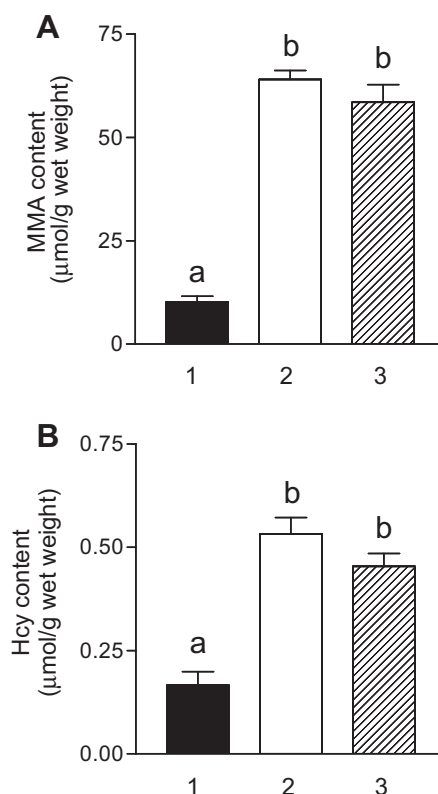

Fig. 3. Methylmalonic acid and homocysteine contents of worms treated with the CN-Cbl dodecylamine derivative. (A) Methylmalonic acid (MMA) and (B) homocysteine (Hcy) were measured in cell homogenates of worms grown in the presence of CN-Cbl (control; 1, black bar), the CN-Cbl dodecylamine derivative (2, white bar), or Cbl-deficient worms (3, shaded bar). Data represent the mean ± SD of five independent experiments. The different letters indicate values that are significantly different, $p < 0.05$.

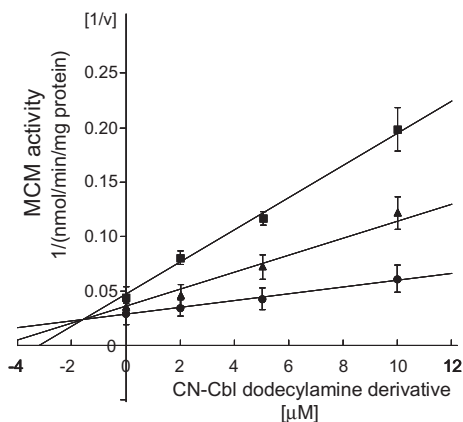## 2.2. Effects of the CN-Cbl dodecylamine derivative on Cbl-dependent enzymes in C. elegans

The increased levels of MMA and Hcy in worms exposed to the CN-Cbl dodecylamine derivative indicate that the activities of MCM and MS were significantly inhibited by the accumulation of the CN-Cbl derivative. To determine whether the dodecylamine derivative inhibited these Cbl-dependent enzyme activities, homogenates of worms grown in the presence (control) or absence of Cbl or in the presence of the derivative were assayed for MCM and MS activities. The holo-MCM and MS activities of the worms treated with the dodecylamine derivative decreased to approximately 8% (Fig. 4A) and 18% (Fig. 4B), respectively, compared with those of control worms.

To define the mechanism of inhibition of MCM by the CN-Cbl derivative, we determined the effects of the CN-Cbl derivative on apo-MCM activity of worms in the presence or absence of AdoCbl (Fig. 5). The apparent K_m value of the worm apoenzyme was 4.7 μM for AdoCbl. The CN-Cbl dodecylamine derivative competitively inhibited the apoenzyme. The K_i value of the worm apoenzyme was 1.5 μM for CN-Cbl dodecylamine derivative, which has increased affinity for the apoenzyme relative to that of AdoCbl. CN-Cbl did not reduce the apo-MCM activity in the presence of AdoCbl.

Our previous study shows that the holo-MS present in Cbl-supplemented or -deficient worms accounts for most MS activity [8]. To determine whether holo-MS activity was inhibited by the addition of CN-Cbl dodecylamine derivative, MS activity was assayed in a homogenate of the control worms in the presence of
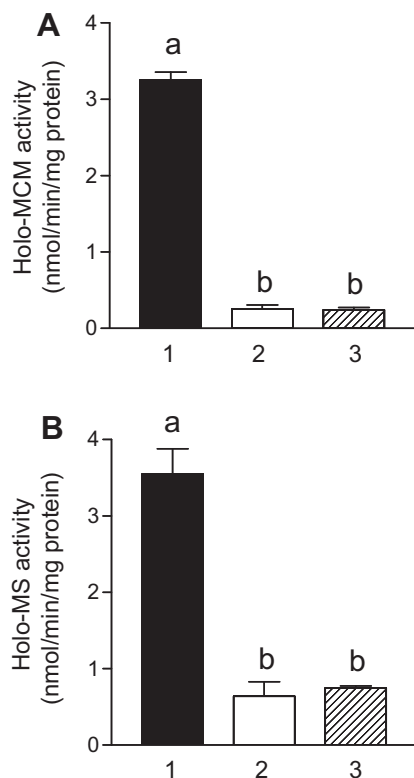
Fig. 4. Cbl-dependent enzyme (methylmalonyl-CoA mutase and methionine synthase) activities in worms treated with the CN-Cbl dodecylamine derivative. Holo-methylmalonyl-CoA mutase (MCM) (A) and holo-methionine synthase (MS) (B) activities were measured in cell homogenates prepared from worms grown in the presence of CN-Cbl (control; 1, black bar), the CN-Cbl dodecylamine derivative (2, white bar), or Cbl-deficient worms (3, shaded bar). Data represent the mean ± SD of five independent experiments; the different letters indicate values that are significantly different, $p < 0.05$.

Fig. 5. Effects of the CN-Cbl dodecylamine derivative on the enzyme activity of *C. elegans* apo-MCM in the presence of AdoCbl. A cell homogenate of worms grown under conditions of Cbl deficiency was used as an apo-MCM preparation. MCM activity was assayed in the reaction mixture containing various concentrations (0, 2, 5, 10 μM) of the CN-Cbl dodecylamine derivative in the presence of ■ 2.5 μM, ▲ 5.0 μM, or ● 10.0 μM of AdoCbl. MCM activity data were analyzed using the Dixon plot. Data represent the mean ± SD of three independent experiments.

the dodecylamine derivative (Fig. 6). CN-Cbl did not inhibit holo-MS activity. In contrast, the dodecylamine derivative significantly inhibited enzyme activity in a dose-dependent manner, and enzyme activity was completely inhibited in the presence of
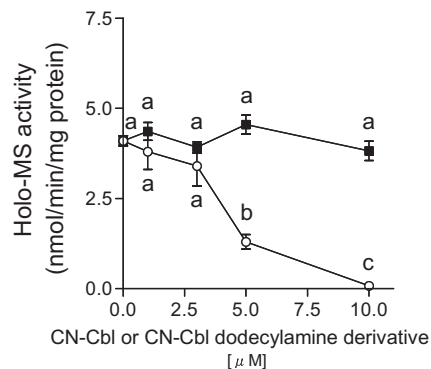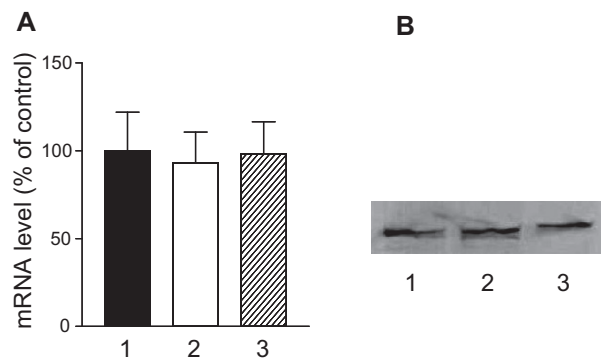

Fig. 6. Effects of the CN-Cbl dodecylamine derivative on the activity of the *C. elegans* holo-MS. A cell homogenate of worms grown in the presence of CN-Cbl (control) was used as a holo-MS preparation. MS activity was assayed in the presence of various concentrations (0, 1, 3, 5, and 10 μM) of CN-Cbl (■) or the CN-Cbl dodecylamine derivative (○). Data represent the mean ± SD of three independent experiments; the different letters indicate values that are significantly different, $p < 0.05$.

10 μM dodecylamine. These results suggest that the CN-Cbl derivative is exchanged for CH_3-Cbl bound to MS, inhibiting enzyme activity.

These results indicate that the affinity of CN-Cbl dodecylamine derivative is higher for MCM compared with that of AdoCbl and that the inhibitions of MCM and MS activities are attributable to the formation of inactive CN-Cbl derivative-enzyme complexes.

Yamada et al. demonstrated that most MS activity is derived from the holo-enzyme in normal or Cbl-deficient rats because the apo-enzyme is very unstable [10]. Although MS protein levels in liver extracts of Cbl-deficient rats are significantly decreased compared with those of control rats, the level of the mRNA encoding MS is not affected by the Cbl concentration of rat livers [10]. To determine the changes in MS protein and mRNA levels (*metr-1*) of worms treated with the CN-Cbl derivative, we conducted western blot and qPCR analyses and found that the level of mRNA was unchanged (Fig. 7A), whereas the level of MS protein in Cbl-deficient worms was reduced to approximately 32% compared with that of controls. This result differs from the detection of an extremely faint band of MS in the livers of Cbl-deficient rats, suggesting that the worm apo-enzyme is not very unstable. The

Fig. 7. Levels of mRNA and protein of MS in worms treated with the CN-Cbl dodecylamine derivative. (A) qPCR analysis of the mRNA levels of MS in homogenates of worms exposed to CN-Cbl (control; 1, black bar), the CN-Cbl dodecylamine derivative (2, white bar) or Cbl-deficient worms (3, shaded bar). (B) Western blot analysis using an anti-MS antibody of homogenates (100 μg of protein) prepared from worms exposed to CN-Cbl (control; 1), the CN-Cbl dodecylamine derivative (2), or Cbl-deficient worms (3) was treated by SDS–polyacrylamide slab gel electrophoresis followed by immunoblotting with an anti-MS antibody. Immunoreactive components were visualized using an immunoblot-staining kit for peroxidase.

intensity of the MS protein band in extracts prepared from worms treated with the CN-Cbl derivative was identical to that of the controls (Fig. 7B). These results suggest that the binding of the CN-Cbl derivative to MS completely inhibited MS activity and did not affect the level of MS protein.

2.3. Effects of the CN-Cbl dodecylamine derivative on the levels of mRNAs encoding proteins involved in Cbl metabolism in C. elegans

To evaluate the effects of the CN-Cbl derivative on the levels of mRNAs encoding proteins involved in Cbl metabolism, we performed qPCR analyses to detect mRNAs encoding MCM (*mmcm-1*), methylmalonic acidemia Cbl A complementation group; MMAA (*mmaa-1*), cob(I)alamin adenosyltransferase; MMAB (*mmab-1*), methylmalonic aciduria cblC type; MMACHC (*cblc-1*), and methionine synthase reductase; MSR (*mtrr-1*). The mRNA levels of *mmaa-1*, *cblc-1*, and *mtrr-1* did not differ significantly among three experimental conditions (Fig. 8A, B, D, and E). However, the levels of *mmab-1* mRNA of worms treated with the CN-Cbl derivative was significantly increased compared with that of the control worms, and the increased level was identical to that of Cbl-deficient worms (Fig. 8C).

2.4. Effects of the CN-Cbl dodecylamine derivative on the C. elegans egg-laying capacity and life cycle

Our previous study demonstrates that Cbl deficiency decreases the egg-laying rates and prolongs the life cycle of *C. elegans* [8]. In the present study, the egg-laying rates of worms exposed to the CN-Cbl dodecylamine derivative were significantly decreased (Fig. 9A), and the life cycle was increased, but the difference was not statistically significant (Fig. 9B). These results suggest that increased Cbl deficiency, long exposure to Cbl-deficient conditions, or both are necessary to significantly prolong life cycle.

These results indicate that the CN-Cbl dodecylamine derivative acts a potent inhibitor of Cbl-dependent MCM and MS activity, which leads to metabolic disorders caused by Cbl deficiency.

Chandler et al. [11] described that MMA level was approximately 3 times greater in *C. elegans* treated with RNA interference against *mmcm-1*, *mmaa-1*, and *mmab-1* than in the wild-type worms and that the *mmcm-1* deletion mutant accumulated 17 times more MMA than the wild-type worms after propionic acid loading. In this study, MMA level was approximately 4 times greater in Cbl-deficient and CN-Cbl derivative-treated worms than in the control worms (Fig. 3A); the magnitude of MMA elevation was similar to that of the worms treated with RNA interference against *mmcm-1*.

Our previous study [8] indicated that MS is more sensitive to cellular Cbl concentrations of *C. elegans* than MCM, suggesting that the phenotype observed in this and previous studies would rather be attributable to the results of impaired remethylation *via* the MS reaction than those of perturbed propionyl-CoA oxidation in the MCM pathway.

CN-Cbl [*c*-lactam], a CN-Cbl analog with a modified C-ring side chain inhibits the MS activity of cultured HL-60 cells to induce cell death but does not affect MCM activity [12]. However, Sponne et al.
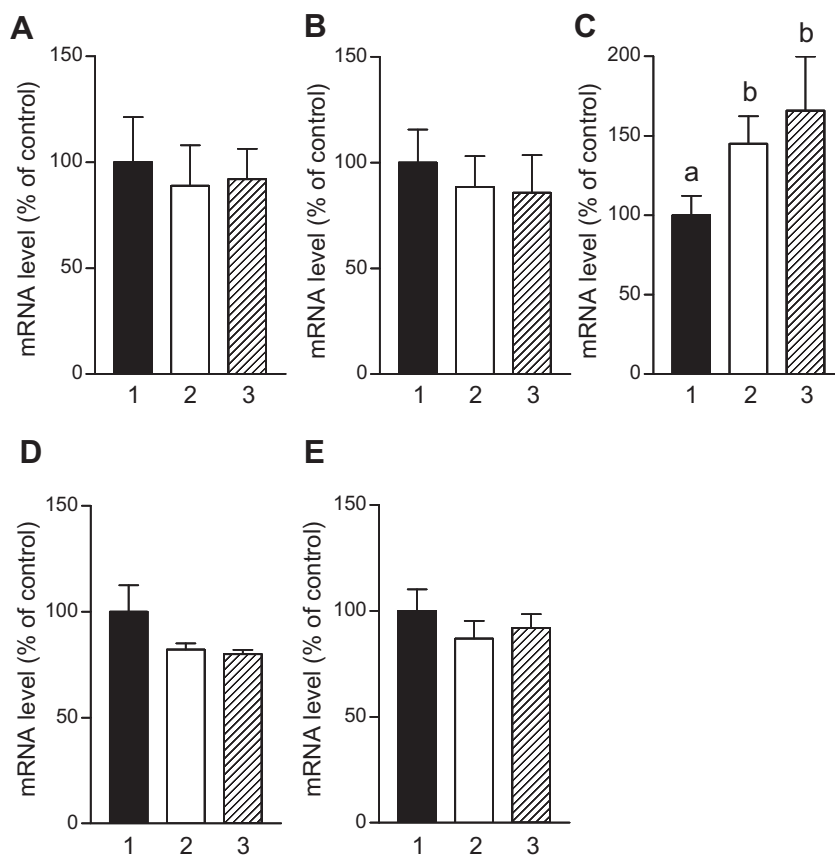
Fig. 8. Levels of mRNAs encoding proteins involved in Cbl metabolism in worms treated with the CN-Cbl dodecylamine derivative. Using qPCR, we determined the levels of mRNAs encoding (A) methylmalonyl-CoA mutase, MCM; (B) methylmalonic aciduria type A protein, MMAA; (C) methylmalonic aciduria type B,MMAB (mmab-1 encodes an ortholog of human cob(I)alamin adenosyltransferase in *C. elegans*); (D) Cbl deficiency C complementation group (human) homolog, MMACHC; and (E) methionine synthase reductase, MSR in worms treated with CN-Cbl (control; 1, black bar), the CN-Cbl dodecylamine derivative (2; white bar), and Cbl-deficient worms (3; shaded bar). Data represent the mean ± SD of three independent experiments. The letters indicate values that are significantly different, *p* < 0.05.
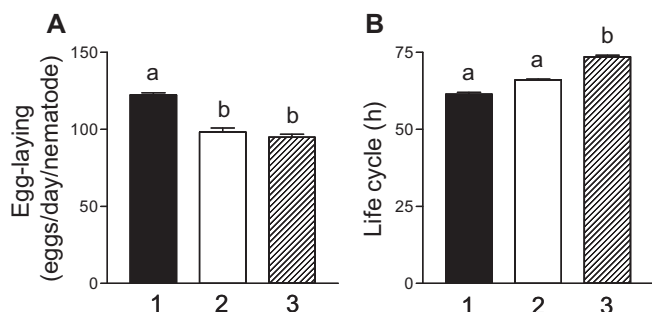
Fig. 9. Egg-laying capacity and the length of the life cycle of worms treated with the CN-Cbl dodecylamine derivative. Egg numbers per day per worm (A) and length (h) of the life cycle (B) of worms treated with CN-Cbl (control; 1, black bar), CN-Cbl dodecylamine derivative (2, white bar), and Cbl-deficient worms (3, shaded bar). Data represent the mean ± SD of five independent experiments. The letters indicate values that are significantly different, $p < 0.05$.

[13] indicated that when 10 mg/L OH-Cbl [*c*-lactam] was added to a culture medium of rat oligodendrocytes and incubated for 25 days, MMA and Hcy were approximately 200 times and twice greater, respectively, in OH-Cbl [*c*-lactam]-treated cells than in the control cells.

Subcutaneous administration of OH-Cbl [*c*-lactam] to rats causes Cbl deficiency because plasma MMA was approximately 331 times and twice greater in the treated rats than in the control and Cbl-deficient rats, respectively [14]. Hepatic holo-MCM and MS activities of OH-Cbl [*c*-lactam]-treated rats decreased to approximately 65% and 43% of the control activities, respectively, indicating that OH-Cbl [*c*-lactam] potently inhibits mammalian Cbl-dependent enzymes [15].

However, high concentrations of OH-Cbl [*c*-lactam] must be administered for several weeks using osmotic mini-pumps to produce Cbl-deficient rats [9] because of poor binding to IF [15]. Compared with OH-Cbl [*c*-lactam], the CN-Cbl dodecylamine derivative is easy to prepare and acts a potent inhibitor of both MCM and MS. Moreover, this compound shows high affinity binding of IF [9]. These properties of the CN-Cbl dodecylamine derivative suggest that Cbl deficiency in mammals may be readily induced by oral or intravenous administration of this compound.

3. Materials and methods

Chemicals: CN-Cbl, 1,1′-carbonyldiimidazole, and dodecylamine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Phenyl-Toyopearl 650 M was purchased from Tosoh Corporation (Tokyo, Japan).

3.1. Organisms and culture conditions

The N2 Bristol wild-type *C. elegans* strain was maintained at 20 °C on nematode growth medium (NGM) plates using *E. coli* OP50 as the food source [16]. To induce dietary Cbl deficiency, worms were grown on 1.7% (w/v) agar plates containing M9 medium (3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 1 mmol/L $MgSO_4$, 50 μmol/L $CaCl_2$, 2 g/L glucose, 4 mg/L thiamine hydrochloride, and 5 mg/L cholesterol) in l L water. Each plate containing M9-medium supplemented with Cbl (100 μg/L CN-Cbl) received one egg obtained from worms grown on NGM plates with Cbl-deficient *E. coli* OP50 [8]. Eggs were allowed to hatch and develop into reproductively active adults. The adults were then removed from each plate, eggs were collected, and each egg was transferred onto a new control plate. After this procedure was repeated at least 10 times, the worms were used as controls. Cbl-deficient worms were prepared as described previously [8].

3.2. Preparation of the CN-Cbl dodecylamine derivative

The CN-Cbl dodecylamine derivative was prepared according to the method by McEwan et al. [9]. In brief, solid 1,1′-carbonyldiimidazole (26 mg) was added to CN-Cbl (0.1 g) dissolved in dimethyl sulfoxide (1.2 mL) at 30 °C, and the mixture was stirred for 25 min. Dodecylamine (0.27 mmol) was added to the mixture and then stirred for 24 h at 25 °C. The Cbl compound was extracted twice from the mixture with 2 mL of phenol/dichloromethane (1:2, v/v) and then re-extracted twice from the combined phenol fractions with 2 mL of distilled water. The water-soluble fractions were combined and chromatographed using a TSK gel-Toyopearl 650 M column (2.4 × 24 cm) equilibrated with 250 mL of 25% (v/v) ethanol. Unreacted and modified CN-Cbl compounds were eluted with 250 mL of 25% (v/v) and 60% (v/v) ethanol. The fractions containing products were combined, the solvent was evaporated under reduced pressure, and the residue was dissolved in 5 mL of 80% (v/v) ethanol. The product was further purified using silica-gel 60 TLC with the solvent 1-butanol:2-propanol:water (10:7:10, v/v/v). After drying the TLC sheet, the CN-Cbl derivative was collected, extracted with 80% (v/v) methanol, and the solvent was evaporated under reduced pressure. The residue was dissolved in a small amount of 18% (v/v) acetonitrile and then purified using a reversed-phase high performance liquid chromatography (HPLC) column (Wakosil-II 5C18RS, φ 4.6 × 150 mm) and a Shimadzu HPLC system (SCL-10A VP System controller, DGU-20A 3R Degassing unit, LC-20AB Liquid chromatograph, SPD-20A UV/VIS detector, and CTO-20AC Column oven). The CN-Cbl derivative was eluted with a linear gradient of acetonitrile (18–28% for 8.5 min and 28–100% for 13 min, 1.0 mL/min) at 40 °C and monitored by measuring absorbance at 254 nm. The peak fraction was collected, the solvent was evaporated under reduced pressure, and the residue was used for the following experiments. The purified dodecylamine derivative of CN-Cbl (approximately 94% purity) was dissolved in distilled water, and its concentration was determined at λ_{361} ($\varepsilon = 13,000$) as described previously [9].

3.3. Preparation of C. elegans treated with CN-Cbl dodecylamine derivative

Control worm eggs were transferred to and hatched on an M9 plate containing the CN-Cbl derivative (100 μg/L) and *E. coli* OP50 and grown to maturity.

3.4. Determination of the levels of CN-Cbl and its derivative in C. elegans

Worms grown in the absence or presence of CN-Cbl or its dodecylamine derivative were harvested and incubated for 1 h at 20 °C in fresh M9 medium to remove any residual *E. coli*. The washed worms (1.0 g wet weight) were homogenized in 0.5 mL of 100 mmol/L potassium phosphate buffer (pH 7.0) at 4 °C using a manual homogenizer (AS ONE Corp., Osaka, Japan) and sonicated (6 kHz for 60 s) 3 times on ice. The worm homogenate was resuspended in 25 mL of 57 mmol/L sodium acetate buffer (pH 4.8) containing 0.05% (w/v) KCN, boiled for 30 min, and centrifuged at 15,000×g for 15 min at 4 °C. The Cbl content of the supernatant fraction was determined using a microbiological assay employing *L. delbrueckii* subsp. *lactis* ATCC 7830 as described previously [17].

CN-Cbl and the Cbl derivative were extracted from treated worms using the same conditions as described above. The supernatant fraction (20 mL) that was chromatographed using a Sep-Pak Plus C18 cartridge (Waters Corp., Milford, MA, USA) to separate the CN-Cbl derivative from CN-Cbl. After the cartridge was washed with 10 mL of distilled water, CN-Cbl and the CN-Cbl derivative were eluted sequentially with 10 mL of 20% (v/v) ethanol solution

and 60% (v/v) ethanol, respectively. The solvent of each fraction was evaporated under reduced pressure and then dissolved in 100 μL of distilled water. CN-Cbl was determined using the microbiological Cbl assay method described above, and the CN-Cbl derivative was assayed using a Shimadzu HPLC system (SCL-10A VP System controller, DGU-20A 3R Degassing unit, LC-20AB Liquid chromatograph, SPD-20A UV/VIS detector, and CTO-20AC column oven) with a CDS ver. 5 chromato-data processing system (LAsoft Ltd., Chiba, Japan). Samples (20 μL) were chromatographed using a reversed-phase HPLC column (Wakosil-II 5C18RS, φ 4.6 × 150 mm) and eluted (1.0 mL/min) with a linear gradient of methanol (20–90% for 30 min) containing 1% (v/v) acetic acid at 40 °C. The Cbl derivative was monitored by its absorbance at 254 nm, and its retention time was 30.3 min.

3.5. Determination of coenzyme forms of the Cbl-dodecylamine derivative

All procedures were performed in the dark. Hydro (OH)- and adenosyl (Ado)-forms of the Cbl-dodecylamine derivative were prepared from the CN-Cbl derivative. In brief, the CN-Cbl dodecylamine derivative was dissolved in distilled water, bubbled with nitrogen gas for 30 min, reduced with sodium tetrahydroborate until the solution turned dark brown, and neutralized with 1.0 mol/L HCl. Most of the CN-Cbl derivative was converted to the OH-Cbl dodecylamine derivative. To prepare AdoCbl dodecylamine derivative, a small amount of 5′-iodo-5′-deoxyadenosine was added to the reduced form of the Cbl dodecylamine derivative, and this solution was neutralized as described above. The neutralized preparations were desalted using a Sep-Pak Vac 20 cc (5 g) C18 cartridge (Waters Corp.), and the desalted and concentrated solutions were treated with silica-gel 60 TLC as described above to separate the OH- or Ado-form of the dodecylamine derivative and unmodified CN-Cbl dodecylamine derivative. After drying the TLC sheet, the OH- or Ado-form of the dodecylamine derivative was collected, extracted with 80% (v/v) ethanol, and the solvent was evaporated to dryness under reduced pressure. Each residue was dissolved in a small amount of distilled water and used as the authentic OH-Cbl dodecylamine or AdoCbl dodecylamine derivatives. We were unable to prepare a CH_3-Cbl dodecylamine derivative with high purity.

To extract Cbl derivatives, including its coenzyme forms from worms, 100 mL of 80% (v/v) ethanol was added to lyophilized samples of worms treated with the CN-Cbl dodecylamine derivative. The samples were heated at 98 °C for 30 min under reflux and cooled to room temperature [18], centrifuged at $10,000 \times g$ for 10 min, and the supernatant was evaporated under reduced pressure. The residue was dissolved in 5 mL of distilled water and centrifuged at $10,000 \times g$ for 10 min to remove insoluble material. The supernatant fraction was chromatographed a Sep-Pak Plus C18 cartridge, which was washed with 10 mL of distilled water and eluted with 2 mL of ethanol. The ethanol elute was evaporated under reduced pressure and the residue was dissolved in 100 μL of distilled water. The levels of coenzyme forms of the Cbl dodecylamine derivative in worms treated with the CN-Cbl dodecylamine derivative were determined using HPLC as described above. The retention times of OH-, CN, and Ado-forms of Cbl dodecylamine derivatives were 22.0, 30.6, and 36.2 min, respectively.

3.6. Assays for Cbl-related biomarkers

The worms (1.0 g wet weight) grown as described above, washed with fresh M9 medium, homogenized in 0.5 mL of 100 mmol/L potassium phosphate buffer (pH 7.0) at 4 °C using a manual homogenizer (AS ONE), and sonicated 3 times on ice. Each homogenate was centrifuged at $15,000 \times g$ for 15 min at 4 °C, and

the supernatant was used as a crude homogenate for assaying Cbl-related biomarkers. The levels of methylmalonic acid (MMA) [19] and homocysteine (Hcy) [20], two indicators of Cbl deficiency, were determined using HPLC as described above. Cbl-dependent enzyme (MCM and MS) activities were assayed using published methods [21,22]. Total enzyme (holoenzyme and apoenzyme) and holoenzyme activities were determined in the presence or absence of Cbl coenzymes (AdoCbl for MCM and CH_3-Cbl for MS).

3.7. Inhibition of MCM and MS activities by the CN-Cbl dodecylamine derivative

Our previous study [8] showed that the levels of apo-MCM are increased up to approximately 97% of the total MCM (holo- and apo-MCMs) in Cbl-deficient worms. Thus, a cell homogenate of Cbl-deficient worms was used as an apo-MCM preparation in the following experiments. Lineweaver–Burk analysis was used to determine the K_m value of worm MCM for AdoCbl in reaction mixtures containing 0, 2.5, 5, and 10 μM of AdoCbl. Dixon plots [23] were used to determine the apparent K_i value of MCM in reaction mixtures containing 0, 1, 3, 5, and 10 μM of CN-Cbl or its dodecylamine derivative.

A cell homogenate of Cbl-supplemented (control) worms was used as a holo-MS enzyme preparation, because most MS activity is derived from the holoenzyme in Cbl-supplemented or -deficient worms [8]. Holo-MS activity was determined in reactions containing 0, 1, 3, 5, and 10 μM of CN-Cbl or its dodecylamine derivative.

3.8. Western blotting

Worms grown in the absence of CN-Cbl or in the presence of CN-Cbl or the CN-Cbl dodecylamine derivative were homogenized in 100 mmol/L potassium phosphate buffer (pH 7.0) at 4 °C. Each homogenate was centrifuged at $15,000 \times g$ for 10 min and the supernatant fraction was analyzed. We used a precast slab gel (PAGEL, type NPG-520L, ATTO Corporation, Tokyo, Japan) for electrophoresis of samples through a 5–20% (w/w) linear gradient of polyacrylamide in the presence of SDS. After electrophoresis, proteins were transferred to a PVDF membrane (Immuno-Blot PVDF, Bio-Rad Laboratories Inc., Hercules, CA, USA) in a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad). The PVDF membrane was probed with an anti-MS antibody (ab66039, abcam®, Cambridge, MA, USA). We performed the immunodetection reactions using an anti-mouse IgG antibody secondary antibody (Promega KK, Tokyo, Japan) coupled to horseradish peroxidase and an immunoblot-staining kit for peroxidase (EzWestBlue, ATTO), according to the manufacturer's instructions. A Protein Ladder One Triple-color kit (Nacalai Tesque Inc., Kyoto, Japan) was used to determine molecular mass. After the treated PVDF membrane was photographed using a digital camera (Coolpix 4300, Nikon, Japan), the intensities of the protein bands were calculated of Image J software [24].

3.9. Quantitative PCR analysis (qPCR)

Total RNA was prepared from worms using Sephasol®-RNA1 (Nacalai Tesque). Poly(A)$^+$ mRNA prepared from total RNA using the Poly (A)$^+$ Isolation kit from Total RNA (Nippon Gene, Tokyo, Japan) was used to synthesize cDNA using PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara Bio, Otsu, Japan). Primer pairs used for qPCR analysis were designed using GENETYX software (GENETYX Corporation, Tokyo, Japan) (Table 2). Gene-specific primers were selected such that the resulting PCR products were approximately 100 bp. A CFX Connect™ Real-Time System (Bio-Rad) with SYBR Premix Ex Taq (Takara Bio) was used to perform qPCR. The level of the mRNA encoding β-actin was used as

Table 2
Primer pairs used for the qPCR analysis.

Gene symbol	Primer sequences (5′–3′)
mmcm-1	ATTAAAGCAGGGCAGCAAGGAC and GGCACGGATATGGTTCAACTTGG
metr-1	AAAGACCACGACAAGCCACTGA and CGCTTGATGATTCGAGCACGTT
mmaa-1	TTGGAAAGAAGGCGACAGGA and TTGTTTCTGCAGCCAGACCA
mmab-1	GCTCTTCTGATTTGGGAAGAGG and CGACGCTCATTGTTGTACAGAG
cblc-1	ATGTTGCTGGAGCAGCTTTC and AGTGTCCACCGTAGATTGGATG
mtrr-1	AAGACCGCTGATTCGTGTACTC and GCATGTCAGCCAATGACAATCC
act-1	TCCAAGAGAGGTATCCTTACCC and CTCCATATCATCCCAGTTGGTG

Primer pairs for the qPCR were designed using GENETYX software. Each gene is involved in the metabolisms of Cbl, including MCM (*mmcm-1*), MS (*metr-1*), methylmalonic acidemia cobalamin A complementation group (*mmaa-1*), cob(I)alamin adenosyltransferase (*mmab-1*), methylmalonic aciduria cblC type (*cblc-1*), and MS reductase (*mtrr-1*). For normalization, β-actin (*act-1*) mRNA levels served as the internal standard.

an internal standard. The qPCR experiments were repeated at least 3 times for each cDNA prepared from three preparations of worms.

3.10. Analysis of egg-laying capacity and life cycle

Measurements of egg-laying capacity were performed according to the method by Byerly et al. [25]. Individual worms grown in the presence of CN-Cbl, its dodecylamine derivative or in the absence of Cbl were transferred to fresh plates containing the respective culture media and incubated for 1 day at 20 °C. After laying eggs, each worm was removed from the plate, and the eggs were counted in triplicate. The life cycle of worms grown under each experimental condition was determined at 20 °C using the synchronization method by Johnson and Wood [26].

3.11. Protein quantitation

Protein concentrations were determined using the Bradford method [27] with ovalbumin as a standard.

3.12. Statistical analysis

The effects of the CN-Cbl dodecylamine derivative on *C. elegans* phenotypes were evaluated using one-way ANOVA with Tukey's multiple comparison test (GraphPad Prism 3 for Windows version 2.01; GraphPad Software Inc., La Jolla, CA, USA). All data are presented as the mean ± standard deviation (SD). Significant differences were defined as $p < 0.05$.

Author contributions

T.B. and F.W. planned experiments; T.B. performed experiments; T.B. and Y.Y. analyzed data; T.I. and T.K. contributed reagents or other essential material; and T.B. and F.W. wrote the paper.

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