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Cobalamin Analogues Modulate the Growth of Leukemia Cells *in Vitro*[1]

Gary R. McLean, Pradip M. Pathare, D. Scott Wilbur, A. Charles Morgan, Clive S. Woodhouse, John W. Schrader, and Hermann J. Ziltener[2]

The Biomedical Research Centre [G. R. M., J. W. S., H. J. Z.] and Departments of Medicine [J. W. S.] and Pathology and Laboratory Medicine [H. J. Z.], University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3; Department of Radiation Oncology, University of Washington, Seattle, WA 98103 [P. M. P., D. S. W.]; and Receptagen Corp., Edmonds, WA 98020 [A. C. M., C. S. W.]

ABSTRACT

Analogues of cyanocobalamin (CN-Cbl), with functional groups attached to either the various propionamide groups of the corrin ring or to the ribose-nucleotide linker arm, have been evaluated in a cobalamin (Cbl)-dependent *in vitro* cell growth assay. In this bioassay, CN-Cbl supported, in a dose-dependent manner, the growth of the murine lymphoma BW5147 and the Cbl carrier protein, human apo-transcobalamin II, reduced the required concentration of Cbl by 100-1000-fold. Any chemical modification of Cbl decreased its ability to support cellular viability and proliferation, with several of the modifications abrogating activity completely. All of the Cbl analogues that promoted growth required the presence of apo-transcobalamin II for the optimal support of cell growth. Generally, Cbl analogues modified at the *d*-position of the corrin ring and, to a lesser degree, analogues modified at the *b*- position supported cell growth, whereas analogues with modifications at the *e*-position did not support cell growth. Mixing experiments demonstrated an inverse order of potency of Cbl analogues to inhibit cell growth. Thus, Cbl analogues with modifications at the *e*-position were potent inhibitors, whereas *b*-analogues exhibited only partial inhibitory activity at high molar excess, and *d*-analogues had no inhibitory activity at all. These results indicate that modifications at the *e*-position of Cbl abolish the ability of Cbl to support cell growth and generate potent inhibitors of Cbl-dependent cell growth.

INTRODUCTION

CN-Cbl[3] is a water-soluble vitamin (vitamin B_{12}) that is essential for cell growth. Naturally occurring Cbl analogues are required as coenzymes by two mammalian enzymes that catalyze metabolically critical monocarbon transfer reactions (1). One reaction involves the methylation of homocysteine in the *de novo* synthesis of methionine and is catalyzed by methionine synthase. The other reaction rearranges L-methylmalonyl-CoA to succinyl-CoA and is catalyzed by L-methylmalonyl-CoA mutase. Cbl-binding proteins (R-binders and intrinsic factor) aid in its absorption from food and in its transport (2). The cellular uptake of Cbl is facilitated by the plasma protein TCII (3), which, when complexed to Cbl, binds to specific high affinity receptors on the surface of cells (4). The Cbl-TCII complex is internalized by receptor-mediated endocytosis, and Cbl is thought to be released from TCII via lysosomal action, followed by enzymatic modification to the forms that are active as coenzymes (1).

In humans, deficiencies of the vitamin or perturbations of its intracellular metabolism can result in a variety of cell growth-related disorders, including megaloblastic anemia, methylmalonic aciduria, and central nervous system abnormalities due to the improper functioning of the Cbl-dependent enzymes (5, 6). Cbl deficiency may be brought on by a lack of dietary Cbl, dysfunction of Cbl uptake via abnormalities in the binding proteins, including TCII (7), or errors of intracellular Cbl metabolism (8). Because Cbl deficiency can result in decreased cell proliferation, as evidenced in megaloblastic anemia, we have been investigating new methods to interfere with Cbl metabolism as part of a program to develop antiproliferative agents.

There are many naturally occurring analogues of Cbl (9), as well as a variety of Cbl analogues that have been synthesized by different laboratories (10, 11). Analysis of Cbl analogues *in vitro* and *in vivo* have shown that some interfere with Cbl metabolism, as evidenced by increased levels of homocysteine and methylmalonic acid, the substrates of the two mammalian enzymes dependent on Cbl (10, 12). More recently, it has been shown that relatively high doses of the c-lactam of CN-Cbl can inhibit the *in vitro* growth of HL60 cells (13), further promoting Cbl metabolism as a potential antiproliferative target.

In vitro cultures in which growth is dependent on Cbl have been reported (14, 15). Recently, we have described *in vitro* growth conditions in which the proliferation of human and murine leukemia cells were dependent on Cbl and recombinant human TCII (16). We have used this Cbl/TCII-dependent proliferation assay to evaluate the changes in growth characteristics of leukemic cells brought about by modifications in the chemical structure of Cbl. Here, we show that the modification of Cbl generally resulted in reduced ability to support cell growth. In particular, modifications of the propionamide side chains of the Cbl corrin ring resulted in reduced or complete loss of activity. In many cases, the loss of bioactivity of Cbl analogues correlated with a capacity to inhibit Cbl-dependent cell proliferation in a dose-dependent manner.

MATERIALS AND METHODS

Materials. BW5147 mouse lymphoma cells were obtained from American Type Culture Collection (Rockville, MD). RPMI 1640 culture medium and RPMI 1640 culture medium deficient in Cbl and folate were obtained from Stem Cell Technologies (Vancouver, Canada). FCS was from Life Technologies, Inc. (Grand Island, NY). QUSO was a gift from Degussa Corp. (Ridgefield, NJ). CN-Cbl, 5-methyl tetrahydrofolate, MTT, and D,L-homocysteine were obtained from Sigma Chemical Co. (St. Louis, MO).

Recombinant Human TCII. Recombinant protein (apo form), kindly provided by E. V. Quadros (Veteran Affairs Medical Center and State University of New York Health Science Center, Brooklyn, NY), had been produced by infection of SF9 cells with baculovirus containing human TCII cDNA and purified as described (17).

Cell Culture. BW5147 cells were maintained in complete RPMI 1640 medium supplemented with 10% FCS. Cells were grown in 60 × 15 mm culture dishes (Fisher Scientific Co., Nepean, Canada) in a humidified atmosphere (5% CO_2, 95% air) at 37°C. Cells used in the Cbl/TCII bioassay were grown to late logarithmic phase, then washed three times in PBS before resuspension in Cbl-deficient bioassay medium.

Cbl/TCII Bioassay. To measure Cbl/TCII-dependent cell growth, we used RPMI 1640 deficient in Cbl and in which the folic acid was replaced with 1 μM 5-methyltetrahydrofolate and 1 μM homocysteine. FCS was pretreated with QUSO to reduce interference of endogenous bovine TCII/Cbl in the bioassay (16). In brief, 30 mg of QUSO were added per ml of FCS, mixed well, and removed by centrifugation as described previously (18). Washed cells were

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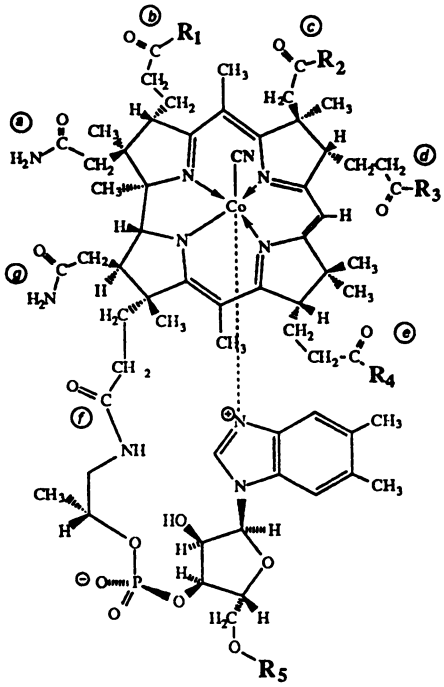
[2] To whom requests for reprints should be addressed, at The Biomedical Research Centre, 2222 Health Sciences Mall, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3. Phone: (604) 822-7834; Fax: (604) 822-7815; E-mail: hermann@brc.ubc.ca.

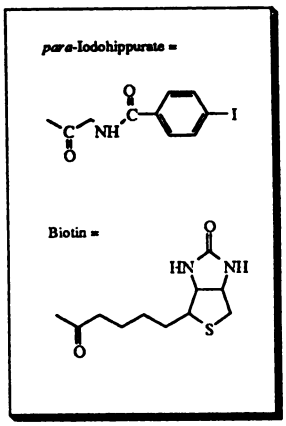
[3] The abbreviations used are: CN-Cbl, cyanocobalamin; Cbl, cobalamin; TCII, transcobalamin II; QUSO, microfine precipitated silica; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Ihipp, iodohippurate.

plated out in 96-well microtiter plates at 2000 cells per well in 100 μl of the above medium, supplemented with 10% QUSO-treated FCS. Following 5 days in culture with various concentrations of CN-Cbl or Cbl analogues in the presence and absence of 25 ng/ml apo-TCII, cell viability was assessed with MTT reduction by measuring the absorbance at 550 nm ($A_{550\ nm}$), as described previously (19, 20).

Inhibition Bioassay. To test the inhibitory effects of the Cbl analogues on Cbl-dependent cell growth, BW5147 cells were cultured in the above bioassay medium. The tested Cbl analogues were titrated in the presence of a constant concentration of CN-Cbl (0.5, 5, or 50 nM) in the absence or presence of 25 ng/ml apo-TCII. Following 5 days in culture, cell viability was assessed by MTT reduction.

Cbl Derivatives. The syntheses of Cbl analogues **2–24** have been described previously (21–23), and their structures are shown in Figs. 1 (monomeric analogues) and 2 (dimeric analogues). The Cbl analogues were purified to >99% by high-performance liquid chromatography separation (21). Importantly, no free Cbl could be detected in any of the purified Cbl analogue preparations. Cbl analogues (2 mg) were dissolved in 100–200 μl of DMSO

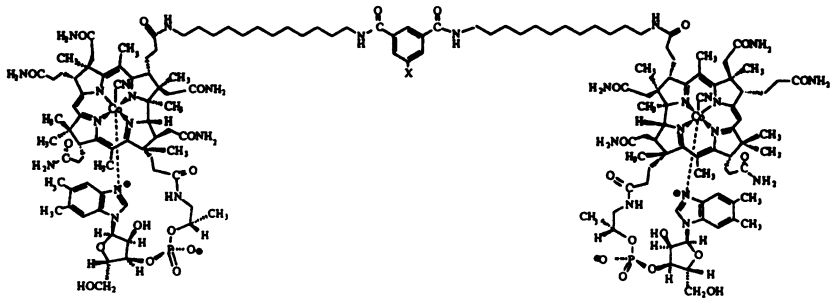




Compd #	Sidechain Structures (R_1-R_5)	Designation
1	$R_1 = NH_2$; $R_2 = NH_2$; $R_3 = NH_2$; $R_4 = NH_2$; $R_5 = H$	(CN-Cbl)
2	$R_1 = OH$; $R_2 = NH_2$; $R_3 = NH_2$; $R_4 = NH_2$; $R_5 = H$	(*b*-COOH)
3	$R_1 = NH_2$; $R_2 = OH$*; $R_3 = NH_2$; $R_4 = NH_2$; $R_5 = H$	(*c*-lactone)
4	$R_1 = NH_2$; $R_2 = NH_2$; $R_3 = OH$; $R_4 = NH_2$; $R_5 = H$	(*d*-COOH)
5	$R_1 = NH_2$; $R_2 = NH_2$; $R_3 = NH_2$; $R_4 = OH$; $R_5 = H$	(*e*-COOH)
6	$R_1 = NH(CH_2)_{12}NH_2$; $R_2 = NH_2$; $R_3 = NH_2$; $R_4 = NH_2$; $R_5 = H$	(*b*-NH_2)
7	$R_1 = NH_2$; $R_2 = NH(CH_2)_{12}NH_2$; $R_3 = NH_2$; $R_4 = NH_2$; $R_5 = H$	(*c*-NH_2)
8	$R_1 = NH_2$; $R_2 = NH_2$; $R_3 = NH(CH_2)_{12}NH_2$; $R_4 = NH_2$; $R_5 = H$	(*d*-NH_2)
9	$R_1 = NH_2$; $R_2 = NH_2$; $R_3 = NH_2$; $R_4 = NH(CH_2)_{12}NH_2$; $R_5 = H$	(*e*-NH_2)
10	$R_1 = NH(CH_2)_{12}$NH-Biotin; $R_2 = NH_2$; $R_3 = NH_2$; $R_4 = H$; $R_5 = CN$	(*b*-biotin)
11	$R_1 = NH_2$; $R_2 = NH(CH_2)_{12}$NH-Biotin; $R_3 = NH_2$; $R_4 = NH_2$; $R_5 = H$	(*c*-biotin)
12	$R_1 = NH_2$; $R_2 = NH_2$; $R_3 = NH(CH_2)_{12}$NH-Biotin; $R_4 = NH_2$; $R_5 = H$	(*d*-biotin)
13	$R_1 = NH_2$; $R_2 = NH_2$; $R_3 = NH_2$; $R_4 = NH(CH_2)_{12}$NH-Biotin; $R_5 = H$	(*e*-biotin)
14	$R_1 = NH(CH_2)_{12}$NH-*p*-Iodohippurate; $R_2 = NH_2$; $R_3 = NH_2$; $R_4 = NH_2$; $R_5 = H$	(*b*-Ihipp)
15	$R_1 = NH_2$; $R_2 = NH_2$; $R_3 = NH(CH_2)_{12}$NH-*p*-Iodohippurate; $R_4 = NH_2$; $R_5 = H$	(*d*-Ihipp)
16	$R_1 = NH_2$; $R_2 = NH_2$; $R_3 = NH_2$; $R_4 = NH(CH_2)_{12}$NH-*p*-Iodohippurate; $R_5 = H$	(*e*-Ihipp)
17	$R_1 = NH_2$; $R_2 = NH_2$; $R_3 = NH_2$; $R_4 = NH_2$; $R_5 = COCH_2CH_2COOH$	(5′-COOH)
18	$R_1 = NH_2$; $R_2 = NH_2$; $R_3 = NH_2$; $R_4 = NH_2$; $R_5 = CO(CH_2)_2CONH(CH_2)_{12}NH_2$	(5′-NH^2)

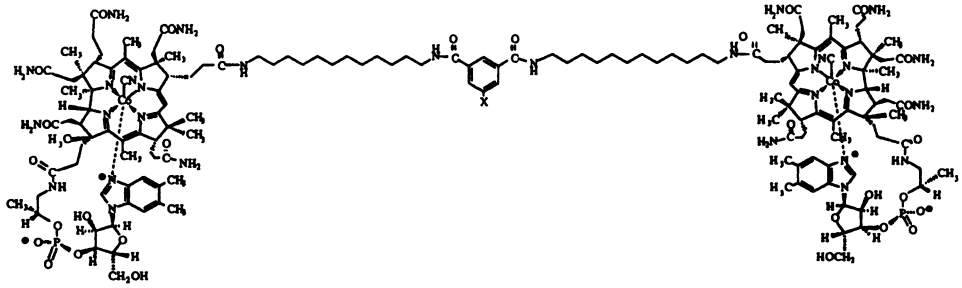
Fig. 1. Monomeric CN-Cbl derivatives evaluated in cell growth assays. *Circled letters*, corrin ring side chain nomenclature system

b-propionamide dimer



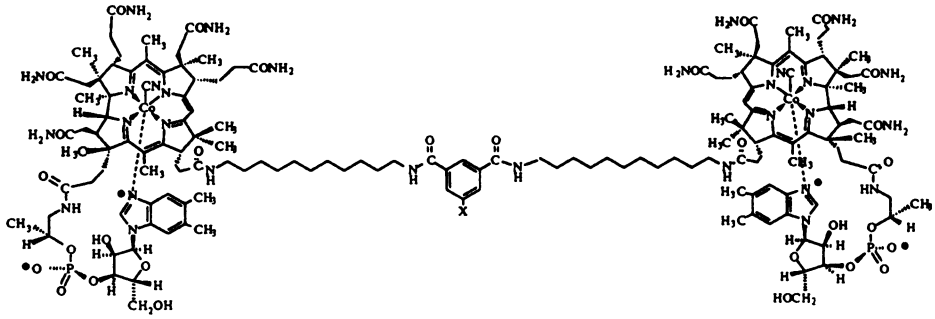
19: X = H
20: X = *p*-iodobenzoate (Ibz)

d-propionamide dimer



21: X = H
22: X = *p*-iodobenzoate

e-propionamide dimer



23: X = H
24: X = *p*-iodobenzoate

Fig. 2. Dimeric CN-Cbl derivatives evaluated in cell growth assays.

and then diluted to 1 ml with double-distilled water, and aliquots were stored at 4°C in the dark until tested. The Cbl concentration of these preparations was determined by measuring $A_{360\ nm}$ using the experimentally derived mM extinction coefficient, as described previously (21). The highest final concentration of DMSO in the bioassay medium was approximately 0.2%. This concentration and lower concentrations of DMSO do not interfere with Cbl/TCII-dependent cell growth.

RESULTS

Cell Growth Supported by CN-Cbl. The *in vitro* cell growth assay we previously established (16) has allowed us to monitor the effects of Cbl and its plasma transport protein, TCII, on cell growth. Figs. 3 and 4 show that CN-Cbl supports the growth of the murine leukemic cell line BW5147 in a dose-dependent manner and that this growth is enhanced by the addition of 25 ng/ml of recombinant human apo-TCII. Maximal cell growth was achieved with 200–1000 nM CN-Cbl, with detectable effects on cell growth at concentrations as low as 1–10 nM CN-Cbl. When optimal amounts of apo-TCII were added, the concentrations of CN-Cbl required were approximately 1000-fold lower. The levels of Cbl/TCII that were present were comparable to those found *in vivo*, indicating that, as expected, the natural Cbl transport protein TCII aids in delivering Cbl to proliferating cells.

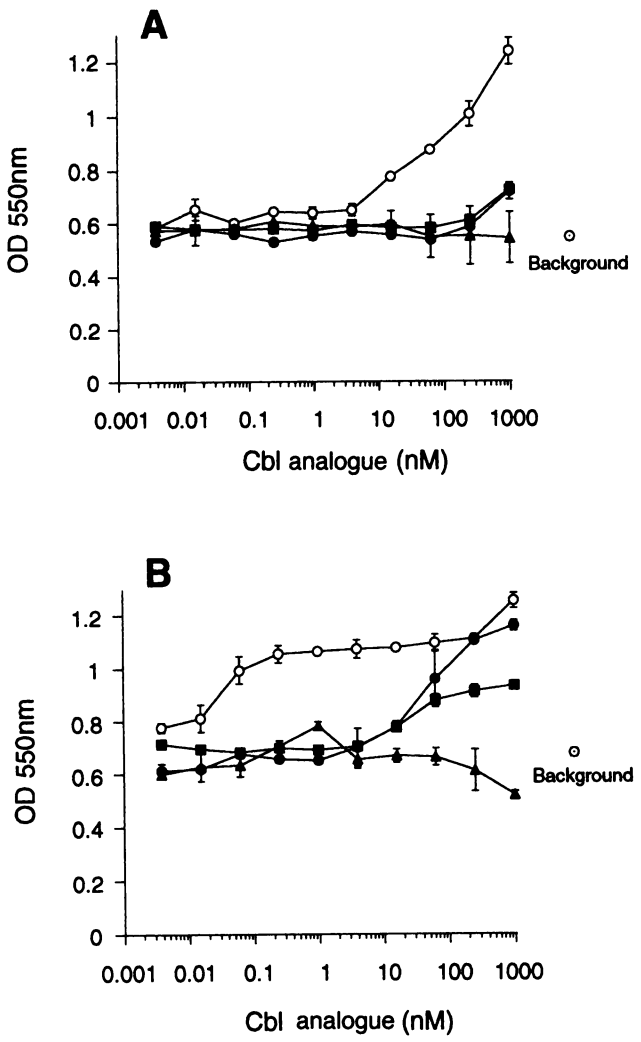


Fig. 3. Cbl-Ihipp analogues *b*-Ihipp and *d*-Ihipp support cell proliferation in the absence and presence of TCII. BW5147 cells were seeded at 2000 cells/well in bioassay medium containing the indicated concentrations of CN-Cbl (**1**; ○), *d*-Ihipp (**15**; ●), *b*-Ihipp (**14**; ■), and *e*-Ihipp (**16**; ▲) in the absence (*A*) and presence (*B*) of 25 ng/ml apo-TCII. ⊙, background cell viability in the absence of CN-Cbl and Cbl analogue. After 5 days in culture, cell viability was assessed by MTT reduction. *Data points*, means of three replicate results; *bars*, SE.

Cell Growth Supported by Cbl Analogues. Various analogues of Cbl were tested in this assay for their ability to replace CN-Cbl, in both the absence and presence of apo-TCII, and their dose-response curves correlated with those of CN-Cbl (**1**). The results are shown in summary form in Table 1. None of the analogues were as potent in supporting growth as was CN-Cbl, either in the absence or presence of apo-TCII. Moreover, even at the highest concentration tested and in the presence of apo-TCII, none were able to support growth to the level achieved by CN-Cbl.

In the absence of apo-TCII, analogues with substituents at the ribose-5′-OH (**17** and **18**) were the most active in supporting cell growth (Table 1). Fig. 3 shows bioassay data for CN-Cbl and Cbl analogues with Ihipp substitutions at the *d*-, *b*-, and *e*- positions of the corrin ring (**15**, **14**, and **16**, respectively). Two of this group of Cbl analogues were capable of supporting cell growth in the absence of TCII but only to 20% of the level observed with CN-Cbl and only at the highest concentration tested. Thus, at the concentration range tested, only four of the Cbl analogues retained bioactivity in the absence of TCII.

In contrast, in the presence of apo-TCII, many analogues exhibited detectable activity (Table 1), indicating that TCII-mediated internalization occurred. These include the *c*-lactone (**3**) and *d*- and *b*-carboxylate conjugated analogues with various substitutions (**6**, **8**, **14**, and **15**) and the ribose-conjugated analogues (**17** and **18**). Dimeric analogues of Cbl-*b*-carboxylates and Cbl-*d*-carboxylates, linked through an isophthaloyl moiety (**19–22**), were able to support cell growth to a low level. However, the Cbl-*b*-carboxylate and Cbl-*d*-carboxylate analogues conjugated with biotin (**10** and **12**) were unable to support cell growth, either in the absence or presence of apo-TCII. Analogues with monocarboxylic acid substitutions at the *d*-, *b*-, and *e*-positions of the corrin ring (**2**, **4**, and **5**) were also ineffective supporters of cell proliferation. Generally, any change of the Cbl structure at the *e*-position of the corrin ring (**5**, **9**, **13**, **16**, **23**, and **24**) resulted in a complete loss of ability to support cell growth, even in the presence of TCII, suggesting this position of the molecule may be important in the intracellular function of Cbl. Also, ring opening conjugations with the *c*-lactone resulted in the loss of cell growth (**7** and **11**).

The growth response of BW5147 cells in the presence of Cbl-isophthaloyl-linked dimers (**19**, **20**, and **23**) compared to that with CN-Cbl is shown in Fig. 4. Again, apo-TCII was absolutely required for dimeric Cbl analogue support of cell growth at the concentrations tested (Fig. 4*B*). Interestingly, the *d*- and *b*-linked Cbl dimers (**21**, **19**)

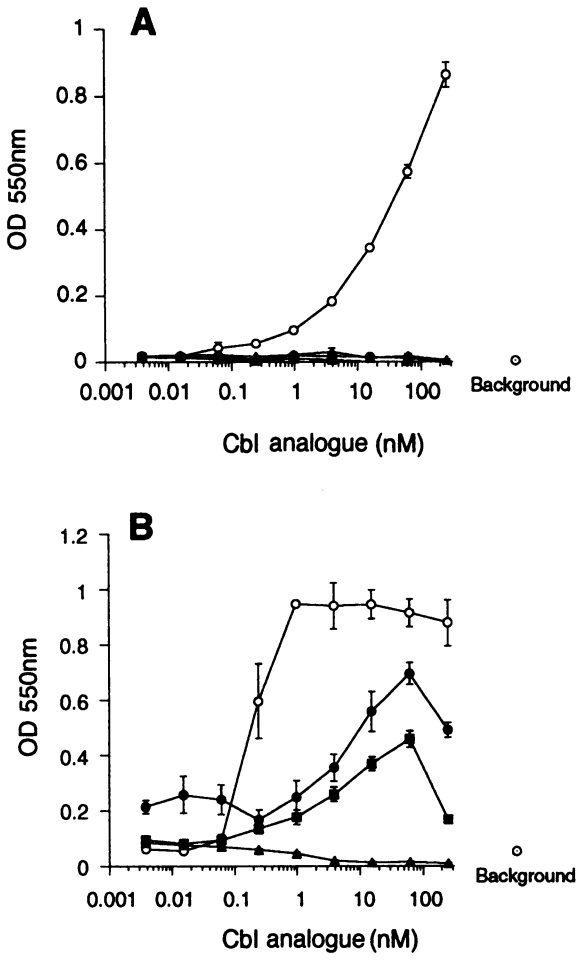


Fig. 4. *b*- and *d*-Cbl-isophthaloyl dichloride dimer analogues support cell proliferation in the presence of TCII. BW5147 cells were seeded at 2000 cells/well in bioassay medium containing the indicated concentrations of CN-Cbl (**1**; ○), *b*-dimer (**19**; ●), *d*-dimer (**21**; ■), and *e*-dimer (**23**; ▲) in the absence (*A*) and presence (*B*) of 25 ng/ml apo-TCII. ⊙, background cell viability in the absence of CN-Cbl (**1**) and Cbl analogue. After 5 days in culture, cell viability was assessed by MTT reduction. *Data points*, means of three replicate results; *bars*, SE.

Table 1 *Cell growth with CN-Cbl derivatives in the presence or absence of recombinant human apo-TCII*[a]

Cobalmin derivative		Cell Growth	
Compound no.	Designation	−apo-rhTCII	+apo-rhTCII
1	(CN-Cbl)	++++	++++
2	*b*-COOH	−	−
3	*c*-Lactone	−	++
4	*d*-COOH	−	−
5	*e*-COOH	−	−
6	*b*-NH_2	−	+
7	*c*-NH_2	−	−
8	*d*-NH_2	−	++
9	*e*-NH_2	−	−
10	*b*-Biotin	−	−
11	*c*-Biotin	−	−
12	*d*-Biotin	−	−
13	*e*-Biotin	−	−
14	*b*-Ihipp	+	++
15	*d-Ihipp*	+	+++
16	*e*-Ihipp	−	−
17	5′-COOH	+	+++
18	5′-NH_2	++	+++
19	*b*-Dimer	−	+
20	*b*-Dimer-Ibz[b]	−	++
21	*d*-Dimer	−	++
22	*d*-Dimer-Ibz	−	++
23	*e*-Dimer	−	−
24	*e*-Dimer-Ibz	−	−

[a] Effect on cell growth relative to CN-Cbl. The cell growth in presence of Cbl analogue is represented as fraction of maximal growth: −, no cell growth; and ++, 50% cell growth, relative to CN-Cbl.
[b] Ibz, iodobenzoate.

exhibited a biphasic dose response, with reduced growth-promoting activity at high concentrations.

Inhibition of Cell Growth by Cbl Analogues. Three groups of Cbl analogues, each with a specific side chain coupled to the *d*-, *b*-, or *e*-propionamide group, were tested for their ability to inhibit the growth-promoting effects of CN-Cbl. Serial dilutions of each of the Cbl analogues were tested at three concentrations of CN-Cbl in the presence or absence of apo-TCII. Data showing the inhibitory effectiveness of analogues modified at each of these three positions are shown for the Cbl-Ihipp analogues (**14–16**; Fig. 5), for the Cbl-biotin analogues (**10**, **12**, and **13**; Fig. 6), and for the Cbl-isophthaloyl-linked dimers (**19**, **21**, and **23**; Fig. 7). The Cbl analogues with either type of substitution at the *e*-position (**13**, **16**, and **23**) proved to be effective inhibitors of Cbl/TCII-dependent cell growth, inhibiting cell growth in the presence of 5 nM Cbl and apo-TCII, with an IC_{50} of 5–15 nM. Increased concentrations of the Cbl analogues were required to inhibit cell growth supported with higher concentrations of CN-Cbl. Interestingly, the *e*-analogues were only effective as inhibitors of Cbl-dependent cell growth in the presence of TCII. This requirement for TCII is shown in data with the *e*-Ihipp analogue **16**, demonstrating that, at all of the concentrations tested, this analogue had no inhibitory activity in the absence of apo-TCII (Fig. 5*D*). Cbl *b*-analogues (**10**, **14**, and **19**) exhibited inhibitory effects on cells maintained in low concentrations of CN-Cbl (0.5 nM) and TCII, but this was reversible with the addition of higher CN-Cbl concentrations. The *d*-analogues of Cbl were ineffective as inhibitors; only a single analogue (**21**) partially inhibited Cbl-dependent cell growth and only at low Cbl concentrations.

DISCUSSION

Here, we have evaluated the changes in Cbl bioactivity following conjugation of chemical groups to different sites on the Cbl molecule. Five sites on the Cbl molecule, the *b*-, *c*-, *d*-, and *e*- amide side groups of the corrin ring, and the ribose-5′-OH group were modified by different substitutions (Fig. 1). These Cbl analogues were designed in a manner such that each site of modification allowed for conjugation of moieties with different properties (21–23). These included monocarboxylic acid functionalities (**2**, **4**, **5**), a lactone (**3**), ribose succinate adducts (**17** and **18**), carboxylate adducts of diaminododecane (**6–9**), biotin conjugates (**10–13**), and *p*-Ihipp groups (**14–16**). Dimeric analogues of Cbl, linked through the *b*- (**19** and **20**), *d*- (**21** and **22**), and *e*- (**23**, **24**) carboxylic acids of the corrin ring, were also evaluated.

All Cbl analogues that supported cell growth were more potent in the presence of apo-TCII, indicating that they retain some ability to bind to TCII and are transported into cells via the TCII receptor. In earlier work (21–23), we have shown that modifications at either the Co metal, the 5′-OH of the ribose, or the *e*-propionamide side group of the corrin ring had little effect on binding to TCII, whereas Cbl analogues with substitutions at the *c*- and *d*-side chains of the corrin B-pyrroline ring bound only poorly with TCII, and *b*-analogues displayed intermediate TCII binding. The fact that the presence of apo-TCII nevertheless increased the cellular growth supported by the *b*- and *d*-analogues suggests that, although they do not bind TCII as well as *e*-analogues, they do bind sufficiently well and are internalized via the receptor-mediated process. We did not detect free Cbl in any of the Cbl analogue preparations, excluding the possibility that small amounts of Cbl contributed to this support of cell growth.

The fact that the *e*-analogues, which bind well to TCII, exhibited no growth-promoting activity, either in the absence or presence of TCII (Table 1, Fig. 3, and Fig. 4) may indicate that the structure at the *e*-propionamide group is crucial for the ability of Cbl to support cell growth. In contrast, substitutions at the *d*- and *b*-positions, although negatively affecting interaction with TCII, appear to interfere less severely with Cbl function. Furthermore, our data indicate that alterations of any type at the *d*-position have less effect on Cbl function than do alterations at the *b*-position of the corrin ring. Derivatives linked via the 5′-OH of the ribose (**17** and **18**) behaved comparably to CN-Cbl. However, this may reflect the fact that the ester linker is susceptible to hydrolysis, resulting in the release of Cbl into the medium or inside of the cell.

The inhibitory activity of Cbl analogues correlated positively with their ability to bind TCII but negatively with their ability to promote growth. Thus, *e*-analogues such as **13**, **16**, and **23** were potent inhibitors of Cbl-dependent cell growth, whereas *d*- and *b*-analogues were relatively ineffective inhibitors. The fact that *e*-analogues require TCII to show inhibitory effects indicates that they may act as competitive inhibitors of TCII-mediated Cbl uptake. These data are consistent with previous reports that *in vivo* application of Cbl analogues with modifications at the *e*-position resulted in the inhibition of methylmalonyl CoA mutase and methionine synthase activities, whereas *in vivo* treatment with *d*- and *b*-analogues did not affect the activity of these enzymes (10). Also concurring are data showing that *in vitro* treatment of human fibroblasts with an *e*-analogue of Cbl increased homocysteine levels 5-fold and methylmalonic acid levels up to 200-fold in the culture medium (12). These increases in metabolites occurred after 3–4 weeks in culture and in the presence of high (10 μg/ml) concentrations of the Cbl analogue. The *c*-lactam of Cbl has also been shown to antagonize Cbl *in vivo* (10) and, more recently, *in vitro* (13). However, the *in vitro* effects were only seen in the absence of methionine and at analogue concentrations approximately 1000-fold greater than we show for the *e*-analogues in the present study. Taken together, it appears that the inhibitory Cbl analogues may act, both *in vitro* and *in vivo*, by depleting intracellular Cbl. In the case of *e*-analogues, which retain high affinity for TCII but do not support cell proliferation, it is likely that they act as simple competitors with CN-Cbl for binding to TCII and thus reduce the intracellular level of functional Cbl. It has been shown previously that many analogues of Cbl (including *b*-, *c*-, *d*-, and *e*-analogues) bind to and activate human

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API

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Build custom dashboards for your attorneys and clients with live data direct from the court.

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Sync your system to PACER to automate legal marketing.