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ORIGINAL PAPER

Vitamin B_{12} as a carrier for targeted platinum delivery: in vitro cytotoxicity and mechanistic studies

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Abstract It is attractive to use vitamin B_{12} as a carrier for targeted delivery of cytotoxic agents such as platinum complexes owing to the high demand for vitamin B_{12} by fast proliferating cells. The basic $\{B_{12}–CN–Pt^{II}\}$ conjugates are recognized by intracellular enzymes and converted to coenzyme B_{12} in an enzymatic adenosylation assay. The reductive adenosylation of $\{B_{12}–CN–Pt^{II}\}$ conjugates leads to the release of the Pt^{II} complexes; thus, $\{B_{12}–CN–Pt^{II}\}$ conjugates can be considered as prodrugs. It is important not only to elucidate the activity of the cisplatin–B_{12} conjugates, but also to understand the mode of action on a molecular level. Chemical reduction of $\{B_{12}–CN–Pt^{II}\}$ conjugates with cobaltocene yielded cob(II)alamin and induced release of the corresponding Pt^{II} species. Kurnakov tests and coordination of 2′-deoxyguanosine or GMP to the released Pt^{II} complexes allowed isolation and characterization of Pt^{II} complexes as released during enzymatic adenosylation. The biological activity of these Pt^{II} complexes was evaluated. Since the cleaved Pt^{II} complexes show cytotoxicity, the $\{B_{12}–CN–Pt^{II}\}$ conjugates can be used for specific targeting of cancer cells and therapeutic drug delivery. Preliminary in vitro cytotoxicity studies indicated lower activity (IC_{50} between 8 and 88 μM) than found for pure cisplatin. Since active transport and receptor-mediated uptake limits the intracellular $\{B_{12}–CN–Pt^{II}\}$ concentration, comparison with pure cisplatin is of limited use. We could show that the Pt^{II} complexes cleaved from B_{12} exerted a cytotoxicity comparable to that of cisplatin itself. Cytotoxicity studies in vitamin B_{12} free media showed a dependence on the addition of transcobalamin II for B_{12}–Pt(II) conjugates.

Keywords Vitamin B_{12} · Cisplatin · Targeting · Medicinal inorganic chemistry · Cytotoxicity

Introduction

The treatment of diseases in current medicine widely relies on the administration of target-specific but cell-receptor-unspecific pharmaceuticals. Cell-specific targeted drug delivery has, however, become a major issue in modern molecular approaches to drug design and discovery [1]. Ideally, a pharmaceutical exclusively accumulates at the target site and is readily eliminated from the rest of the body. Much current research is, thus, currently performed in the field of targeted drug delivery, based on nanoparticles, organic copolymers, but also with small receptor binding molecules [2–6]. The targeted drug delivery concept relies on natural or artificial molecules which carry their bioactive load to a specific site. At this site, the pharmaceutical is released and exerts its activity. The feature of targeting is particularly crucial in molecular imaging since the quality of the images depends strongly on the target to nontarget ratio; thus, the more specific the targeting, the better the interpretation of the images [7, 8]. There are numerous modalities

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for targeting, encompassing in, e.g., nanomedicine, artificial macromolecules such as dendrimers, carbon nanotubes, or polyoxometallates derivatized with cell-specific receptor binding functions such as antibodies, peptides, or small molecules (and the bioactive substance). Metabolic tracers are another class of targeting molecules in molecular imaging but not in therapy; they accumulate owing to rapid proliferation of cells. A prototype for such compounds is ^{18}F-deoxyglucose [9], which is trapped in cells by irreversible inhibition of hexokinase. With the same concept, bioactive drugs guided by targeting (small) molecules could be delivered to specific sites provided that these bioconjugates still follow the same metabolic pathway. Furthermore, intracellular biochemical processes have to be involved in the release of the drug after delivery to the cell but not elsewhere. Whereas targeted cancer therapy with antibodies is well developed, small carrier molecules are still rare. One of the major problems related to small-molecule conjugation of bioactive substances is the affection of the biological properties of the carrier after conjugation to the imaging agent or pharmaceutical.

Cobalamin (Cbl, vitamin B_{12}) is essential for mammals. Different cancer cell lines express a strongly increased demand for Cbl. Cbl has been radiolabeled at various sites on the corrin framework, generally on the b, d, or e propionamide chain of Cbl after hydrolysis to the corresponding acids (Scheme 1) [10–17]. Those radiolabeled Cbl showed high tumor accumulation, confirming their adequacy as metabolic tracers and suitability for imaging [18]. We showed recently that ^{99m}Tc-labeled Cbl derivatives not binding to the transport protein transcobalamin II (TCII) showed high site-specific accumulation and very low kidney retention [19].

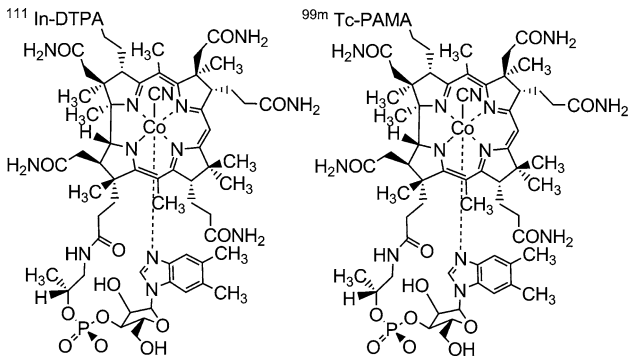
Whereas derivatizations with metal binding functionalities or bioactive molecules were generally performed at the propionamide groups of the coring ring, at the 2′-OH group in the ribose [20–22], or by alkylation of Co^{I} [23–26], we have demonstrated that metal complexes can be bound directly to the cyanide group in Cbl, thereby yielding a heterodinuclear complex $\{B_{12}–CN–M\}$ in which Cbl acts as a ligand [27]. In particular, cyanide in Cbl coordinated to various square-planar Pt^{II} complexes such as cisplatin [28]. With Pt^{II} complexes in particular, the central $\{B_{12}–CN–Pt^{II}\}$ motif was formed. An enzymatic assay showed that these B_{12}–Pt conjugates were still recognized by intracellular adenosylation enzymes and were converted to adenosylcobalamin (AdoCbl) [29]. During adenosylation, the Pt^{II} complex was released by reduction of Co^{III} to Co^{II} in Cbl. Hence, conjugates of the general structure $\{B_{12}–CN–Pt^{II}\}$ can be considered as prodrugs, suitable for Cbl-targeted Pt^{II}-complex delivery to rapidly proliferating cells in which the cytotoxic agent is released. In this paper, we present the findings of in vitro cytotoxicity studies of the $\{B_{12}–CN–Pt^{II}\}$ conjugates *cis*-$[PtCl(NH_3)_2B_{12}]$ (**1**), *trans*-$[PtCl(NH_3)_2B_{12}]$ (**3**), and *cis*-$[PtCl_2(NH_3)B_{12}]$ (**2**) (Scheme 2) as a proof of concept for vitamin B_{12} based metal-drug delivery.

Numerous Pt^{II} complexes have been screened for cytotoxicity during the search for cisplatin analogues with cytotoxic activity [30]. However, the Pt^{II} complexes as released from **1**–**3** (Scheme 2) are cisplatin-like but not cisplatin. Besides NH_3 and Cl, they contain one coordinated cyanide. To our knowledge, the cytotoxicity of these basic Pt^{II} complexes is not known since they are synthetically difficult to access and have not been described in literature. Accordingly, we present the bioactivity of these complexes to assess their cytotoxicity after release from the carrier B_{12} together with studies of the reactivity of the released complexes toward nucleotides as models for the DNA binding sites of cisplatin (analogues).

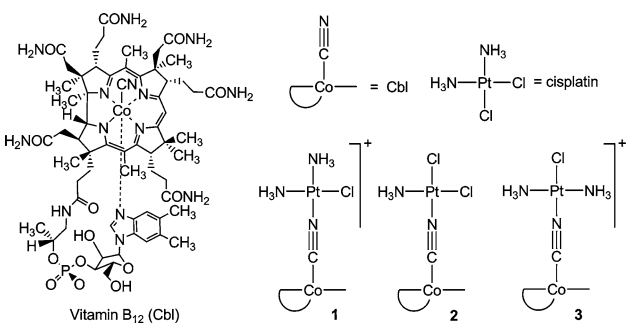
Materials and methods

General

All chemicals were purchased from Sigma, Fluka, and Strem. Chemicals were of reagent grade and used without further purification. The vitamin B_{12} complexes **1**–**3** were



Scheme 1 Basic structure of vitamin B_{12} derivatized at the b-position with metal complexes [10–17]



Scheme 2 Structures of vitamin B_{12}–Pt^{II}–complex conjugates $\{B_{12}–CN–Pt^{II}\}$ **1**–**3** used in this study

synthesized according to published procedures [29]. Ultraperformance liquid chromatography (UPLC) analyses were performed using a Waters Acquity^{TM} connected to an HCT ion trap mass spectrometer (Bruker Daltonics). The following liquid chromatography (LC) column, solvent systems, and gradient were used: column, Macherey–Nagel C-18ec Nucleosil RP (5-μm particle size, 100-Å pore size, 250 mm × 3 mm); solvent system 1, 0.1% trifluoroacetic acid (solvent A), methanol (solvent B); solvent system 2, 0.1% formic acid (solvent A), methanol (solvent B); solvent system 3, 10 mM Et3Nac, pH 4.6 (solvent A), methanol (solvent B); gradient 1, 0–5 min (75% solvent A), 5–30 min (75% solvent A → 0% solventA); gradient 2, 0–5 min (100% solvent A), 5–30 min (100% solvent A → 0% solvent A).

Chemical reduction of complexes **1–3**

Reduction of cob(III)alamin to cob(II)alamin of derivatives **1–3** was performed in methanol, under a N_2 atmosphere, using 2 equiv of cobaltocene. Formation of cob(II)alamin was monitored by UV–vis spectroscopy. The formation of platinum complexes in the reaction mixture after release from derivatives **1–3** was monitored by electrospray ionization (ESI) mass spectrometry (MS) and high performance LC (HPLC) measurements (solvent system 1, gradient 1, flow rate 0.5 mL min^{-1}) and the formation of HOCbl.

Chemical characterization of released Pt complexes **4–6**

Isolation and characterization of *cis*-[Pt(CN)Cl(NH_3)$_2$] (**4**), *cis*-[Pt(CN)Cl$_2$(NH_3)]$^-$ (**5**), and *trans*-[Pt(CN)Cl(NH_3)$_2$] (**6**) was not directly possible. Alternative tests were performed to elucidate their chemical structures:

- Kurnakov test. Thirty minutes after chemical reduction of **1–3** as described earlier, the solvent was evaporated under vacuum. Ten equivalents of thiourea dissolved in water was added to the mixture. After 5 min, the solution was filtered and analyzed by UPLC–MS (solvent system 2, gradient 2, flow rate 0.3 mL min^{-1}). Platinum released from conjugates **1** and **2** gave the same product with a retention time of 11.5 min and [M]$^+$ of 499.0, indicating the formation of [Pt(CN)(thiourea)$_3$]$^+$ (**7**). Platinum released from complex **3** gave a product with a retention time 4.2 min and [M]$^+$ of 331.0, indicating the formation of [Pt(CN)(NH_3)$_2$(thiourea)]$^+$ (**8**).
- 2′-Deoxyguanosine interaction. **1** (6.5 μmol) was reduced with cobaltocene (2 equiv) in methanol. After the mixture had been stirred for 30 min, the solvent was evaporated and the crude mixture was dissolved in 1.5 mL phosphate-buffered saline (PBS.) The solution was filtered and added to 8 equiv of 2′-deoxyguanosine. Formation of complexes *cis*-[Pt(CN)(NH_3)$_2$(2′-deoxyguanosine)]$^+$ (**9**) and *cis*-[Pt(CN)(NH_3)(2′-deoxyguanosine)$_2$]$^{2+}$ (**10**) was followed by UPLC–MS (solvent system 2, gradient 2, flow rate 0.3 mL min^{-1}), with a retention time of 12.5 min and [M]$^+$ of 772.2. As a control experiment, 6.5 μmol cisplatin was dissolved in 1.5 mL PBS containing 8 equiv of 2′-deoxyguanosine. Formation of cis-[PtCl(NH_3)$_2$(2′-deoxyguanosine)]$^+$ and *cis*-[Pt(NH_3)$_2$(2′-deoxyguanosine)$_2$]$^{2+}$ was followed by UPLC–MS (solvent system 2, gradient 2, flow 0.3 mL min^{-1}), with a retention time of 13.5 min and [M]$^+$ of 762.2.

Kinetic interaction of GMP with cisplatin, **4**, and **5**

Complexes **1** and **2** (both 6.5 μmol) were reduced with 2 equiv of cobaltocene in methanol. After 30 min, the solvent was evaporated and the crude mixture was dissolved in 1.5 mL PBS. The solution was filtered and added to 50 equiv of GMP. The reaction was followed by UPLC–MS every 4 h (solvent system 3, gradient 2, flow rate 0.3 mL min^{-1}). As a control experiment, the reaction of 6.5 μmol cisplatin with 50 equiv of GMP in 1.5 mL PBS was followed by UPLC–MS every 4 h under the same conditions.

Cell lines

The human breast carcinoma MCF7 cell line was maintained in minimum Eagle's essential medium (MEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}). The human ovarian cancer A2780 cell line was maintained in RPMI (Gibco) supplemented with 10% FBS (Gibco), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}).

Cbl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

One thousand cells per well were seeded in 96-well plates 1 day before treatment. Cells were treated with cisplatin, B_{12}, cobaltocenium, **1**, **2**, **3**, **4**, **5**, or **6** for 5 days, the drug was removed, and cells were kept in fresh medium. Upon addition of 20 μL per well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg mL^{-1}), the plates were incubated for 4–5 h at 37 °C. One hundred microliters per well of lysis buffer (20% sodium dodecyl sulfate, 50% dimethylformamide, 50% double-distilled H_2O, pH < 4.7) was added and the plates were incubated overnight at 37 °C. The solubilized

formazan was quantified at 570 nm using a SpectraMax M5 microplate reader. Optical density values were plotted against the logarithm of drug (or derivative) concentration and IC_{50} values were calculated from the regression curve.

Cbl/TCII MTT assay

To ensure a Cbl-dependent cytotoxic effect, RPMI deficient in Cbl and folic acid (medium A) or MEM (Gibco) (medium B), which does not contain Cbl in the original formulation, was used. FBS was pretreated with fumed silica to reduce interference of endogenous TCII/Cbl in the assay [31]. One thousand cells per well were seeded in 96-well plates 1 day before treatment using standard medium. After removal of the growth medium, the cells were treated with cisplatin or with **1**, **2**, or **3**, or with **1**, **2**, or **3** in the presence of 1 equiv of TCII for 1 day in medium A or medium B containing 10% treated FBS. The drug was removed and the cells were kept in fresh medium for an additional 4 days. Upon addition of 20 μL per well of MTT solution (5 mg mL^{-1}), the plates were incubated for 4–5 h at 37 °C. One hundred microliters per well of lysis buffer (20% sodium dodecyl sulfate, 50% dimethylformamide, 50% double-distilled H_2O, pH < 4.7) was added and the plates were incubated overnight at 37 °C. The solubilized formazan was quantified at 570 nm using a SpectraMax M5 microplate reader.

Immunofluorescence

Indirect immunofluorescence experiments were performed with MCF7 cells grown on glass coverslips. Upon fixation in methanol (15 min, −20°C), the cells were washed with PBS. Blocking was carried out in PBS supplemented with 3% nonfat dry milk (blocking solution). The coverslips were incubated overnight at 4 °C in a humidified chamber with γ-H2AX ($pSer_{139}$) antibody (mouse monoclonal, Upstate Biotechnology) diluted 1:200 in blocking solution. After they had been washed with blocking solution, the cells were incubated with a Texas Red conjugated anti-mouse antibody at 1:200 dilution in blocking solution for 1 h at 37 °C. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (0.1 μg mL^{-1}, Sigma). Images were acquired using an Olympus IX81 fluorescence microscope.

Results and discussion

Reduction of Co(III) to Co(II) and isolation of the released Pt(II) complexes

The enzymatic conversion of $\{B_{12}–CN–Pt^{II}\}$ derivatives to AdoCbl by CobA enzyme has been reported previously [29]. The corrinoid adenosylation pathway proposed required the electron-transfer protein flavodoxin FldA and the ferrodoxin Fpr as reductases for the $Co^{III} \rightarrow Co^{II}$ step. Only in the presence of the corresponding enzyme and ATP did further reduction of Co^{II} to Co^{I} take place with concomitant formation of AdoCbl [32, 33]. For the purpose of targeted metal complex delivery in our study, the first reduction step is decisive. The Pt^{II} complexes from Cbl derivatives **1**–**3** (Scheme 2) are released into the intracellular space after the Cbl has entered the cells by receptor-mediated endocytosis via the TCII receptor, but not before. The release of the platinum complexes from **1**–**3** occurs after enzymatic reduction of Co^{III} to Co^{II}. The remaining cob(II)alamin is then reduced to Co^{I} and adenosylated to coenzyme AdoCbl by adenosyltransferase [29]. After reduction to Co^{II}, the composition of the released Pt^{II} complexes is an important question and, in particular, if the cyanide ligand is still present on the released Pt^{II} complex or is lost during reduction. It should be emphasized that the cyanide is N-bound to Pt^{II} in $\{B_{12}–CN–Pt^{II}\}$ and it would be uncommon to find an {Pt–NC} motif in the released complex. Compounds **1**–**3** were electrochemically reduced to release the β-axial complexes **4**–**6**. After reduction, HPLC evidenced the presence of exclusively aquo-Cbl, assessing the cleavage of the complexes together with the cyanide ligand. Although this is a very smooth and straightforward method, the presence of large amounts of supporting electrolyte as compared with the tiny amounts of Pt^{II} complex made their characterization or isolation impossible. Chemical reduction was more appropriate since inert salts are absent and the process is faster. An appropriate reducing agent is cobaltocene. Cobaltocene is a noncoordinating molecule with a redox potential of about −750 mV (vs. Ag^{+}/AgCl) in methanol. This agent is ideal for reduction of cob(III)alamin to cob(II)alamin and does not further reduce Co^{II} or the released platinum complexes.

Quantitative chemical reduction of **1**–**3** was smoothly achieved with 2 equiv of cobaltocene in methanol and under N_2. The reaction was complete after 30 min at room temperature. A typical UV–vis spectrum of the cob(II)alamin produced together with an HPLC trace for HOCbl formation is shown in Fig. 1.

The isolation of complexes **4** from **1**, **5** from **2**, and **6** from **3** (Scheme 3), released after cobaltocene reduction, was not directly possible owing to the respective low concentrations and insolubilities. Owing to their electroneutrality, compounds **4** and **6** as well as pure cisplatin were not observed in ESI-MS. However, it has been shown in the literature that characterization of Pt^{II} complexes containing a ^{15}N-enriched $^{15}NH_3$ ligand is possible by 2D [^{1}H,^{15}N] heteronuclear single quantum coherence (HSQC) NMR spectroscopy [34]. The chemical shift δ_N is indicative for the type of ligand *trans* to $^{15}NH_3$ and it has been shown that stronger *trans* ligands shift the ^{15}N signal

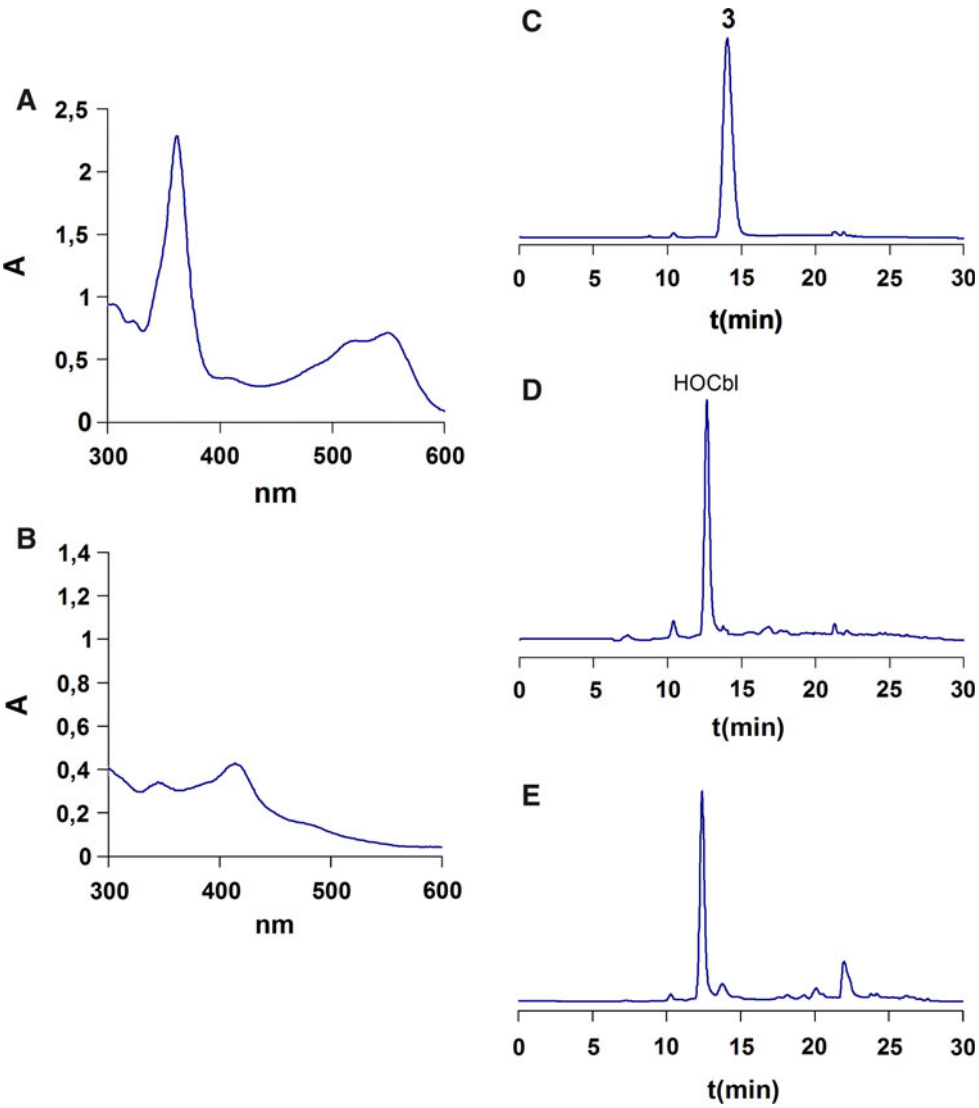
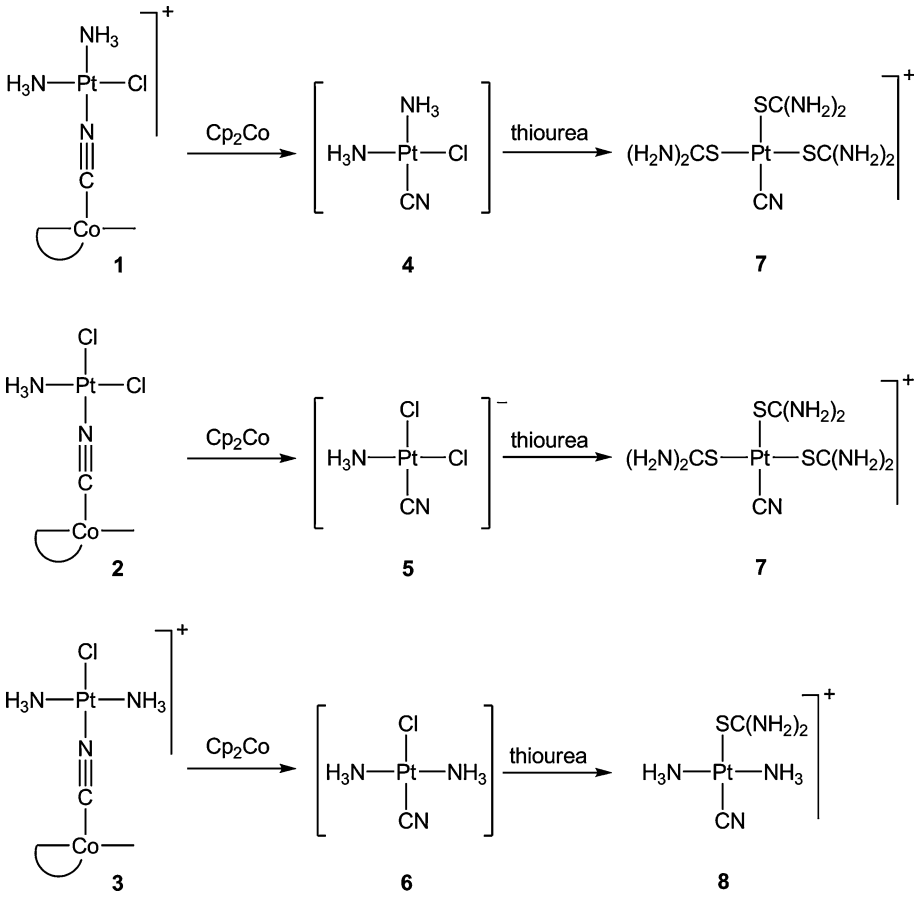


Fig. 1 UV–vis spectra associated with the reduction of **3** from cob(III)alamin (**a**) to cob(II)alamin (**b**) by cobaltocene. High performance liquid chromatography traces monitored at 360 nm of a solution of **3** (**b**), hydroxocobalamin (*HOCbl*) (**c**), and the cobalamin product obtained after reduction of **3** with cobaltocene and reoxidation in air (**d**)

toward lower field. Thus, $^{15}NH_3$ *trans* to H_2O can clearly be differentiated from $^{15}NH_3$ *trans* to Cl^- [35–37]. Taking the $^{15}NH_3$ signal from cisplatin as a reference at $\delta_N = -68.0$ ppm, [^{1}H,^{15}N] HSQC NMR spectroscopy of the B_{12} conjugate **1** showed two nearby ^{15}N signals, one at −68.5 ppm, corresponding to $^{15}NH_3$ *trans* to Cl^-, and one at −72.9 ppm, assigned to the $^{15}NH_3$ group *trans* to the Pt–NC–Co moiety. When **4** was chemically cleaved from **1**, the NMR spectrum again showed two, now well-separated signals. The $^{15}NH_3$ signal *trans* to Cl^- remained almost constant (−73.9 pm), whereas the second signal (−40.7 ppm) shifted significantly to lower field. Since cyanide is a very strong *trans* ligand, the latter signal indicated the presence of a cyanide *trans* to $^{15}NH_3$, thereby further rationalizing the authenticity of **4** (see the electronic supplementary material).

Dimethyl sulfoxide (dmso) is known to substitute Cl^- in Pt^{II} complexes [38]. Addition of dmso to the reaction solution of **3** after cobaltocene reduction allowed for the clear mass-spectrometric detection of *trans*-$[Pt(CN)(dmso)(NH_3)_2]^+$. Although the signals were weak, the mass spectra indicated that cyanide is still coordinated to the released Pt^{II} complexes after reduction of **1**–**3** (see the electronic supplementary material). An alternative method for the characterization of the released platinum complexes relied on the Kurnakov test, a method which allows one to distinguish *cis* and *trans* isomers of dihalide–diamine Pt^{II} complexes [39]. The test is based on the relative *trans* effect of thiourea > Cl^- > NH_3 and involves, after reaction with thiourea, the formation of $[Pt(thiourea)_4]^{2+}$ from *cis*-$[PtCl_2(NH_3)_2]$ and *trans*-$[Pt(NH_3)_2(thiourea)_2]^{2+}$ from *trans*-$[PtCl_2(NH_3)_2]$. Since cyanide is known to be strongly coordinated in platinum complexes, any substitution reactions with thiourea in derivatives **4**–**6** should not affect the cyanide ligand. Furthermore, according to the *trans*-effect series CN^- > thiourea > Cl^- > NH_3, any ligand *trans* to the cyanide should be replaced by thiourea following the principles of the Kurnakov test.



Scheme 3 Reaction of complexes **4–6** with thiourea. *Cp2Co* cobaltocene

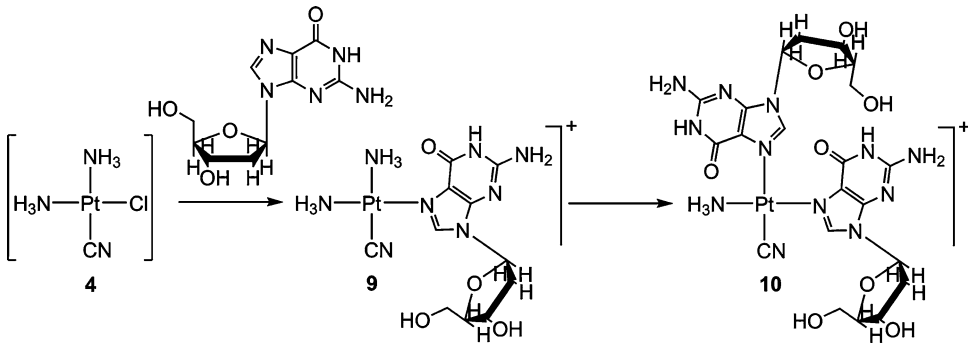
After reduction of **1–3** with cobaltocene, mixtures of HOCbl and the respective Pt^{II} complexes **4–6** were formed. The solvent was evaporated and the red residues were dissolved in water containing a tenfold excess of thiourea. After 30 min, the sample was filtered and analyzed by LC–MS. In all three cases, HOCbl was observed. The substitution reactions were fast and product formation was complete after 1 h at a platinum-to-thiourea ratio of 1:10. Since cyanide has a very strong *trans* effect, the reaction of **4** and **5** with thiourea should give the triply substituted complex $[Pt(CN)\{SC(NH_2)_2\}_3]^+$ (**7**) and the reaction of thiourea with **6**, a transplatin-type complex, should give the monosubstituted complex *trans*-$[Pt(CN)(NH_3)_2\{SC(NH_2)\}]^+$ (**8**) (Scheme 3). This predicted behavior was confirmed and complexes **7** and **8** could clearly be identified as the major products by HPLC and ESI-MS (see the electronic supplementary material).

Furthermore, complex **7** could be isolated by preparative HPLC as a yellow powder. The ^{195}Pt NMR spectrum of **7** gave a sharp signal at −4,130 ppm, a region in which resonances of Pt^{II} complexes bound to three sulfur atoms and one carbon atom are usually observed [40, 41]. The infrared spectrum of **7** displayed a weak but characteristic ν_{CN} band at 2,187 cm^{-1}, indicating that the platinum complex was bound to the cyanide ligand via the carbon atom [42]. Inspecting the basic structures of **1–3**, we expect that the platinum complex released after reduction will bind the cyanide group via the nitrogen atom. Since all our released complexes **4–6** showed the cyanide to be C-bound, we conclude that isomerization of the cyanide from N- to C-coordination took place very rapidly.

The platinum complexes **4–6** as released after chemical or enzymatic reduction of cob(III)alamin in the intracellular space are not cisplatin but cisplatin analogues. The reactivity of these complexes toward their targets, guanine or other nucleobases in DNA, is unknown. To assess the affinity for nucleobases, we studied the interaction of **4–6** with 2′-deoxyguanosine (Scheme 4).

According to the *trans*-effect series, in complex **4** one chloride and eventually one NH_3 should be replaced by a base. To mimic physiological conditions, the reaction of, e.g., **4** with 2′-deoxyguanosine was performed in 10 mM phosphate buffer (pH 7.4) with 0.9% NaCl as an electrolyte; eightfold excess of 2′-deoxyguanosine was applied. After chemical reduction of **1**, the formation of products was monitored by LC–MS as a function of time. Single substitution should result in complex **9**, and double substitution in complex **10** (Scheme 4). Figure 2 shows

Scheme 4 Interaction of complex **4** with 2′-deoxyguanosine



representative UPLC chromatograms obtained from the extrapolation of mass spectra of the substitution reactions at the time points indicated. After 6 h at room temperature, it is immediately obvious that both complex **9** and complex **10** were formed. Over time, the amount of **9** slowly decreased, whereas the amount of the final product **10** increased. Thus, double substitution on Pt^{II} took place, confirming the cisplatin-like behavior of the complexes cleaved from their Cbl carriers.

To compare the reactivity of **4** and **5** with that of cisplatin, we pursued the same strategy with the dianion 5′-GMP (Scheme 5). The Cbl conjugates **1** and **2** were reduced with cobaltocene, dried, and redissolved in 10 mM phosphate buffer and 0.9% NaCl. For a qualitative comparison of the reaction rates, a 50-fold excess of GMP was applied to ensure conditions for pseudo-first-order kinetics. Product formation was monitored by LC–MS as a function of time. The mobile phase used for these experiments was crucial. With 0.1% formic acid in methanol as the mobile phase, protonation of coordinated 5′-GMP resulted in loss of signals of the monosubstituted complexes $[PtCl(5'\text{-GMP})(NH_3)_2]^-$, **11**, and **12**. In 10 mM $[HNEt_3]^+[CH_3CO_2]^-$ (pH 7) and methanol, the signals for $[Pt(5'\text{-GMP})_2(NH_3)_2]^{2-}$ and **13**, respectively, could not be observed anymore (Scheme 4). All platinum intermediates and products could, however, be observed in UPLC–MS when using $[HNEt_3]^+[CH_3CO_2]^-$ (pH 4.6) and methanol as a mobile phase.

Qualitative kinetic measurements were performed for **4** and **5** after release from **1** and **2** and were compared with those for cisplatin. Formation of 5′-GMP-substituted derivatives was plotted versus time by extrapolation of the area obtained by UPLC–MS measurements for the individual species.

Other studies typically used the monoaqua and diaqua analogues of cisplatin for kinetic analysis of the reaction of nucleotides and Pt^{II} complexes [43–45]. In our study, we did not examine the aquation process since it was not observed by ESI-MS. Bancroft et al. [44] observed no evidence of either *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ or *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ during the reaction of cisplatin with DNA, indicating that the hydrolyzed species reacted with DNA as rapidly as they were formed. Furthermore, they found that the rate constant of hydrolysis was equal to the rate of DNA–Pt formation.

Under our conditions, cisplatin interacted with 5′-GMP to yield *cis*-$[PtCl(GMP)(NH_3)_2]^-$ and *cis*-$[Pt(GMP)_2(NH_3)_2]^{2-}$, respectively. After about 5 h, the monosubstituted complex reached its maximum concentration (Fig. 3). The amount of disubstituted complex steadily grew, with complete formation after about 20 h. The behavior of **4** and **5** was qualitatively comparable. Both complexes were substituted by one 5′-GMP very rapidly. After less than 1 h, the maximum concentration of monosubstituted compounds **11** and **12** was achieved (see the electronic supplementary material). The kinetics of the second substitution were similar for **11**, **12**, and *cis*-$[PtCl(GMP)(NH_3)_2]^-$. Formation of the corresponding doubly substituted complex **13** was complete after about 20 h. These kinetics measurements followed qualitatively a consecutive A → B → C reaction sequence. The very rapid formation of the monosubstituted adducts **11** and **12** is a very important feature since it enables retention of the cleaved platinum complexes in the cells. Since the kinetics is comparable to that for cisplatin, one could, at least from a chemical point of view, expect similar biological behavior for **4**–**6**.

Cytotoxicity studies

Having established the binding of the new platinum complexes **4**–**6** with guanine derivatives, we considered it important to investigate the cytotoxicity of these complexes. Complexes **4**–**6** are the active drugs carried into the cells by Cbl and released after reduction. Reactivity toward 5′-GMP is an indication of but not a guarantee for cytotoxicity since other coordination sites may compete for Pt^{II} binding in vivo. We therefore investigated the in vitro cytotoxicity of compounds **4** and **5** with the cancer cell lines A2780 and MCF7. IC_{50} values are given in Table 1. Owing to the essentially nontoxic behavior of **3**, the cleavage product **6** was not further investigated. Compound **6** can form only DNA monoadducts, and complexes

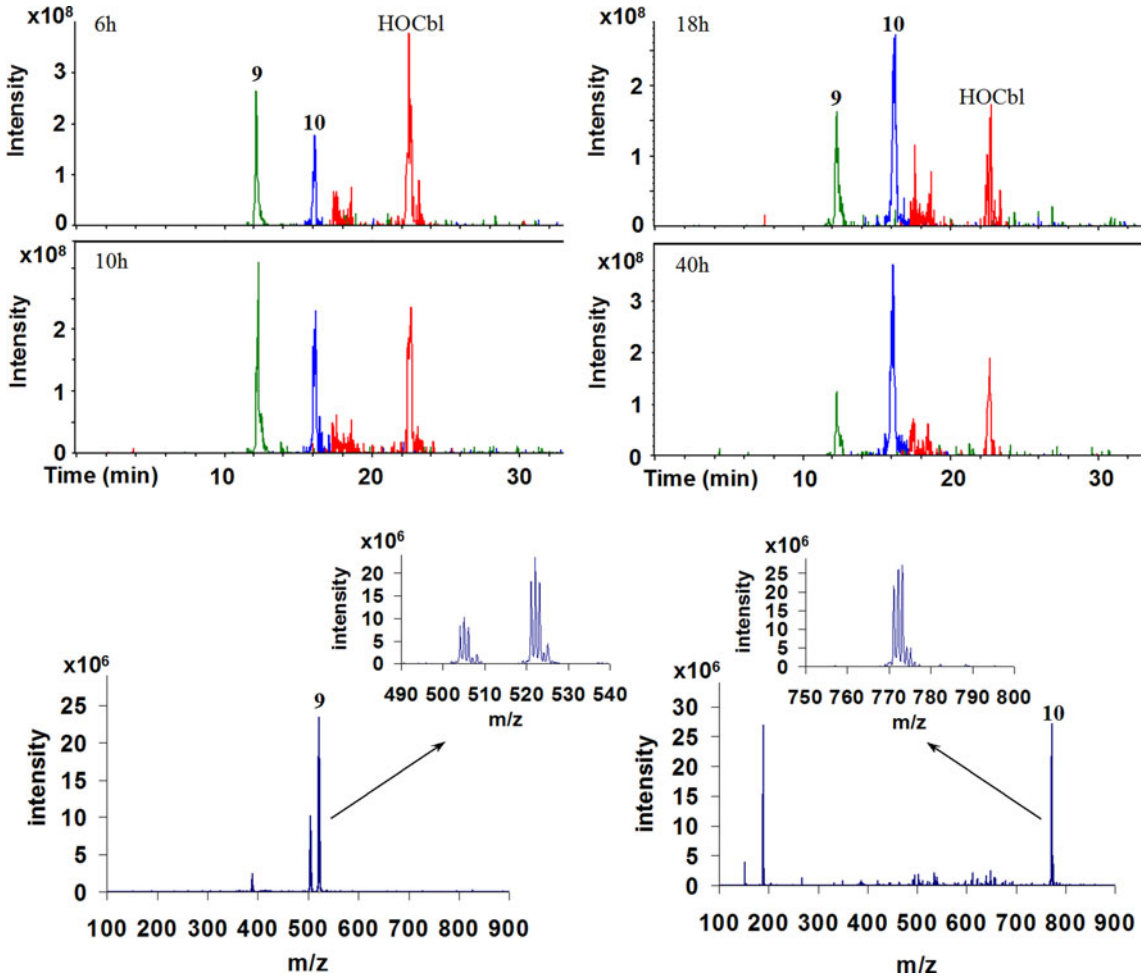


Fig. 2 Ultraperformance liquid chromatography chromatogram and corresponding mass spectrometry spectra of the substitution reaction of **4** with 2′-deoxyguanosine

exerting a cisplatin-like behavior with DNA such as **4** and **5** are immediately more promising. However, it is known that some exceptional, mono-DNA-adduct-forming Pt^{II} complexes are highly cytotoxic [46].

Although the IC_{50} values of complexes **4** and **5** are higher than those of cisplatin, they are comparable to those of other cytotoxic or static agents [47]. One should keep in mind that the IC_{50} values are not the only quality criteria for successful therapeutic agents and are even sometimes misleading. To evaluate the targeted drug delivery of the $\{B_{12}–CN–Pt^{II}\}$ conjugates, we studied the in vitro cytotoxic behavior of **1**–**3** in the human breast carcinoma cell line MCF7 and in the human ovarian cancer cell line A2780. We showed in previous studies that the Pt^{II} complexes remain stably bound to Cbl in human serum; hence, any cytotoxicity found must be related to intracellular release of the complexes rather than to decomposition in the media [29]. It should be emphasized that Cbl is bound in vivo to the transport protein TCII [48]. Cellular uptake and internalization of Cbl occurs upon binding of holo-TCII to the membrane TCII receptor (rTCII). After degradation, Cbls are released and enter the enzymatic cycles. Reports in the literature describe the uptake of Cbl derivatives that are TCII nonbinders along so far not fully elucidated pathways [19]. The targeted approach is, however, based on holo-TCII, able to bind rTCII. We initially compared **1**–**3** with cisplatin despite the fact that the former complexes are prodrug-like compounds, probably unable to freely diffuse into the cell, in contrast to cisplatin. Therefore, in the case of **1**–**3**, the concentration of active platinum complexes in the intracellular space is governed by the rate of internalization and might be significantly lower than what is achieved with cisplatin. As outlined later, the amount of Cbl–Pt conjugates bound to TCII available in the medium is decisive for cytotoxicity, since conjugates not bound to TCII are unlikely to enter the cells.

Cells were plated in 96-well plates at 5,000 cells per well in 100 μL RPMI 1640 or MEM containing 10% of FBS. After 24 h, the cells were treated with cisplatin, **1**, **2**, and **3** at different concentrations. To assess the potency of

Scheme 5 Interaction of cisplatin, **4**, and **5** with GMP

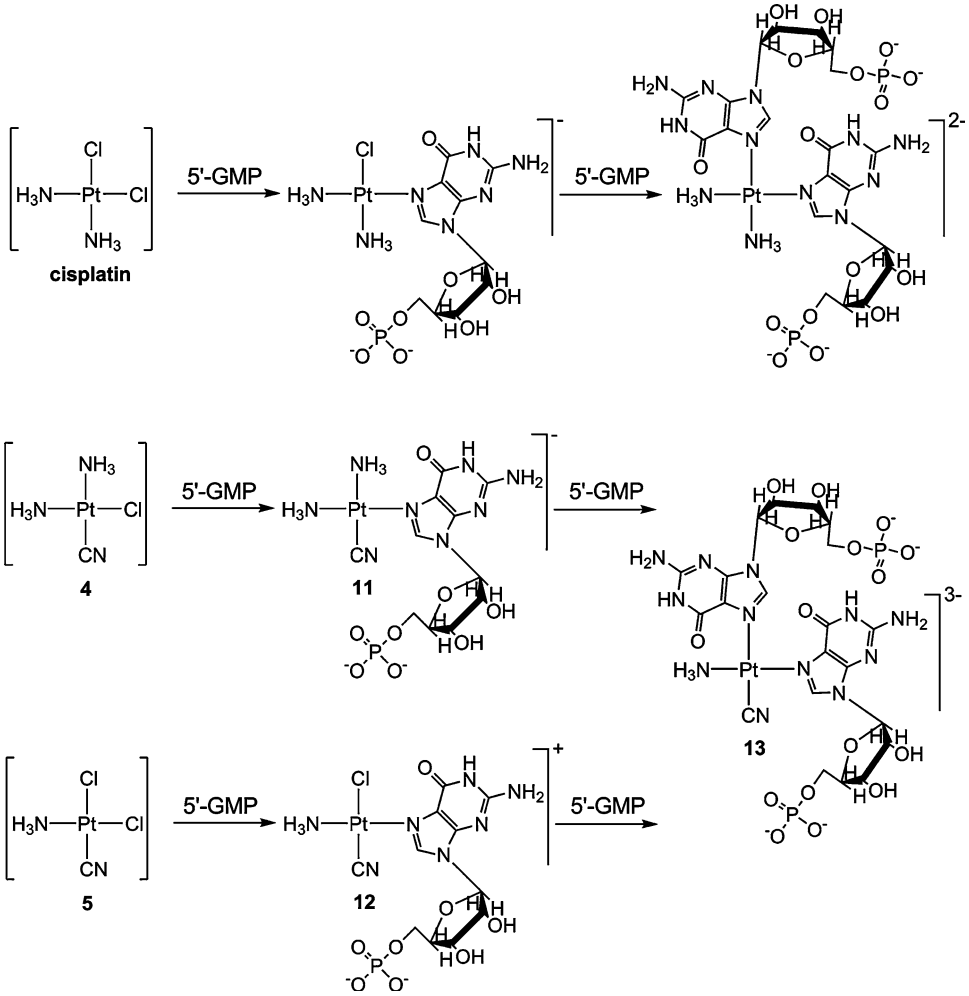


Fig. 3 Time-dependent formation of mono-GMP- and bis-GMP-substituted complexes, starting from cisplatin, **4**, and **5**

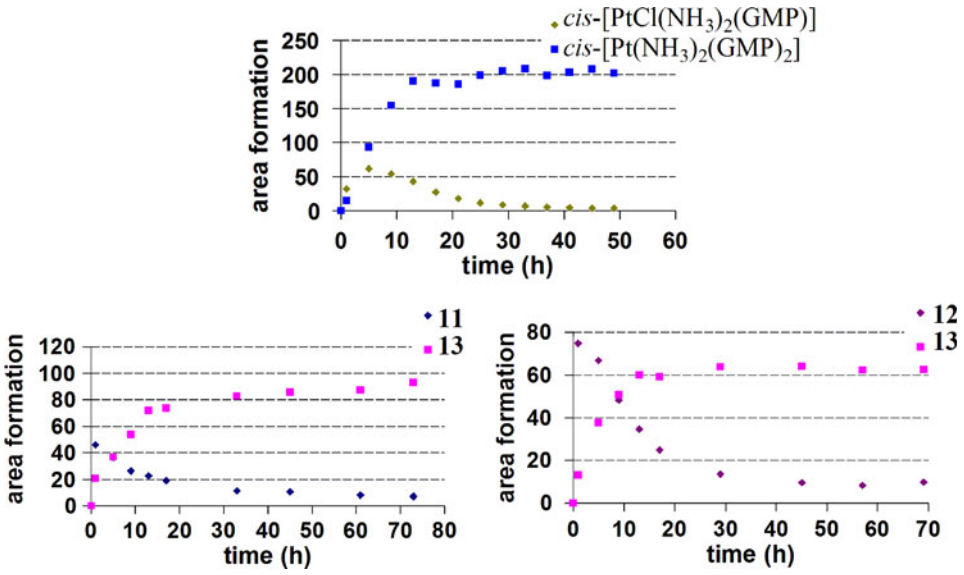


Table 1 Comparison of the IC_{50} values of cisplatin and the $\{B_{12}$–CN–$Pt^{II}\}$ conjugates **1**–**3** and the cleaved complexes **4** and **5**

	IC_{50} (μM)	
	A2780	MCF7
Cisplatin	0.3	1.2
1	8	13
2	48	>100
3	54	88
4	6	5.5
5	4	7

1–**3**, cell viability after 5 days of treatment with the compounds was evaluated by MTT assays.

We found that the Cbl conjugates **1**–**3** were less active than cisplatin against MCF7 and A2780 cells. Figure 4 shows a concentration–activity comparison. The IC_{50} values are given in Table 1. Conjugate **1** is the most active $\{B_{12}$–CN–$Pt^{II}\}$ compound, whereas **2** and **3** are essentially inactive. Since the Pt^{II} complex **6** as cleaved from **3** can form only monoadducts with DNA, its nonactivity is reasonable. However, since **4** and **5** as released from **1** and **2,** respectively, show the same behavior toward thiourea and 5′-GMP (vide infra), the nonactivity of **2** is not immediately obvious. We speculate that there is a diffusion-controlled uptake mechanism (e.g., via cation channels), which would favor the uptake of cationic **1** over neutral **2**, resulting in an enhanced cytotoxic effect as observed for **1**. For the time being, questions about quantification and the pathway of uptake remain to be elucidated.

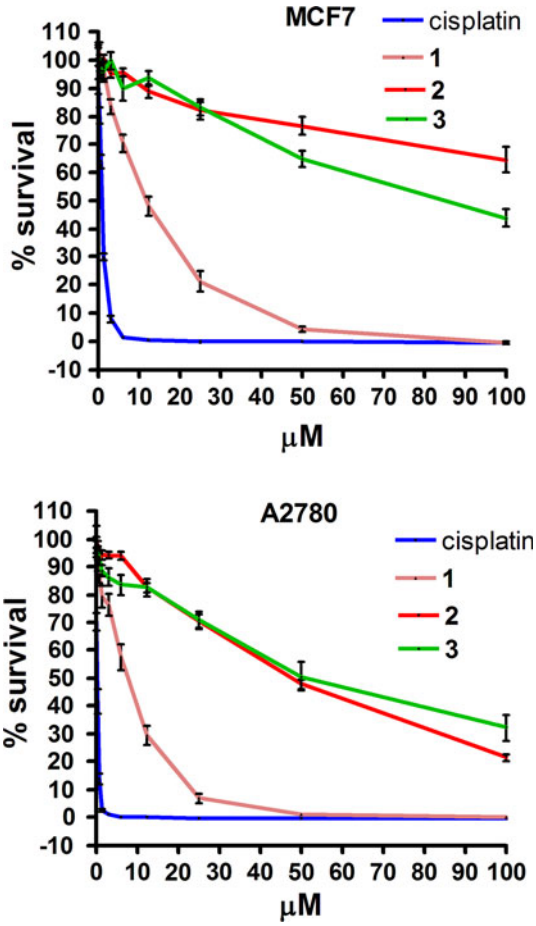


Fig. 4 Cytotoxicity of cisplatin, **1**, **2**, and **3** for MCF7 and A2780 after 5 days of treatment

Role of TCII

As mentioned before, the plasma transport of Cbl or Cbl derivatives to all tissues or cells is mediated by TCII. Holo-TCII is taken up by receptor-mediated endocytosis via rTCII [49]. Expression of rTCII is important for the cellular uptake of Cbl, although information on the level of rTCII expression in A2780 and MCF7 cell lines is not available to our knowledge. In addition, the concentration of TCII in the cell culture medium determines the level of Cbl–TCII complexes that will undergo internalization [31]. The results of the cytotoxicity assays described earlier might have been influenced by these factors and passive internalization of **1**–**3** also have to be taken into account. To assess this issue, we set out to determine the effect of TCII on cytotoxicity upon addition of apo-TCII to the media, using very low concentrations of **1**–**3**. To measure TCII/Cbl-dependent cytotoxicity, RPMI 1640 deficient in Cbl and folic acid (medium A) was used for A2780 and commercial MEM (medium B) was used for for MCF7, since the formulation of MEM is deficient in Cbl. The medium was supplemented with 10% FBS pretreated with fumed silica to reduce interference of bovine TCII–Cbl in the bioassay.

Cells were plated in 96-well plates at 1,000 cells per well in 100 μL of medium A or medium B containing 10% pretreated FBS. After 24 h, the cells were treated with cisplatin, compounds **1**, **2,** apo-TCII + **1**, or apo-TCII + **2** at 3.125, 1.56, and 0.78 μM. After 24 h the compounds were removed and the cells were kept in standard medium for 4 days. Cell viability was assessed by MTT assays. Figure 5 shows that compounds **1** and **2** were not active at all in the absence of apo-TCII; thus, passive diffusion played a minor role in the observed cytotoxicity. In contrast, in the presence of apo-TCII, analogues **1** and **2** exhibited detectable toxicities even at these low concentrations, indicating that TCII-mediated internalization had occurred.

Interaction with DNA: immunofluorescence studies

Whereas cytotoxicity can be induced by many biological events, it has been thoroughly established for Pt^{II} complexes that DNA strand cross-links are the major mode of

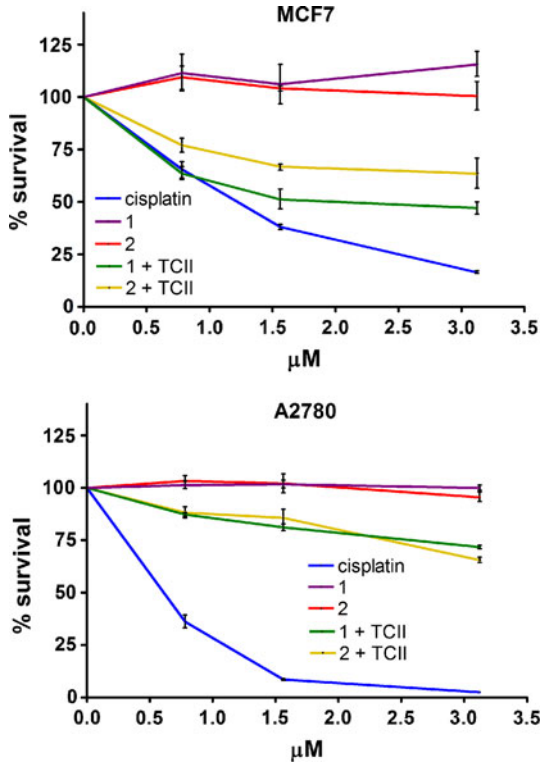


Fig. 5 Cytotoxicity of cisplatin, **1**, **2**, the apo form of transcobalamin II (*TCII*) + **1**, and apo-TCII + **2** for MCF7 and A2780 after 1 day of treatment

action [38, 44, 50, 51]. The ability of compounds **1, 2**, and **5** to interact with DNA and induce DNA damage was investigated by the formation of γ-H2AX foci in MCF7 cells. Cells were grown on glass coverslips and treated with 50 μM compound **1**, a concentration that corresponds to its IC_{90} in the MTT assay and that exerted an effect comparable to that of cisplatin at its IC_{90}. As positive control, cells were irradiated with 10 Gy or treated with 3 μM cisplatin (IC_{90}). The cells were examined at the time points indicated (24, 72, and 144 h). Since the maximum concentration used in the MTT assay was 100 μM, corresponding to IC_{35} for compound **2**, immunofluorescence studies of compound **1**, **2**, **5**, and cisplatin were carried out using their corresponding IC_{35} values (see the electronic supplementary material). As shown in Fig. 6, 24 h of treatment with compound **1** induced γ-H2AX foci displaying an intensity similar to that of cisplatin-treated cells. However, the signal decreased more rapidly in cells treated with compound **1** than in cisplatin-treated cells, likely indicating a more efficient repair in the former case.

Conclusions

We have studied the mode of action of the vitamin B_{12}–Pt^{II} conjugates **1–3** for targeted drug delivery. To mimic

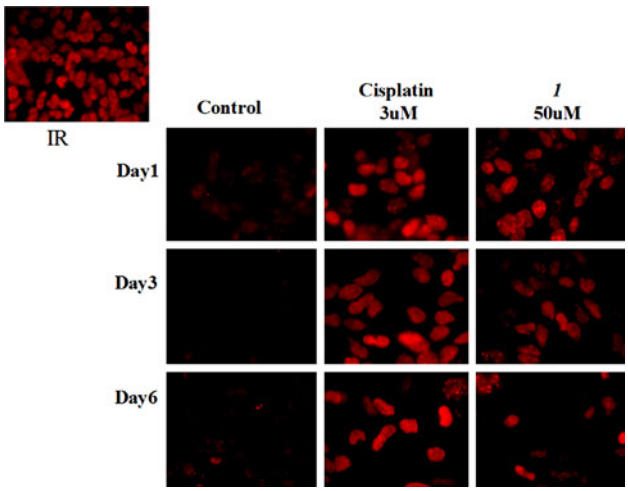


Fig. 6 Immunofluorescence detection of γ-H2AX foci as an indication of the DNA damage caused by cobalamin derivative **4** compared with cisplatin at the IC_{90} value. Positive control: cells irradiated with 10 Gy and examined 1 h after treatment

biological reduction in cells, chemical reduction of **1–3** with cobaltocene yielded HOCbl and the corresponding Pt^{II} complexes **4–6** released from B_{12}, The reactivity of these cisplatin analogues was studied with thiourea, 2′-deoxyguanosine, and GMP. The reactivities of **4** and **5** with GMP compared well with the reactivity of cisplatin. Accordingly, isolated **4** and **5** after B_{12} release displayed cytotoxicity comparable to that of cisplatin. Preliminary cytotoxicity studies of **1–3** showed a lower effect than for cisplatin, probably owing to a low, receptor-mediated uptake of the {B_{12}–CN–Pt^{II}} conjugates. Addition of apo-TCII to the cell media substantially increased the cytotoxic effect, underlining the limiting aspect of uptake and correlation to cytotoxicity. We conclude that {B_{12}–CN–Pt^{II}} conjugates can be considered as prodrugs since they release their cytotoxic cargo, the Pt^{II} complexes in this case, only in the intracellular space. Currently, we are investigating the amount of uptake of {B_{12}–CN–Pt^{II}} and therapeutic action.

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