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Article

A Comparative Study of ROS Production and Cytotoxicity Induced by Combinations of Thiols with HOCbl and CNCbl

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Abstract: Cobalamin is an essential nutrient required for the normal functioning of cells. Its deficiency can lead to various pathological states. Hydroxocobalamin (HOCbl) and cyanocobalamin (CNCbl) are the forms of vitamin B12 that are most commonly used for supplementation. There is substantial evidence indicating that cobalamins can both suppress and promote oxidative stress; however, the mechanisms underlying these effects are poorly understood. Here, it was shown that the oxidation of thiols catalyzed by HOCbl and CNCbl is accompanied by ROS production and induces, under certain conditions, oxidative stress and cell death. The form of vitamin B12 and the structure of thiol play a decisive role in these processes. It was found that the mechanisms and kinetics of thiol oxidation catalyzed by HOCbl and CNCbl differ substantially. It was discussed how these differences may explain different levels of ROS production and cytotoxicity induced by combinations of thiols with HOCbl and CNCbl. On the whole, the data obtained provide a new insight into the redox processes in which cobalamins are involved and might be helpful in developing new approaches to the treatment of some cobalamin-responsive disorders in which oxidative stress is an important component. In addition, these data may be useful for a better understanding of mechanisms underlying induction of different types of death of cancer cells and in a search for new targets for anticancer therapy.

Keywords: hydroxocobalamin; cyanocobalamin; thiolatocobalamins; thiol oxidation; ROS production; cytotoxicity

1. Introduction

Vitamin B12 (cobalamin) is a water-soluble vitamin; its presence in human diet is necessary for normal cell function. It exists in several related forms (vitamers). All forms of vitamin B12 are complexes, each consisting of a central cobalt ion and an organic component called the corrin ring (Fig. 1). The corrin ring has four pyrrole moieties. Two of them are directly bonded to each other, whereas the other pyrrole groups are linked through a methene bridge. The cobalt ion is bonded to four pyrrole nitrogen atoms and can form additional bonds on the lower and upper surfaces of the corrin ring (axial positions). The formation of a coordination bond at these positions depends on the redox state of the cobalt ion and environmental conditions. Thus, [Co^{1+}]Cbl is predominantly four-coordinate and has no axial ligands. [Co^{2+}]Cbl and [Co^{3+}]Cbl are typically five- and six-coordinate, with one and two axial ligands, respectively. In free cobalamin, the lower axial position (α-position) is often occupied by the nitrogen atom of 5,6-dimethylbenzimidazole (Bzm), which is covalently bound to the corrin ring via an intramolecular loop. However, the Co-N bond is not strong, and Bzm can dissociate depending on pH, temperature, and environment [1–3]. Depending on whether Bzm forms a Co-N bond or not, two conformations are recognized, which are called base-on and base-off, respectively. The upper axial position (β-position) can be occupied by cyanide (CN^-), water (H_2O or HO^- depending on pH), 5′-deoxyadenosine, or a methyl group, as well as some other ligands. There

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are also structural analogues of cobalamins that lack the dimethylbenzimidazole ribonucleotide group (cobinamides).

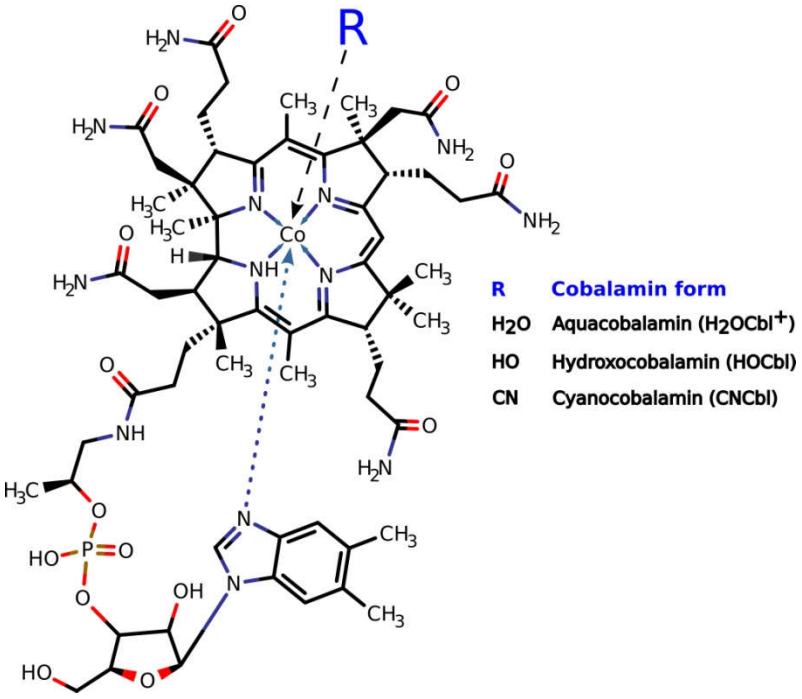

Figure 1. Structure of Vitamin B12.

In mammals, methylcobalamin and adenosylcobalamin are the cofactors of metabolically important enzymes such as methionine synthase and methylmalonyl-CoA (MM-CoA) mutase, respectively. The cytosolic methionine synthase catalyzes the transfer of a methyl group to homocysteine from N5-methyltetrahydrofolate. As a result, methionine and tetrahydrofolate are formed. The latter plays an important role in the metabolism of amino acids and nucleotides. In particular, it participates in the synthesis of purines and one pyrimidine of thymine, which are needed for the synthesis of nucleic acids. Methylmalonyl-CoA mutase is a mitochondrial matrix enzyme, which catalyzes the isomerization of L-methylmalonyl-CoA into succinyl-CoA, an important reaction in the metabolism of amino acids and fatty acids. Succinyl CoA is also required for the synthesis of the heme and hence of hemoglobin.

A deficiency of vitamin B12 can lead to various pathological states, including neurological, psychiatric, and haematological disorders. It is usually caused by the malabsorption of the vitamin or its inadequate dietary intake. There are also genetic defects leading to the impairment of the functioning of cobalamin-dependent enzymes and proteins involved in the transport and metabolism of cobalamin. In the latter case, the deficiency of cobalamin also causes an inactivation of methionine synthase and methylmalonyl-CoA mutase. In most cases, for the treatment of vitamin B12 deficiency, hydroxolcobalamin (HOCbl) and cyanocobalamin (CNCbl) are used [4–6]. HOCbl is a naturally occurring intracellular form of cobalamin, whereas CNCbl is a synthetic form. Meanwhile, it is in the CNCbl form that vitamin B12 has been first isolated and which received wide recognition for the treatment of vitamin B12 deficiency [6]. The low cost and stability of CNCbl contribute to its wide use today as a dietary supplement and an additive for food fortification. However, in some countries, HOCbl has completely replaced CNCbl as a drug of first choice for vitamin B12 replacement therapy [5,6], because it is retained in the body longer and can be administered at greater intervals than CNCbl [5]. In addition, due to the capacity to tightly bind the cyanide ion, HOCbl is also used as an antidote to cyanide poisoning [4,5]. In this case, HOCbl is administered at high doses (5–10 (g) intravenously [7]), which leads to a significant elevation in its concentration in the blood serum [7,8]. In some

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cases, maximum hydroxocobalamin concentration in the serum can reach several hundred micromoles and more (267-1011 μM [8] vs. 0.17-0.92 nM (normal range)). This makes it possible to more effectively bind cyanide ions; as a result, CNCbl is formed endogenously [4].

Nowadays, the main pathways of absorption, transport, internalization, and metabolism of cobalamins have been clarified [9,10]. The interaction of cobalamins with high- and low-molecular-weight sulfur-containing compounds, including thiols, represents an important part of their redox and coordination chemistry in health and disease [9,11-14]. In particular, glutathion (GSH), the most abundant low-molecular-weight thiol in cells [15], is involved in cobalamin metabolism. Glutathionylcobalamin (GSCbl) is an intermediate that is formed in cells and, probably, represents another active form of vitamin B12 [16–18]. GSH is involved in the dealkylation of AdoCbl and MeCbl, as well as, under anaerobic condition, in the decyanation of CNCbl and the removal of the OH group in HOCbl mediated by the methylmalonic aciduria and homocystinuria type C protein (CblC). Defects in CblC (cblC gene) result in the impaired conversion of dietary vitamin B12 to its coenzyme forms (AdoCbl and MeCbl) [19,20]. A functional deficiency of MeCbl and AdoCbl leads to the inactivation of cobalamin-dependent enzymes and the accumulation of homocysteine and methylmalonic acid. CNCbl is inefficient in the treatment of patients with cobalamin C disease [21,22], whereas HOCbl at high doses has a positive effect by enhancing the activity of both cobalamin-dependent enzymes [21–26]. It is important that, in some patients with cblC, the concentration of cobalamin in the serum should be near to micromolar to attain an optimal metabolic response to OHCbl therapy [25,27,28]. Treatment can last several months [25,29]. In turn, whether it is safe to use high-dose HOCbl therapy for a long time has still to be examined [27].

The causes of the differences between treatment outcomes still remain unclear. Meanwhile, it was shown that HOCbl activates methionine synthase in vitro [18]. According to one of the suggestions, the exposed nature of the Co ion in HOCbl facilitates its nonspecific chemical reduction to [Co^{2+}]Cbl, which can be subsequently converted to the coenzyme forms [1]. GSH is considered as a potential reducing agent [1]. However, it should be noted that the oxidation of thiols by cobalamins is accompanied by ROS production and may induce oxidative stress. In particular, the formation of hydrogen peroxide during the cobalamin-catalyzed oxidation of 2-mercaptoethanol and dithioerythritol has been reported [30]. We have previously shown that HOCbl in combination with thiols such as GSH, NAC, and DTT catalyzes the formation of hydrogen peroxide in culture medium, leading to cell death [31,32]. Catalase completely prevents oxidative stress and cell death induced by combinations of HOCbl with these thiols, demonstrating that H_2O_2 plays an important role in these processes. It may be proposed that the oxidation of thiols by cobalamins, accompanied by ROS production, contributes to the development of oxidative stress associated with the CblC disease. It is remarkable that the pathological variants of CblC (R161G/Q) exhibit thiol oxidase activity, which is suppressed in wild-type human CblC. These mutants oxidize GSH to GSSG and reduce dissolved oxygen to superoxide anion [33,34]. Taking into account the aforesaid, it may be assumed that, in CblC defects, the oxidation of GSH by cobalamins is mediated by mutant CblC, and/or in the case of the high-dose OHCbl treatment, it can occur spontaneously. In both cases, the redox process is accompanied by ROS production and may contribute to oxidative stress and a depletion of the glutathione pool in patients treated with high doses of OHCbl. In addition, in patients with CblC deficiency, a depletion of cysteine, an amino acid containing a sulfhydryl group, was also found [35]. This may also be due to direct interaction of thiol with cobalamin [35].

On the other hand, the available literature data indicate that combinations of thiols (GSH and NAC) with standard cobalamin derivatives (HOCbl, MeCbl, CNCbl) protect cells against H_2O_2-induced oxidative stress [36]. At the same time, the thiolatocobalamin complexes GSCbl and NACCbl demonstrate significantly greater protective capabilities than the combinations mentioned above or the thiols alone. Probably, due to the high antioxidant activity of thiolatocobalamins, these complexes may have potential in treating a

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number of pathological conditions accompanied by oxidative stress [36]. In particular, thiolatocobalamins are considered as a possible alternative to HOCbl in the treatment of patients with the cblC disease [11,35].

It remains unclear how different forms of vitamin B12 affect the development/suppression of oxidative stress. The key modulators of cell survival and death (such as products of the cobalamin-catalyzed oxidation) also remain unknown. Our previous results indicate that combinations of HOCbl with GSH, NAC, DTT, and AA lead to apoptotic death in cancer cells [31,32,37]. At the same time, a combination of HOCbl with DDC leads to entosis [38] and/or paraptosis-like cell death [38,39]. For a more complete understanding of mechanisms underlying the effects of cobalamin-reducing agent combinations, first of all a deeper insight into ongoing chemical processes is needed. The aim of the present work was a comparative study of the interaction of HOCbl and CNCbl with some low-molecular-weight thiols, as well as a comparative analysis of ROS production during the cobalamin-catalyzed oxidation of thiols and of the cytotoxic effects of thiol–cobalamin combinations.

2. Materials and Methods

2.1. Chemicals

Horseradish peroxidase (HRP), glutathione (GSH), N-acetylcysteine (NAC), dithiothreitol (DTT), ascorbic acid (AA), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), hydroxocobalamin (HOCbl), cyanocobalamin (CNCbl), 5,5′-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent, DTNB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was from Gibco (Carlsbad, CA, USA). Sodium diethyldithiocarbamate (DDC) was purchased from MP Biomedicals (Irvine, CA, USA). Phosphate buffered saline (PBS) was obtained from Paneco (Moscow, Russia). All reagents were of analytical grade purity. Water used for the preparation of solutions was purified USING a Milli-Q system (Millipore, Burlington, MA, USA).

2.2. Cell culture

MCF-7 cells were obtained from the Russian Cell Culture Collection (Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia). Cells were grown in DMEM (#5648, Sigma, USA) supplemented with 10% FBS (Gibco, USA), 80 mg/l of gentamycin, and 20 mM sodium bicarbonate at 37°C in a humidified atmosphere with 5% CO_2.

2.3. Chemiluminescence measurements

To estimate ROS production during the oxidation of the compounds in the presence of different forms of vitamin B12, the method of luminol-dependent chemiluminescence (LCL) was used for the first time. Briefly, a reaction mixture contained NaH_2PO_4/NaOH buffer (20 mM, pH 7.2), luminol (5 μM), horseradish peroxidase (1.25 U), cobalamin (25 μM), and the compounds being tested (GSH, NAC, DTT, DDC, TNB, AA) at different concentrations (15.6–1000 μM). A stock solution of TNB (10 mM) was prepared by adding a stoichiometic amount of DTNB to DTT in PBS (pH 7.2). Cobalamin (HOCbl or CNCbl) was added to the solutions immediately prior to the registration of the signal. LCL was recorded in 96-well plates (Greiner, Kremsmunster, Austria) at 37°C using a multi-plate reader (Infinite 200 Tecan, Grodig, Austria). The integral LCL response was calculated as a sum of LCL values recorded during the measurements.

2.4. Kinetic studies

2.4.1. Reactions of cobalamins with GSH, NAC, and DTT

The concentration of thiols was determined using the Ellman's reagent (DTNB). Briefly, solutions containing NaH_2PO_4/NaOH buffer (20 mM, pH 7.2), cobalamin (25 μM), and thiol (GSH, NAC, DTT) (100 μM) were incubated at 37°C for different lengths of time (0, 5, 10,20, 30, 40, 50, 60, 90, 120, 240, 360 min). Then, 2 μl of DTNB (10 mM) dissolved in

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DMSO was added to 100 µl of a mixture. TNB formed in the reaction of DTNB with thiols was detected at 412 nm. The concentration of thiols was evaluated by a calibration curve. Measurements were carried out on a multi-plate reader (Infinite F200 Tecan) in 96-well plates (Greiner).

2.4.2. Reaction of cobalamins with TNB

The oxidation of TNB in the presence of HOCbl and CNCbl was studied under different conditions.

In the first case, a reaction mixture contained NaH_2PO_4/NaOH buffer (20 mM, pH 7.2), cobalamin (25 µM), and NAC (100 µM). The solution was incubated at 37°C for 5 min. After that, 2 µl of DTNB (10 mM) dissolved in DMSO was added to 100 µl of the mixture. TNB formed in the reaction between DTNB and NAC was detected at 412 nm. The measurements were performed until the absorbance returned to the baseline. This made it possible to monitor changes in the TNB concentration with time in the presence of cobalamins. The concentration of TNB was evaluated by a calibration curve. Measurements were carried out on a multi-plate reader (Infinite F200 Tecan) in 96-well plates (Greiner).

In the second case, a reaction mixture contained NaH_2PO_4/NaOH buffer (20 mM, pH 7.2), cobalamin (25 µM), and TNB (100 µM). A stock solution of TNB (10 mM) was prepared by adding a stoichiometic amount of DTNB to DTT in PBS (pH 7.2). TNB was added to the solution immediately prior to the measurements. The absorbance at 412 nm, which corresponds to the absorption maximum of TNB, was recorded until absorbance values returned to the baseline (the catalytic cycle of TNB oxidation). After that, to reform TNB, 1 µl of 5mM DTT was added to 100 µl of the mixture and the absorbance at 412 nm was further recorded. The measurements were performed during four catalytic cycles. The concentration of TNB was evaluated by a calibration curve. Measurements were carried out on a multi-plate reader (Infinite F200 Tecan) in 96-well plates (Greiner) at 37°C.

2.4.3. Reactions of cobalamins with DDC and AA

Changes in the concentrations of AA and DDC in the presence of cobalamins were assessed by absorption spectrophotometry. Briefly, a reaction mixture contained NaH_2PO_4/NaOH buffer (20 mM, pH 7.2), cobalamin (25 µM), and DDC (100 µM) or AA (100 µM). The UV-Vis spectra were recorded at 37°C every 5 minutes for four hours. Changes in absorption at 258 and 265 nm were used to determine changes in the concentrations of DDC and AA, respectively.

2.4.4. Determination of rate constants

The concentration of compounds was plotted versus the time. Pseudo-first-order rate constants were determined by a nonlinear least squares curve fit using the QtiPot software.

2.5. UV-Visible spectral studies

UV–Vis spectra were recorded in a 1-cm quartz cuvette on a Cary100 Scan spectrophotometer (Varian, Australia) at room temperature. A reaction mixture contained NaH_2PO_4/NaOH buffer (20 mM, pH 7.2), cobalamin (50 µM), as well as a sulfur-containing compound (100 µM) or AA (100 µM). To detect any spectral changes during the reaction between cobalamins and DTT, the final concentration of DTT was increased up to 500 µM. The spectra were recorded every 5 minutes as long as significant changes were observed.

2.6. Cytotoxicity assay

Cells were seeded in 96-well microplates (Corning, NY, USA) at an amount of 10^4 cells in 100 µL per well and incubated for 24 h. After that, cobalamins and a compound under test (GSH, NAC, DTT, TNB, DDC, AA) were added to the cells. The concentration of cobalamins in the mixture was 25 µM, and the concentration of the compounds was

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varied from 0.1 μM to 5 mM. The cells were incubated with added substances for 48 h. After incubation, the number of viable cells was estimated using the crystal violet cytotoxicity assay [40]. The absorbance at 620 nm was measured using a multi-plate reader (Infinite F200, Tecan, Austria).

2.7. Statistical analysis

Each experiment was performed at least five times. All the values represent the means ± standard deviation. The statistical significance of the results was analyzed using the Student's test for paired experiments, and the values of $p < 0.05$ were considered statistically significant.

3. Results

Under physiological conditions, HOCbl is converted to H_2OCbl^+ (H_2OCbl^+/HOCbl, pKa ≈ 7.8 [41]). As a result, an equilibrium between these two forms (HOCbl and H_2OCbl^+) is established. Therefore, the solutions of HOCbl in PBS (pH 7.2) are subsequently designated as H_2OCbl^+/HOCbl. It should be noted that, as opposed to HOCbl, the β-axial ligand in H_2OCbl^+ can be readily replaced by other ligands [11,41–45], indicating that H_2OCbl^+ enters into the reaction with thiols.

3.1. ROS production during the oxidation of thiols catalyzed by H_2OCbl^+/HOCbl and CNCbl

To detect ROS formed during the cobalamin-catalyzed oxidation of sulfur-containing compounds, the method of LCL was used for the first time. It was found that the oxidation of thiols (GSH, NAC, DTT) by cobalamins (H_2OCbl^+/HOCbl and CNCbl) gives rise to the emission of chemiluminescence, indicating the formation of ROS in the system (Fig. 2, Table S1). An increase in the concentration of thiols caused an increase in the integral LCL response up to some maximum value (∫LCLmax) (Fig. 2, insert). A further increase in the concentration of thiols led to a decrease in integral LCL response, which may be due to the antioxidant properties of these compounds (Fig. S1). A comparison of ∫LCLmax values (H_2OCbl^+/HOCbl vs. CNCbl) indicates that the oxidation of DTT catalyzed by H_2OCbl^+/HOCbl results in a higher LCL response than the oxidation of this dithiol catalyzed by CNCbl (Fig. 2A and B). In contrast, the oxidation of the monothiols GSH and NAC catalyzed by H_2OCbl^+/HOCbl leads to lower LCL responses than the oxidation of these compounds catalyzed by CNCbl (Fig. 2C–F). Among reactions catalyzed by H_2OCbl^+/HOCbl, the highest LCL response is observed during the oxidation of the dithiol DTT, whereas the oxidation of monothiols GSH and NAC results in lower LCL responses. Among CNCbl-catalyzed reactions, the oxidation of the dithiol DTT and monothiol GSH leads to comparable LCL responses, whereas the oxidation of NAC results in the lowest LCL response.

In systems containing either of the vitamin B12 forms and DDC, LCL response was not detected (Fig. S2). In systems containing AA, which was used in the study as a standard reducing agent, a high LCL response was observed only in the presence of H_2OCbl^+/HOCbl (Fig. 2). In the presence of CNCbl, LCL was not detected in the concentration range used, which may be due to both the antioxidant properties of AA and a slow rate of its oxidation.

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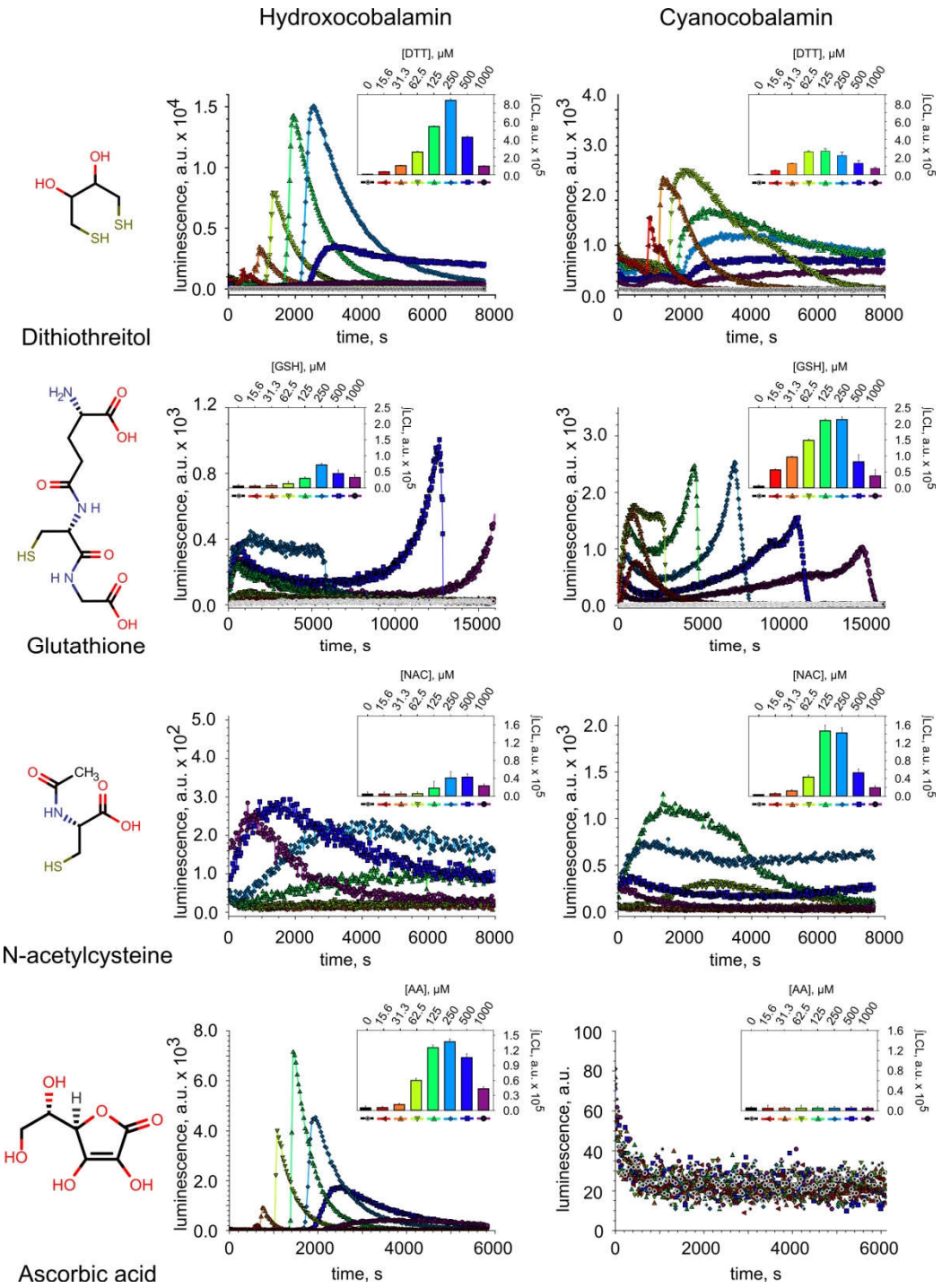

Figure 2. Chemiluminescence recorded during the oxidation of compounds tested in the presence of cobalamins. Inserts: Dependence of integral chemiluminescence response (JLCL) on the concentration of the compounds.

3.2. Kinetics of thiol oxidation catalyzed by H_2OCbl^+/HOCbl and CNCbl

The concentration of thiols in the presence of different forms of vitamin B12 was evaluated using the Ellman's reagent (DTNB). Changes in the concentrations of thiols in the presence of cobalamins are shown in Figure 3.

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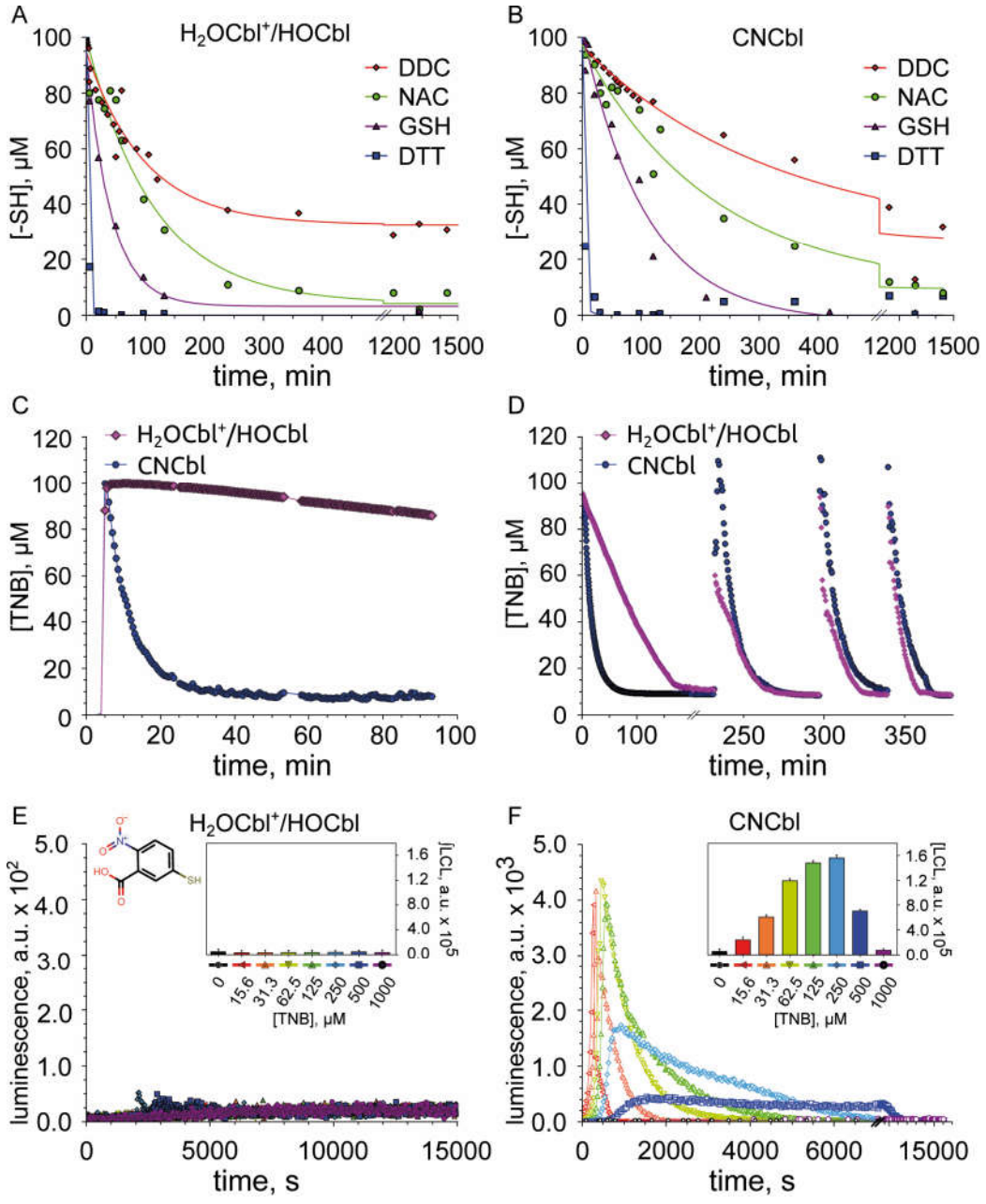

Figure 3. The oxidation of thiols in the presence of cobalamins. (A), (B) Changes in the concentrations of sulfur-containing compounds in the presence of cobalamins. (C) Changes in the concentration of TNB formed in the reaction of DTNB with NAC in the presence of cobalamins. (D) Changes in the concentration of TNB preliminarily formed in the reaction of DTNB with DTT in the presence of cobalamins during four catalytic cycles. (E), (F) - Chemiluminescence recorded during the oxidation of TNB in the presence of cobalamins. Inserts: Dependence of integral chemiluminescence response (∫LCL) on the concentration of TNB.

It is seen that the oxidation of thiols DTT, GSH, and NAC catalyzed by H_2OCbl^+/HOCbl proceeds at a higher rate than their oxidation by CNCbl (Fig. 3A and B). Both H_2OCbl^+/HOCbl and CNCbl catalyze the oxidation of dithiol DTT at a higher rate than the oxidation of GSH (Table 1). In turn, the oxidation of GSH occurs at a higher rate than the oxidation of NAC.

The addition of DTNB to a mixture containing NAC and H_2OCbl^+/HOCbl leads to a fast formation of 2-nitro-5-thiobenzoate (TNB), as indicated by the appearance of the characteristic absorption band with a maximum at 412 nm. Then, a slow oxidation of TNB occurs (the intensity of absorption at 412 nm decreases) (Fig. 3C). The pseudo-first-order rate constant for this reaction (k_1) is 3.24×10^{-5} s^{-1}. On the other hand, the oxidation of TNB,

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which was preliminarily obtained in the reaction of DTNB with DTT, occurs at a somewhat higher rate (Fig. 3D). The pseudo-first-order rate constant for the oxidation of TNB under these conditions (k_2) is 6.42×10^{-5} s^{-1}. In turn, the addition of DTNB to a mixture containing NAC and CNCbl, as in the case of H_2OCbl^+/HOCbl, results in a fast formation of TNB. However, the oxidation of TNB catalyzed by CNCbl proceeds at a much higher rate than its oxidation by H_2OCbl^+/HOCbl (Fig. 3C). The pseudo-first-order rate constant for this reaction catalyzed by CNCbl (k_3) is 2.42×10^{-3} s^{-1}. The CNCbl-catalyzed oxidation of TNB preliminarily formed during the reaction of DTNB with DTT occurs at a somewhat lower rate with a rate constant (k_4) of 1.02×10^{-3} s^{-1} (Fig. 3D).

Table 1. Pseudo-first-order rate constants (k_{obs}, s^{-1}) for the oxidation of sulfur-containing compounds in the presence of cobalamins.

Compounds	HOCbl	CNCbl
DTT	6.33×10^{-3}	5.14×10^{-3}
GSH	3.99×10^{-4}	1.53×10^{-4}
NAC	1.47×10^{-4}	0.75×10^{-4}
DDC[1]	1.70×10^{-4}	0.50×10^{-4}
Cycle of the oxidation of TNB		
1	6.42×10^{-5}	1.02×10^{-3}
2	0.99×10^{-3}	1.33×10^{-3}
3	1.28×10^{-3}	1.38×10^{-3}
4	1.89×10^{-3}	1.40×10^{-3}

[1] Pseudo-first-order rate constants were calculated using UV-Vis spectra recorded during the reactions between cobalamins and DDC.

Thus, it was shown for the first time that both forms of vitamin B12 catalyze the oxidation of TNB, which is formed during the reduction of DTNB by thiols. Meanwhile, CNCbl catalyzes the oxidation of TNB at a much higher rate than H_2OCbl^+/HOCbl (Fig. 3C and D).

After complete oxidation of TNB, the addition of a new aliquot of DTT results in the regeneration of TNB after which the oxidation of this thiol occurs again (Fig. 3D). It was found that the rate of TNB oxidation in the presence of H_2OCbl^+/HOCbl increases more than 15 times after the first catalytic cycle. However, the subsequent increase in the rate is not so significant. The rate of TNB oxidation in the fourth cycle is only two times higher than that in the second cycle. The rate of CNCbl-catalyzed oxidation of TNB slightly increases with each catalytic cycle. The rate of TNB oxidation in the fourth cycle is only 1.37 times higher than that in the first cycle. As a result, in the fourth cycle, the rate of oxidation of TNB catalyzed by H_2OCbl^+/HOCbl begins to exceed the rate of CNCbl-catalyzed oxidation of this thiol (Fig. 3D, Table 1).

It is significant that, during the first cycle of TNB oxidation, a high CL response was observed only in the presence of CNCbl, whereas in the presence of H_2OCbl^+/HOCbl no CL was detected (Fig. 3E and F).

3.3. Examination of the reaction between cobalamins and thiols by UV-visible spectroscopy

Cobalamins absorb light in the UV-visible region of the spectrum. Characteristic bands of the spectra of "typical" cobalamins are designated by α/β (420–600 nm), γ (350–420 nm), and δ (300–330 nm) [46]. The thiols GSH, NAC, and DTT do not absorb light in the wavelength range of 210–700 nm. Therefore, in the wavelength range used in the experiment, the spectral changes of the solutions containing cobalamin and one of the above-mentioned thiols can be assigned to electronic transitions in the structure of cobalamin.

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Our data demonstrate that HOCbl is predominantly converted to H_2OCbl^+ at physiological pH (maximum absorbance at 350, 412 and 525 nm [41,44,47,48]) (Fig. S3).

The addition of a twofold excess of GSH to H_2OCbl^+/HOCbl leads to changes in absorption spectra with time (Fig. 4A).

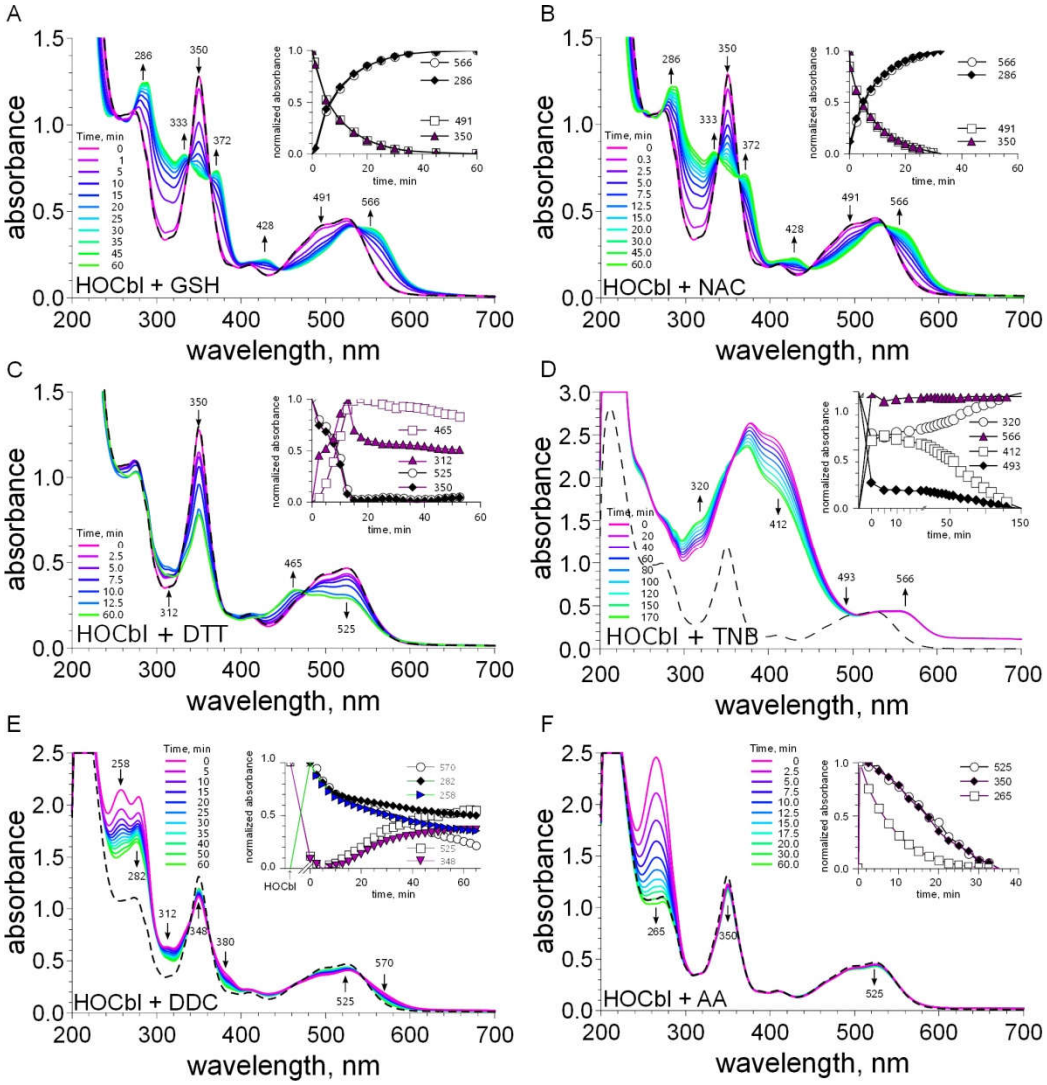

Figure 4. UV-Vis spectra recorded in the reactions between H_2OCbl^+/HOCbl and compounds being tested: (A) GSH, (B) NAC, (C) DTT, (D) TNB, (E) DDC, (F) AA. Dashed lines indicate the spectrum of H_2OCbl^+/HOCbl. Inserts: kinetic traces recorded in the corresponding reactions at selected wavelengths.

In particular, we found a decrease in absorbance at 350 (disappearance of aquacobalamin [11]) and 491 nm as well as an increase in absorbance and the appearance of new peaks at 333, 372, 428, and 566 nm (formation of thiolatocobalamin [11,44,47,48]). Isosbestic points were found at 338, 363, 446, and 536 nm (Fig. S4A). Similar spectral changes were observed in a solution containing H_2OCbl^+/HOCbl and NAC (Fig. 4B, Fig. S5A). Thus, the data obtained indicate the replacement of coordinated water in aquacobalamin by thiols and the formation of thiolatocobalamin complexes [11,44,47,48].

The addition of a twofold excess of DTT to H_2OCbl^+/HOCbl did not lead to any significant changes in absorption spectra with time. However, in the presence of a tenfold excess of this dithiol, the spectrum underwent pronounced changes (Fig. 4C, Fig. S6A). An increase in absorbance at 465 and 312 nm was accompanied by a decrease in absorbance at 350 and 525 nm (disappearance of aquacobalamin). It may be assumed that these spectral changes result from the formation of a complex of cobalamin with DTT or/and

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the reduction of Cbl(III) to Cbl(II). Twelve to seventeen minutes after the start of the reaction, a decrease in absorbance at 465 and 312 nm, as well as an increase in absorbance at 525 nm were observed (Fig. 4C, insert), suggesting the oxidation of Co(II) to Co(III) or/and the decomposition of existing complexes.

The UV-Vis spectra of DDC, TNB, and AA overlapped to some extent with those of cobalamins. Therefore, the spectral changes of the mixtures of cobalamin with DDC, TNB, or AA can be assigned to both electronic transitions in the cobalamin structure and structural changes in these compounds. Figure 4D shows spectral changes observed during the reaction between H_2OCbl^+/HOCbl and TNB. TNB absorbs light at 412 nm. Immediately after the addition of a twofold excess of TNB to a H_2OCbl^+/HOCbl solution, an increase in absorbance at this wavelength occurred (attributed to the overlapping of the bands of TNB and H_2OCbl^+/HOCbl). In addition, the absorbance at 566 nm increased, suggesting the formation of a thiolatocobalamin complex. The time-dependent spectra showed a decrease in the absorbance at 412 nm and an increase in the absorbance at 320 nm (Fig. 4D, Fig. S7A), which indicates that the oxidation of TNB leads to the formation of DTNB. Isosbestic points were observed at 268 and 356 nm (Fig. S7A).

DDC absorbs light at 206, 258, and 282 nm. Immediately after the addition of a twofold excess of DDC to a H_2OCbl^+/HOCbl solution, the absorption spectra of the mixture showed changes: an increase in absorbance at 258, 282 (attributed to the overlapping of bands of DDC and H_2OCbl^+/HOCbl), 312, 380, and 570 nm, as well as a decrease in absorbance at 348, 491, and 525 (Fig. 4E, Fig. S8A). These data suggest the replacement of coordinated water in aquacobalamin (a decrease in absorbance at 348 nm [11]) and, probably, the formation of a cobalamin–DDC complex. Within the next 5 min, the absorbance at 258 and 282 nm decreased, indicating the oxidation of DDC. At the same time, a further minor increase in absorbance at 312 nm and a decrease in absorbance at 348 nm were observed. About 5–7 min after the start of the reaction, absorbance at 258, 282, 312, 380, and 570 nm decreased, and at 348, 491and 525 nm it increased. It may be assumed that, during the oxidation of DDC, a transformation of the cobalamin–DDC complex occurs.

AA also absorbs light in the UV region with a maximum at 265 nm. An increase in absorbance at this wavelength, attributed to the overlapping of bands of AA and H_2OCbl^+/HOCbl, was observed immediately after the addition of AA to H_2OCbl^+/HOCbl (Fig. 4F). The time-dependent spectra showed a rapid decrease in the absorbance at 265 nm, indicating the oxidation of AA. Minor changes in the regions that correspond to the absorption maxima of H_2OCbl^+ (γ, α/β) were observed. The difference absorption spectra showed a decrease in the absorbance at 350 and 525 nm and an increase in the absorbance at 442 nm (Fig. S9A). These data suggest that the oxidation of AA proceeds via the formation of the unstable intermediate aquacobalamin–ascorbate.

After the addition of GSH, NAC, or DTT to a solution of CNCbl, no significant changes in the absorption spectrum were observed (Fig. S4B-6B). In cases that the absorption bands of CNCbl and a compound being tested (TNB, DDC, or AA) overlapped, spectral changes were observed in the regions corresponding to this very compound (Fig. S7B-9B). As an example, Figures 5 shows time-dependent changes in the absorption of a solution containing CNCbl and TNB. It is seen that absorbance at 412 nm (assigned to TNB) decreases, whereas the absorbance at 320 nm (assigned to DTNB) increases. These changes were accompanied by the appearance of the isosbestic point at 356 nm (Fig. 5B). These data indicate that TNB is oxidized to DTNB. Any significant changes in the regions that correspond to the absorption maxima of CNCbl were not observed (γ, α/β). In the whole, the data obtained suggest that, during the reactions of CNCbl with compounds being tested, the replacement of the CN-group in CNCbl does not occur.

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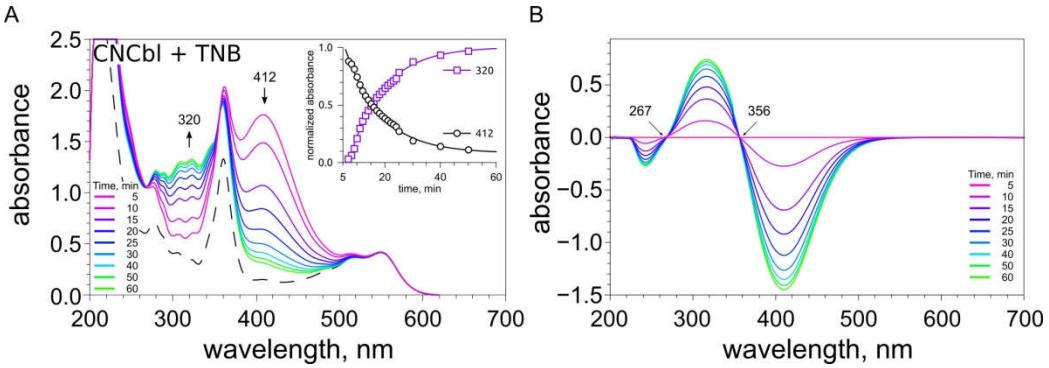

Figure 5. UV-Vis spectra recorded in the reactions between CNCbl and TNB. (A) Absorption spectra. Insert: kinetic traces recorded in the corresponding reaction at selected wavelengths. (B) Difference spectra.

3.4. A comparative examination of the cytotoxic effect of the combinations of thiols with H_2OCbl^+/HOCbl and CNCbl

It was shown that GSH, NAC, TNB, or DTT in the concentration range used (up to 5 mM) do not affect the viability of MCF7 cells. At the same time, a combination of these thiols with B12 derivatives produced a cytotoxic effect. The IC50 values for thiols in combination with 25 μM cobalamin are presented in Figure 6A. It is seen that all monothiols being tested in combination with CNCbl exhibited a more pronounced cytotoxic effect than in combination with H_2OCbl^+/HOCbl. There were no significant differences between combinations DTT+CNCbl and DTT+ H_2OCbl^+/HOCbl.

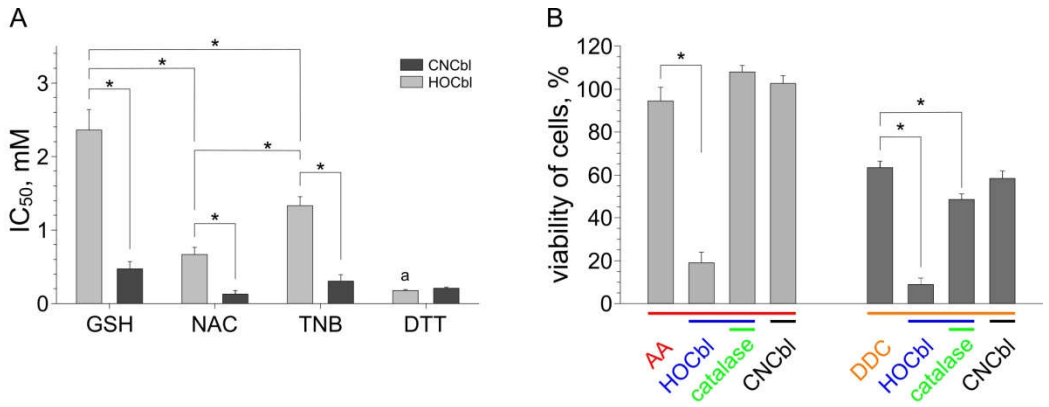

Figure 6. Cytotoxic effect of combinations of cobalamins with compounds being tested. (A) IC50 values for combinations of cobalamins with thiols. (B) Cytotoxicity of combinations of cobalamins with AA and DDC. * significant differences between the combinations; [a] differences are significant compared with combinations HOCbl + monothiols, $p < 0.05$.

A comparison of the cytotoxic action of thiols in the presence of H_2OCbl^+/HOCbl indicates that the dithiol DTT is more cytotoxic than the monothiols. Among the monothiols, NAC produced the highest cytotoxic effect, whereas GSH exhibited the lowest cytotoxicity. In the presence of CNCbl, there was no significant difference between the cytotoxic effects of the dithiol DDT and the monothiols. We did not observe any significant difference in cytotoxicity between the monothiols. Catalase completely prevented the cell death induced by thiol+cobalamin combinations, suggesting that hydrogen peroxide plays an important role in their cytotoxic activity.

DDC, when added alone at a concentration of 1 mM, decreased cell viability to 60–70% of the control (Fig. 6B). The administration of DDC into H_2OCbl^+/HOCbl solutions resulted in the enhancement of its cytotoxicity: the cell viability decreased to 10–13 %. Catalase partially inhibited the cytotoxic effect of this combination. It may be assumed that not only hydrogen peroxide but also other reactive species formed during the

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oxidation of DDC catalyzed by H_2OCbl^+/HOCbl [51] plays an important role in the induction of cell death. There were no significant differences in cell viability between the DDC used alone and its combination with CNCbl.

AA had no cytotoxic effect when administrated alone (0.5 mM) and in combination with CNCbl (25 μM), whereas a combination of AA with H_2OCbl^+/HOCbl led to the significant death of cells (75–80%) (Fig. 6B). Catalase completely prevented the cell death induced by this combination. Similar results were obtained for Hep2 cells (Fig. S10).

4. Discussion

The results of the study indicate that the oxidation of thiols (GSH, NAC, or DTT) catalyzed by both H_2OCbl^+/HOCbl and CNCbl is accompanied by the formation of ROS. The formation of ROS during the oxidation of thiols by cobalamins and cobinamides has been earlier reported in the literature [30,52]. In particular, it was shown that the aerobic oxidation of 2-mercaptoethanol and dithioerythritol by corrinoids results in the formation of hydrogen peroxide and the disulfides of these thiols [30]. In addition, the aquacobalamin-catalyzed oxidation of 2-mercaptoethanol led to the formation of superoxide anion [52]. We have previously shown that thiols GSH, NAC, and DTT in combination with H_2OCbl^+/HOCbl are capable of catalyzing the formation of hydrogen peroxide in culture medium [31,32]. The corrinoid-catalyzed oxidation of thiols in these studies was examined by polarography. This method makes it possible to monitor the consumption of oxygen during the reaction and to determine the amount of hydrogen peroxide accumulated in the system by a particular time (determined from an increase in the oxygen concentration in a solution after the addition of catalase). In the frame of the present work, for the first time, the oxidation of thiols catalyzed by cobalamins was studied by luminol-dependent chemiluminescence. This method was previously applied to determine the content of vitamin B12 in pharmaceutical preparations [53,54] and serum [55]. Here it was used to detect time-dependent changes in ROS production. This enabled us to estimate and compare the total level of ROS formed during thiol oxidation catalyzed by different forms of vitamin B12.

It was found that the oxidation of dithiol DTT in the presence of H_2OCbl^+/HOCbl results in a higher ROS production than in the presence of CNCbl. In contrast, the oxidation of monothiols GSH, NAC, and TNB led to a higher ROS production in the presence of CNCbl, than in the presence of H_2OCbl^+/HOCbl. In the systems containing either of the vitamin B12 forms combined with DDC, ROS production was not being detected. ROS production during the oxidation of ascorbic acid was observed only in the presence of H_2OCbl^+/HOCbl. These data suggest that cobalamins combined with some compounds can induce oxidative stress. For combinations of monothiols with cobalamins, the development of oxidative stress is more likely in the presence of CNCbl than in the presence of H_2OCbl^+/HOCbl. The difference in the level of ROS formed during the H_2OCbl^+/HOCbl- and CNCbl-catalyzed oxidation of thiols may be due to differences in the mechanisms of their oxidation by cobalamins or/and in the kinetics of ROS formation. In addition, ROS, probably, can interact with different intermediates formed during the oxidation of thiols, which leads to the elimination of ROS.

The data on changes in the concentrations of test compounds in the presence of cobalamins showed that H_2OCbl^+/HOCbl catalyzes the oxidation of GSH, NAC, DTT, DDC, and AA at a higher rate than CNCbl. On the other hand, it was found for the first time that TNB in the presence of CNCbl is oxidized faster than in the presence of H_2OCbl^+/HOCbl, indicating that the pathways by which thiols are oxidized by CNCbl and H_2OCbl^+/HOCbl differ substantially. The available literature data demonstrate that coordinated water in aquacobalamin can easily be replaced by sulfur-containing ligands [11,41–45]. The latters are coordinated to the cobalt atom in the β-axial position via the sulfur atom. Our results indicate that the oxidation of thiols in the presence of H_2OCbl^+/HOCbl proceeds via the formation of thiolatocobalamin complexes. Based on

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these findings and the data presented in the literature, the following mechanism of the oxidation of thiols by H_2OCbl^+/HOCbl can be proposed (Fig. 7A).

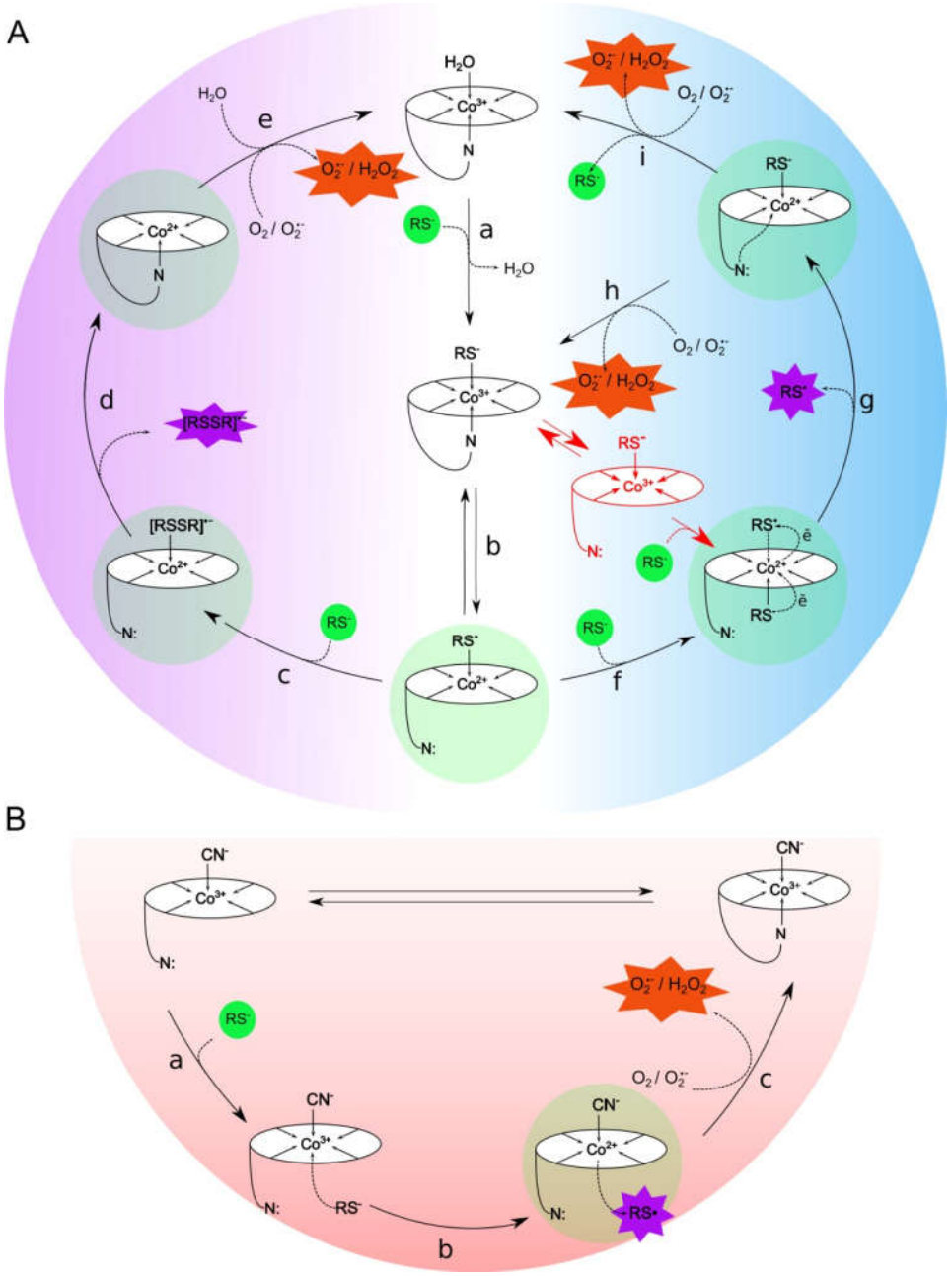

Figure 7. The proposed mechanism for the oxidation of thiols catalyzed by cobalamins. (A) H_2OCbl^+/HOCbl. (B) CNCbl.

Probably, after the replacement of H_2O by thiolate anion (Fig. 7A, step a), intramolecular transfer of the electron from RS^- to Co(III) ion and the dissociation of the 5,6-dimethylbenzimidazole nucleotide occur (Fig. 7A, step b). Thereafter, the intermediate formed interacts with the second molecule of thiol (or thiolate anion) (Fig. 7). Presumably, this process proceeds via two main pathways. The first route involves the formation of the intermediate Cbl(II)$RSSR^{\bullet-}$ (Fig. 7A, step c). The formation of similar structures is supported by the data available in the literature [42]. Subsequent release of $RSSR^{\bullet-}$ (Fig. 7A, step d) and the oxidation of Co(II) by dissolved oxygen (Fig. 7A, step e) lead to the formation of superoxide-anion and the regeneration of aquacob(III)alamin in the system. The second route involves the coordination of the second molecule of thiol at the α-position and subsequent electron transfer (Fig. 7A, step f). This, probably, results in the formation

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of an intermediate in which thiolate-anion is coordinated in the β-position and thiyl radical is coordinated in the α-position. The formation of such complexes during the reaction between HOCbl and GSH under anaerobic conditions has been reported earlier by Ramasamy and co-authors [50]. However, the authors suggested that the next steps after the coordination of the first molecule of GSH to the cobalt(III) ion in the β-axial position would be the dissociation of the 5,6-dimethylbenzimidazole nucleotide (facilitated by a trans effect associated with GSH bound in the β-axial site) and the coordination of a second GSH molecule in the α-axial position. Only after that, GSH coordinated to the α-axial position transfers the electron to Co(III), leading to the formation of a complex with ferromagnetic exchange coupling between the metal ion in the corrin ring and the thiyl radical [50]. It can be assumed that this route takes place during the oxidation of thiols catalyzed by H_2OCbl^+/HOCbl (the red structure in Fig. 7A). Then, the thiyl radical is released from the α-position of the complex, yielding thiolatocob(II)alamin (Fig. 7A, step g). The next step, probably, also proceeds via two routes. The first route involves the oxidation of Co(II) by dissolved oxygen, which leads to the formation of superoxide-anion and the regeneration of thiolatocob(III)alamin in the system (Fig. 7A, step h). The second route leads to the release of the thiyl radical and the formation of cob(II)alamin, which is subsequently oxidized by oxygen to cob(III)alamin (Fig. 7A, step i). The fate of complexes of thiolatocobalamins is mainly determined by their stability. As known, the stability of thiolatocobalamin complexes differs significantly depending on the nature of the sulfur-containing ligand coordinated to cobalt in the β-position. In particular, the constant for the formation of GSCbl from aquacobalamin and the thiolate species of glutathione is ca. 5×10^9 M^{-1}, which suggests that the formation of GSCbl under biological conditions is essentially irreversible [41]. On the other hand, cysteinylcobalamin is by a factor of more than 6×10^4 less stable than GSCbl [56]. The available literature data demonstrate that unstable thiolatocobalamins rapidly decompose, to yield, at least initially, a thiyl radical and cob(II)alamin [56]. It counts in favor of the "path i" (Fig. 7A). On the other hand, in the case of stable thiolatocobalamin complexes, it is more likely that the reaction occurs via "path h".

Cyanocobalamin is more stable than the other forms of vitamin B12 (HOCbl, AdoCbl, and MeCbl) [1,16]. The high enough (10^{12} M^{-1} [57]) stability constant for the binding of cyanide to aquacobalamin suggests that the dissociation of cyanide during thiol oxidation is unlikely. In turn, in a solution containing CNCbl as well as other forms of vitamin B12, an equilibrium between base-on and base-off forms is established. In the base-on form, Bzm is coordinated to cobalt in the α-axial position, inhibiting the binding of a ligand to this site. The intramolecular dissociation of the Bzm ligand leads to the formation of base-off cobalamin, in which the α-axial position is accessible to a ligand. The pKa of base-off cyanocobalamin is 0.1, whereas pKa of base-off aquacobalamin is -2.13 [58]. Therefore, the amount of base-off CNCbl is larger than that of base-off H_2OCbl^+. Thus, it can be suggested that the ligand is coordinated to Co in the α-axial position in base-off CNCbl, after which electron transfer from the ligand to Co(III) ions becomes possible. This suggestion is supported by the literature data [59,60]. In particular, it was proposed that the [59] reduction of cyanocobalamin by sodium hydroxymethanesulfinate, dithionite [59], and $FMNH_2$ [60] occurs in this manner. It should be noted that, in accordance with the data obtained by these authors, the reduction of cob(III)alamin leads to the subsequent dissociation of the cyanide ion from the complexes [59,60]. Spectrophotometric data obtained in the frame of this work indicate that, during the oxidation of thiols by CNCbl, the replacement of the CN-group does not occur. However, the difference absorption spectra showed minor changes at 350 nm during the CNCbl-catalyzed oxidation of DTT, suggests a weakening of the Co(II)-CN bond (Fig. S6B).

The following mechanism of the CNCbl-catalyzed oxidation of thiols can be proposed (Fig. 7B). The first step of this process is a coordination of the molecule of thiol (or thiolate anion) to the cobalt(III) ion in the α-axial position (Fig. 7B, step a). Then, the electron is transferred from RS^- to Co(III) ion, which leads to the formation of the thiyl radical and, probably, a Cbl(II)CN complex (Fig. 7B, step b). The existence of a complex of Cbl(II) and CN^- was confirmed in [61]. Subsequent oxidation of Co(II) by dissolved oxygen

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results in the formation of superoxide-anion and regeneration of CNCbl(III) in the system (Fig. 7B, step c).

It can be concluded that the dissociation of Bzm plays an important part in the oxidation of thiols catalyzed by both CNCbl and H_2OCbl^+/HOCbl. The key role of Bzm in different reactions of cobalamins (including CNCbl and H_2OCbl^+) has been reported in the literature [42,60,62].

Below is given a more detailed discussion of our results that support the proposed mechanisms of the cobalamin-catalyzed oxidation of thiols.

(i) It was shown for the first time that TNB formed during the reduction of the Ellman's reagent (DTNB) by thiols is oxidized in the presence of cobalamins (CNCbl and H_2OCbl^+/HOCbl). In particular, it was found that the addition of DTNB to a mixture containing NAC and cobalamin leads to a fast formation of TNB, which is subsequently oxidized by cobalamins. In addition, it was shown that cobalamins catalyze the oxidation of TNB preliminarily formed in the reaction of DTNB with DTT. The rate of oxidation of TNB by H_2OCbl^+/HOCbl was lower in the first case (the system containing NAC) than in the second case. In contrast, the rate of oxidation of TNB by CNCbl was somewhat higher in the first case (the system containing NAC). It is interesting that, in both systems, CNCbl catalyzed the oxidation of TNB at a 15-fold higher rate than H_2OCbl^+/HOCbl. These data suggest that, in the system containing H_2OCbl^+/HOCbl and NAC, a stable NACCbl complex is formed. The replacement of water in aquacobalamin by thiols and the formation of thiolatocobalamin complexes are evidenced by our spectrophotometric data. Based on the results obtained it can be assumed that the slow oxidation of TNB by H_2OCbl^+/HOCbl in this system may be due to the need to replace the sulfur-containing ligand from the NACCbl complex. We also cannot exclude that the NACCbl complex itself slowly oxidizes TNB. At the same time, the rate of TNB oxidation catalyzed by CNCbl in the system containing NAC was somewhat higher, suggesting that a stable NACCbl complex does not form in this case. Taking into account that CNCbl (the most stable form of vitamin B12) catalyzes the oxidation of TNB at a much higher rate than H_2OCbl^+/HOCbl, it can be assumed that the replacement of the CN-group does not occur and NAC, as well as TNB, is coordinated to the cobalt(III) ion in the α-position of the corrin ring in CNCbl. The spectrophotometric data also indicate that, during the reaction of CNCbl with thiols, no replacement of the CN-group in CNCbl takes place. At the same time, a somewhat higher rate of the CNCbl-catalyzed oxidation of TNB in the system containing NAC remains unclear. It can be only speculated that preliminary interaction between CNCbl and NAC leads to an increase in the amount of base-off CNCbl.

(ii) It was shown that, after a full cobalamin-catalyzed oxidation of TNB, the addition of a new aliquot of DTT results in the regeneration of TNB, after that the oxidation of this thiol occurs over again. It was found that the rate of TNB oxidation catalyzed by H_2OCbl^+/HOCbl increases more than 15 times after the first round of catalysis. It might be suggested that the rate-limiting step is the replacement of the water molecule by thiolate anion and the formation of a thiolatocobalamin complex. However, the spectrophotometric data indicate that the formation of the complex between H_2OCbl^+/HOCbl and TNB proceeds relatively fast. It is also important that the rate of oxidation of TNB catalyzed by CNCbl during the first catalytic cycle was 15 times higher than that catalyzed by H_2OCbl^+/HOCbl. This means that, after the first round of catalysis, the rate of oxidation of TNB catalyzed by H_2OCbl^+/HOCbl approaches the rate of oxidation of TNB catalyzed by CNCbl. Taking into consideration that in solution, the base-off species of CNCbl are present in larger amounts than those of H_2OCbl^+, it can be suggested that the increase in the rate of TNB oxidation catalyzed by H_2OCbl^+/HOCbl after the first catalytic cycle is due to the dissociation of Bzm and an increase in the amount of base-off H_2OCbl^+. Probably, after coordination of the first molecule of the monothiol to the cobalt(III) ion in the β-axial position, the weakening of the Co(II)-Bzm bond takes place, which facilitates the coordination of the second molecule of the monothiol at the α-axial position. After that, the oxidation of the monothiol can occur. Probably, the molecule of TNB coordinates at the α-axial position in such a manner that the nitro group and the carboxyl group interact with cobalt

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ion and Bzm moiety, respectively. These interactions stabilize the structure of the complex formed, leading to the inhibition of TNB oxidation. Nevertheless, monothiol is slowly oxidized to reduce the cobalt ion. These events are probably followed by the release of the thiyl radical and dissociation of Bzm. As a result, a significant increase in the rate of oxidation of TNB catalyzed by H_2OCbl^+/HOCbl is observed after the first catalytic cycle.

In subsequent rounds of catalysis, the increase in the rate of TNB oxidation catalyzed by H_2OCbl^+/HOCbl was not so dramatic. The rate of oxidation in the fourth cycle was two times higher than that in the second cycle. It can be assumed that this effect is also due to an increase in the amount of base-off form. Ramasamy and coauthors have previously proposed that the release of protons during the reduction of cobalamin (HOCbl) by thiol (GSH) may result in a lowering of the pH, which affects the equilibrium between base-on and base-off forms [50]. The increase in the rate of TNB oxidation with subsequent cycles may be due to this effect. This may also explain a slight increase in the rate of CNCbl-catalyzed oxidation of TNB in each round of catalysis. The rate of its oxidation was only 1.37 times higher in the fourth cycle (when compared to the first cycle). Interestingly, during the CNCbl-catalyzed oxidation of thiols (GSH, NAC, DTT), difference absorption spectra showed minor changes in the region of 240–290 nm (Fig. S4B, S5B and S6B). According to the data presented in the literature, changes observed in this region are due to the protonation of 1-a-D-ribofuranosyl-5,6-dimethylbenzimidazole [63,64]. Thus, our findings suggest that the amount of base-off CNCbl increases during thiol oxidation.

(iii) According to the data of UV-vis absorption spectroscopy, the absorbance at 350 and 491 nm decreases during the reaction of aquacobalamin with monothiols (GSH and NAC), indicating the disappearance of aquacobalamin [11]. These spectral changes were accompanied by an increase in absorbance and the appearance of new peaks at 333, 372, 428, and 566 nm, which demonstrates the formation of complexes of thiolatocobalamins [11,44,49,50]. The presence of isosbestic points at 338, 363, 446, 536 nm supports the formation of these complexes [11,50]. In contrast to thiols mentioned above, the absorption bands of cobalamin and TNB, as well as its oxidation product, DTNB, overlapped. TNB and DTNB absorb light with a maximum at 412 and 320 nm, respectively. The difference absorption spectra showed a decrease in the absorbance at 412 nm and an increase in the absorbance at 320 nm, indicating that the oxidation of TNB results in the formation of DTNB. In addition, spectral changes observed at 566 nm suggest that a complex of thiolatocobalamin is formed. Thus, the data obtained indicate that, during the reaction between aquacobalamin and monothiols, the β-axial ligand (water) is replaced by thiolate anion to form thiolatocobalamin complexes.

In contrast, no significant changes in the absorption spectrum were observed in the reaction of CNCbl with GSH or NAC. In the case of TNB, when the absorption bands of cobalamin and the thiol overlapped, spectral changes were found in the region corresponding to this thiol. No significant changes in the regions that correspond to the absorption maxima of CNCbl were observed (γ, α/β). These data indicate that, during the reaction of CNCbl with the compounds being tested, the replacement of the CN-group does not occur, suggesting that these monothiols are coordinated to cobalt ion in the α-position of the corrin ring.

(iv) DTT contains two sulfhydryl groups in the structure. As in the case of GSH and NAC, during the CNCbl-catalyzed oxidation of DTT, no significant changes in the absorption spectrum were observed, which indicates that the replacement of the CN-group does not take place. Most likely, the oxidation of dithiol DTT and monothiols being tested occurs in a similar way (Fig, 7B). This is also supported by the fact that the rate of oxidation of monothiol TNB approaches the rate of oxidation of dithiol DTT.

During the reaction between dithiol and H_2OCbl^+/HOCbl, significant changes in absorption spectra were observed. However, these spectral changes were different from those observed during the oxidation of monothiols (GSH and NAC). One more important thing should be noted. Despite the fact that the rate of oxidation of DTT catalyzed by H_2OCbl^+/HOCbl was higher than that of monothiols, spectral changes during the reaction between DTT and H_2OCbl^+ were observed at a higher molar thiol : cobalamin ratio than

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in the reaction with monothiols. It was shown that, during the cobalamin-catalyzed oxidation of DTT, the absorbance at 350 and 525 nm decreased, indicating the disappearance of aquacobalamin [11], whereas the absorbance at 465 and 312 nm increased. It may be assumed that these spectral changes result from the formation of a complex of cobalamin with DTT or/and the reduction of Cbl(III) to Cbl(II). It is known that, in the latter case, the decrease in absorbance at 350 (γ) and 525 (α) nm is accompanied by an increase in absorbance and the appearance of new peaks at 312, 405, and 475 nm [11,50,65]. Taking into account that, under the experimental conditions used, the changes in absorbance at 312 nm were relatively small and the absorbance peak at 350 nm did not completely disappear, we hypothesized that both forms of cobalamins (Co (II) and Co(III)) are present in the system. Twelve or seventeen minutes after the start of the reaction, further changes in spectra were observed. The absorbance at 465 and 312 nm decreased, whereas absorbance at 525 nm increased, which suggests the oxidation of Co(II) to Co(III) or/and the transformation of existing complexes. It should be noted that these spectroscopic changes were relatively slow. Meanwhile, the oxidation of Co(II) to Co(III) is fast enough. The second-order rate constant for this reaction is 85 $M^{-1}\times s^{-1}$ [66]. Thus, slow spectroscopic changes observed herein may be considered to be in favor of the presence of the complex of DTT with cobalamin in the system.

Taking into account that the oxidation of DTT in the presence of H_2OCbl^+/HOCbl was faster than the oxidation of monothiols and the spectral changes of the solutions containing DTT and H_2OCbl^+/HOCbl were observed in the presence of significant excess of dithiol, it can be proposed that the resulting aquacobalamin–DTT complex is unstable (or at least less stable than complexes formed between cobalamin and monothiols). In general, it can be suggested that the oxidation of DTT by aquacobalamin proceeds as follows: after the replacement of H_2O by a thiolate anion (Fig. 7A, step a), intramolecular transfer of the electron from RS^- to Co(III) ion occurs (Fig. 7A, step b). After that, due to the presence of a second sulfhydryl group in the structure of DTT, a rapid intramolecular cyclization of dithiol proceeds, which leads to the formation of the anion-radical intermediate Cbl(II)RSSR$^{\bullet-}$ (Fig. 7A, step c). Then, the release of the anion radical (Fig. 7A, step d), the oxidation of cobalamin (II) by dissolved oxygen, and the reformation of cob(III)alamin (Fig. 7A, step e) take place.

Thus, the rapid oxidation of DTT by H_2OCbl^+/HOCbl is determined by the presence of two sulfhydryl groups in its structure. Most likely, the second DTT molecule is not involved in this process (Fig. 7A, steps a-e). In turn, the oxidation of monothiols catalyzed by H_2OCbl^+/HOCbl probably proceeds with the participation of two thiol molecules (Fig. 7A, steps a, b, f, g, h). These suggestions are supported by kinetic data, according to which the rate of oxidation of DTT catalyzed by H_2OCbl^+/HOCbl is higher than that of monothiols.

DDC and AA were also oxidized in the presence of cobalamins. An analysis of UV-Vis spectral changes indicated that, during the oxidation of these compounds, the replacement of the CN-group in CNCbl does not occur. Meanwhile, in the reaction between H_2OCbl^+/HOCbl and DDC, the replacement of the β-axial ligand in H_2OCbl^+ by sulfur-containing compounds takes place. The process occurring in the system containing DDC and H_2OCbl^+/HOCbl is probably more complex than that mentioned above. We have previously shown that disulfiram and its oxidation forms, sulfones and sulfoxides, are found among the products of DDC oxidation catalyzed by H_2OCbl^+/HOCbl [51]. It can be assumed that H_2OCbl^+/HOCbl can form complexes with these derivatives. In the course of oxidation of AA catalyzed by H_2OCbl^+/HOCbl, significant spectral changes were observed in the region that corresponds to AA. However, difference absorption spectra showed minor changes in the regions that correspond to the absorption maxima of H_2OCbl^+ (γ, α/β). Thus, it can be suggested that oxidation of AA proceeds through the formation of the unstable intermediate aquacobalamin–ascorbate.

It should be noted that the proposed mechanisms do not allow one to fully explain the difference in the levels of ROS formed during thiol oxidation catalyzed by H_2OCbl^+/HOCbl and CNCbl.

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As was mentioned above, the oxidation of GSH or NAC in the presence of H_2OCbl^+/HOCbl led to lower CL responses than the oxidation of these monothiols in the presence of CNCbl. Meanwhile, the rate of their oxidation was higher in the presence of H_2OCbl^+/HOCbl. This effect may be due to the antioxidant properties of thiolatocob(II)alamin complexes formed during the oxidation of monothiols by H_2OCbl^+/HOCbl. Probably, thiolatocob(II)alamins reduce superoxide anion to hydrogen peroxide, which can be also reduced by these complexes. In the course of these reactions, the regeneration of thiolatocob(III)alamins takes place (Fig. 7A, step h), which results in the closure of the catalytic cycle. This suggestion is supported by literature data according to which cob(II)alamin reduces both $O_2^{\bullet-}$ and H_2O_2 [47,67]. The rate of the oxidation of cob(II)alamin by $O_2^{\bullet-}$ is close to the rate of the $O_2^{\bullet-}$ dismutation by superoxide dismutase (SOD) (7×10^8 versus 2×10^9 $M^{-1}\times s^{-1}$) [47]. Thus, cob(II)alamin may play an important role in the elimination of ROS formed in biological systems. High antioxidant activity of glutathionylcobalamin (GSCbl) and N-acetyl-L-cysteinylcobalamin (NACCbl) has also been reported in the literature [36]. In particular, it was shown that the thiolatocobalamins protect Sk-Hep-1 cells against H_2O_2-induced damage. These complexes are more efficient than thiols by themselves or in combination with standard cobalamin derivatives (CNCbl, HOCbl, and MeCbl) [36]. In addition, it was proposed that glutathionylcob(II)alamin formed during the functioning of CblC reduces $O_2^{\bullet-}$ to H_2O_2 [9,68].

In the course of CNCbl-catalyzed oxidation of monothiols, complexes capable of scavenging ROS did not form. As a result, the integral CL-response, which reflects the total production of ROS in the system, was higher during CNCbl-catalyzed oxidation of the monothiols. The oxidation of the monothiol TNB catalyzed by CNCbl led to a high CL response, whereas during its oxidation in the presence of H_2OCbl^+/HOCbl, CL was not observed. In contrast to GSH and NAC, TNB in the presence of H_2OCbl^+/HOCbl was oxidized slower than in the presence of CNCbl. This may be the chief cause of the lower amount of ROS formed in the system containing H_2OCbl^+/HOCbl. Probably, the subsequent reaction of ROS with thiol and thiolatocob(II)alamin led to the elimination of ROS. As a result, no CL was observed.

The presence of stable complexes of thiolatocobalamins capable of eliminating ROS during the oxidation of monothiols supports the assumption that the second molecule of the monothiol is coordinated to the α-axial position in aquacobalamin and is subsequently oxidized.

The oxidation of DTT catalyzed by H_2OCbl^+/HOCbl led to a higher CL response than the oxidation of this dithiol catalyzed by CNCbl. The rate of DTT oxidation was also higher in the presence of H_2OCbl^+/HOCbl. Probably, the kinetics of this redox reaction is a decisive factor that determines the accumulation of ROS in the systems under study. It should be emphasized once again that, among reactions catalyzed by H_2OCbl^+/HOCbl, the oxidation of DTT proceeded at the highest rate and resulted in the highest CL response. Thus, the results obtained are in good agreement with each other and support the mechanism proposed for DTT oxidation (Fig. 7A, steps a-e), according to which the complex formed between aquacobalamin and DTT is unstable and rapidly decomposes. Therefore, ROS are not eliminated by the DTT–cobalamin complex, which leads to their accumulation in the system.

AA induced CL only when combined with H_2OCbl^+/HOCbl. It can be suggested that the absence of CL in the system containing CNCbl is due to the slow oxidation of this compound (Fig. S9). Most likely, ROS formed in the system react with ascorbic acid. As a result, the elimination of ROS takes place and CL is not observed. The absence of CL in a system containing any of the two vitamin B12 forms and DDC may be due to the absence of ROS production during oxidation of DDC, the antioxidant action of DDC, and/or the rapid transformation of ROS to reactive sulfur species. The fact that disulfiram and its derivatives, sulfones and sulfoxides, are formed during the H_2OCbl^+/HOCbl-catalyzed oxidation of DDC counts in favor of the latter suggestion [51]. It can be proposed that the absence of CL response in the system containing CNCbl is a consequence of the slow oxidation of this compound.

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It should be noted that ROS formed in the systems may subsequently initiate free radical transformations without the involvement of cobalamins [69], which suggests that the oxidation of thiols may be more complex than the processes mentioned above.

Our previous results have indicated that combinations of HOCbl with GSH, NAC, AA [31], DTT [32], and DDC [38,39,51] produce a cytotoxic effect on HEp-2 cells. It was shown using an oxygen electrode that the oxidation of thiols [31,32] and AA [31] catalyzed by H_2OCbl^+/HOCbl is accompanied by the accumulation of hydrogen peroxide in culture medium. Catalase added simultaneously with these combinations completely prevented H_2O_2 accumulation and cell death, indicating that H_2O_2 plays a key role in their cytotoxic activity. Taking into account the differences in ROS production during the oxidation of the compounds catalyzed by H_2OCbl^+/HOCbl and CNCbl, it was interesting to compare the cytotoxic effects of these compounds in combination with cobalamins (H_2OCbl^+/HOCbl and CNCbl).

It was shown that monothiols GSH, NAC, and TNB in combination with CNCbl exhibited a stronger cytotoxic effect than in combination with H_2OCbl^+/HOCbl. In the presence of H_2OCbl^+/HOCbl, dithiol DTT produced a higher cytotoxic effect than the monothiols. The data on the cytotoxicity of these combinations are consistent with the data on ROS production detected by the method of LCL during the oxidation of these thiols in the presence of corresponding cobalamins. Thus, the results obtained suggest that the combination of cobalamins with thiols induces oxidative stress, which leads to cell death. Catalase completely prevented the cell death induced by these combinations.

No significant differences in cytotoxicity between the combinations of DTT with CNCbl or H_2OCbl^+/HOCbl were observed, although ROS production detected by the method of LCL was higher during the oxidation of this dithiol in the presence of H_2OCbl^+/HOCbl. However, in both cases the production of ROS was relatively high and exceeded the production of ROS during the oxidation of monothiols by H_2OCbl^+/HOCbl. It can be assumed that, the amount of ROS formed in both systems is sufficient to trigger processes leading to cell death. This could also explain the equally high cytotoxicity of combinations of CNCbl with all thiols. Catalase completely prevented the cell death induced by the combinations mentioned above, which supports this assumption.

AA at the concentration used had no cytotoxic effect when administrated alone or in combination with CNCbl. However, the incubation of cells with AA combined with HOCbl caused cell death. These data are consistent with the data on ROS production during the oxidation of AA in the presence of cobalamins. In particular, a high production of ROS was found during the oxidation of AA in the presence of H_2OCbl^+/HOCbl, whereas in the presence of CNCbl, ROS production was not detected. Catalase also completely prevented the cell death induced by the combination of AA with H_2OCbl^+/HOCbl.

DDC at the concentration used was slightly cytotoxic when used alone and had a strong cytotoxic effect when added in combination with H_2OCbl^+/HOCbl. In contrast, no significant differences in cytotoxicity between DDC used alone and DDC in combination with CNCbl were observed. Noteworthy, ROS production was not detected by the method of LCL during the oxidation of DDC in the presence of both CNCbl and H_2OCbl^+/HOCbl. No hydrogen peroxide accumulation was also observed using an oxygen electrode during the oxidation of DDC catalyzed by H_2OCbl^+/HOCbl [51]. As was mentioned above, the main products of the reaction of DDC with HOCbl are disulfiram and its oxidation forms, sulfones and sulfoxides [51]. Probably, ROS formed during the oxidation of DDC rapidly transform to reactive sulfur species. This could explain a partial inhibition of the cytotoxic effect of the combination of DDC with H_2OCbl^+/HOCbl by catalase, which was observed here and earlier [39,51]. The absence of any influence of CNCbl on the DDC-induced cell death as well as the absence of CL during the oxidation of DDC in the presence of CNCbl may be due to a slow oxidation of this compound.

The cytotoxic effect of monothiols GSH, NAC, and TNB in combination with H_2OCbl^+/HOCbl is worthy of special attention. It was found that the combination of GSH with H_2OCbl^+/HOCbl caused the lowest cytotoxicity, whereas the oxidation of this thiol in the presence of H_2OCbl^+/HOCbl resulted in the highest ROS production among

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monotiols. A combination of NAC with H_2OCbl^+/HOCbl produced the highest cytotoxic effect, although the oxidation of NAC in the presence of H_2OCbl^+/HOCbl led to a moderate production of ROS. A combination of TNB with H_2OCbl^+/HOCbl exhibited a moderate cytotoxic effect. However, during the first cycle of TNB oxidation in the presence of H_2OCbl^+/HOCbl, ROS production was not detected.

The data obtained are very intriguing and reflect the complexity of processes occurring in cobalamin-containing systems. Taking into account our results and the data presented in the literature, it can be concluded that the reactivity of cobalamins as well as pro- and antioxidant activity of cobalamins combined with different compounds can be influenced by the environment. The question of how this happens is of special interest. Within the framework of this study, only a few points concerning this issue can be specified.

It is known that axial positions in cobalamins can be occupied by different ligands. Coordinated water in aquacobalamin can be easily replaced by other ligands [11,41–45]. The fate of the resulting complexes and the activity they exhibit depend on the nature of the β-axial ligand and environment. In particular, a chemical modification of the β-axial ligand or conformation changes can take place. Thus, the dissociation of Bzm from its lower α-position makes possible the coordination of the second molecule of the ligand at the α-axial position and its subsequent oxidation. It is significant that the weakening of the Co(III)-Bzm bond and the dissociation of Bzm might be due to a trans effect associated with the binding of the ligand in the β-axial site or the reduction of the cobalt ion by the β-axial ligand.

Thus, the occupancy of the β-axial binding site by different ligands in aquacobalamin can influence the reactivity of the pool of cobalamins present in the system. In turn, the β-axial binding site in aquacobalamin may be considered to be responsible for changes in the reactivity of cobalamin in response to environmental changes. In CNCbl, the β-axial position is occupied by a cyanide ion. The CN-Co bond is strong enough [57]. Thus, the complex is inert to ligand substitution reactions, which can limit the influence of environment on the reactivity of CNCbl.

As was mentioned above, Bzm plays a key role in the regulation of activity of cobalamins. It is known that Bzm can dissociate depending on pH, environment, and the oxidation state of the Co-ion [1–3]. This fragment is present in the structure of both aquacobalamin and cyanocobalamin. Meanwhile, the base-off/base-on ratio of CNCbl is higher than that of H_2OCbl^+. In the base-on form, the Bzm ligand is coordinated to the cobalt in the α-axial position of the corrin ring. Therefore, the occupancy of the α-axial binding site by Bzm inhibits the binding of the ligand to this site. In the base-off form, the α-axial position is accessible to a ligand. Thus, it is conceivable that the activity of CNCbl is affected by environment to a lesser extent than the activity of H_2OCbl^+. However, in the whole, an alteration of the conformation of cobalamins (base-on/base-off) influences their activity. This is supported by the literature data [42,60,62] and our results. In particular, we suggest that an increase in the rate of cobalamin-catalyzed TNB oxidation with each catalytic cycle might be due to an increase in the amount of base-off species. A slow oxidation of TNB catalyzed by H_2OCbl^+/HOCbl in the first catalytic cycle is probably associated with the formation of a complex, in which TNB coordinated at the α-position interacts with both cobalt ion and Bzm. These interactions stabilize the structure of the complex formed, which leads to the inhibition of the dissociation of Bzm and the oxidation of thiol. It can be suggested that this mode of inhibition of cobalamin activity is operating in biological systems. Taking into account the aforesaid, it can be assumed that aquacobalamin is more susceptible to various factors than cyanocobalamin. Probably, this could explain different outcomes observed in biological systems containing combinations of cobalamins with thiols.

The difference in the redox behavior of different forms of vitamin B12 may underlie their different efficacy in treating cobalamin deficiency caused by defects in cblC. In these cases, CNCbl is inefficient [21,22]. At the same time, patients respond to high doses of HOCbl [21–26]. Probably, due to the lability of β-axial ligand in H_2OCbl^+(HOCbl), a more

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direct precursor of the coenzyme forms may form. This could partially rescue the cofactor function of this form of cobalamin [1]. Noteworthy, [Co^{2+}]Cbl [1] and thiol-cobalamin adducts including GSCbl [18] are considered as such a precursor. One more important point should be noted. The oxidation of thiols by cobalamins may contribute to the development of oxidative stress associated with cblC disease. Thiolatocobalamins are considered as a potential alternative to HOCbl to treat patients with this disease [11,35]. Our data suggest that thiolatocobalamins are able to maintain the oxidation of thiols accompanied by ROS production, which should be kept in mind. Probably, during co-administration of Vitamin B12 preparations with drugs containing a sulfhydryl group in their structure (NAC, captopril, etc.), it is also necessary to take into account possible interactions between them and exercise care in selecting the appropriate drug.

5. Conclusion

To date, there is growing evidence that cobalamins can both suppress and promote oxidative stress; however, the mechanisms underlying these effects are poorly understood. It also remains unclear how different forms of vitamin B12 affect the development/suppression of oxidative stress. Here, we demonstrate that, under certain conditions, the cobalamin-catalyzed oxidation of thiols leads to ROS production and cell death. These processes depend on the form of cobalamin and the structure of thiol. The mechanisms and kinetics of thiol oxidation by HOCbl and CNCbl differ substantially. In particular, in contrast to CNCbl, HOCbl forms stable complexes with monothiols, which exhibit high antioxidant activity and, probably, are capable of eliminating ROS formed in biological systems. This could explain higher levels of ROS production and cytotoxicity induced by combinations of monothiols with CNCbl. A complex formed between HOCbl and DTT is unstable and rapidly decomposes. As a result, ROS are not eliminated by the complex, which leads to their accumulation in the system and cell death.

On the whole, the data obtained in the frame of the present work provide a new insight into the processes in which cobalamins are involved in health and disease, and might be helpful in developing new approaches to the treatment of some cobalamin-responsive disorders in which oxidative stress is an important component.

Taking into account our previous results according to which a combination of HOCbl with one of the compounds tested leads to apoptotic or paraptosis-like cell death, and/or entosis, it can be concluded that the data obtained herein form the molecular basis necessary for a better understanding of mechanisms underlying the induction of different types of death of cancer cells. This is important for the identification of new targets for anticancer therapy.

Supplementary Materials: Table S1. Dependence of integral chemiluminescence response (JLCL±SD) on the concentration of the compounds; Figure S1: Effect of compounds being tested on chemiluminescence; Figure S2: Chemiluminescence recorded during the oxidation of DDC in the presence of cobalamins; Figure S3: UV/Vis absorption spectra H_2OCbl^+/HOCbl at different pH values; Figure S4: UV-Vis spectra recorded during the reactions between cobalamins and GSH; Figure S5: UV-Vis spectra recorded during the reactions between cobalamins and NAC; Figure S6: UV-Vis spectra recorded during the reactions between cobalamins and DTT; Figure S7: UV-Vis spectra recorded during the reactions between cobalamins and TNB; Figure S8: UV-Vis spectra recorded during the reactions between cobalamins and DDC; Figure S9: UV-Vis spectra recorded during the reactions between cobalamins and AA; Figure S10: Cytotoxic effect of combinations of cobalamins with compounds being tested.

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