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Research paper

Hydroxycobalamin catalyzes the oxidation of diethyldithiocarbamate and increases its cytotoxicity independently of copper ions

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ABSTRACT

It is known that some metals (Cu, Zn, Cd, Au) markedly increase the toxic effect of thiocarbamates. It was shown in the present study that hydroxycobalamin (a form of vitamin B_{12}, HOCbl), which incorporates cobalt, significantly enhances the cytotoxicity of diethyldithiocarbamate (DDC), decreasing its IC_{50} value in tumor cells three to five times. The addition of HOCbl to aqueous DDC solutions accelerated the reduction of oxygen. No hydrogen peroxide accumulation was observed in DDC + HOCbl solutions; however, catalase slowed down the oxygen reduction rate. Catalase as well as the antioxidants N-acetylcysteine (NAC) and glutathione (GSH) partially inhibited the cytotoxic effect of DDC + HOCbl, whereas ascorbate, pyruvate, and tiron, a scavenger of superoxide anion, had no cytoprotective effect. The administration of HOCbl into DDC solutions (> 1 mM) resulted in the formation of a crystalline precipitate, which was inhibited in the presence of GSH. The data of UV and NMR spectroscopy and HPLC and Mass Spectrometry (LC/MS) indicated that the main products of the reaction of DDC with HOCbl are disulfiram (DSF) and its oxidized forms, sulfones and sulfoxides. The increase in the cytotoxicity of DDC combined with HOCbl occurred both in the presence of Cu^{2+} in culture medium and in nominally Cu-free solutions, as well as in growth medium containing the copper chelator bathocuproine disulfonate (BCS). The results indicate that HOCbl accelerates the oxidation of DDC with the formation of DSF and its oxidized forms. Presumably, the main cause of the synergistic increase in the toxic effect of DDC + HOCbl is the formation of sulfones and sulfoxides of DSF.

1. Introduction

SH-containing compounds are involved in the scavenging of reactive oxygen species (ROS) and are widely used as antioxidants. In reactions with ions of transition metals, thiols are capable of generating ROS and producing a damaging action on cells and tissues [1–4]. In addition, in reactions with free radicals, they can transform into thiyl radicals, which can lead to the damage to DNA and other biomolecules [5]. Jacobsen et al. (1984) showed that cobalamin (Cbl) derivatives catalyze the aerobic oxidation of the thiols 2-mercaptoethanol and dithioerythritol, which results in the formation of disulfides and hydrogen peroxide [6]. We have previously shown that hydroxycobalamin (a form of vitamin B_{12}, HOCbl), in combination with the antioxidant thiols GSH, NAC, and DTT is capable of catalyzing the formation of hydrogen peroxide at concentrations up to 30–250 μM. This catalysis considerably enhances the cytotoxic action of these classical thiol compounds and leads to the manifestation of the prooxidant action of DTT, GSH, and NAC [7,8].

Another interesting and widely used group of SH-containing compounds is dithiocarbamates (DTC). Compounds based on DTC are widely used in industry, veterinary science, agronomy, and medicine [9,10]. One of the widely known carbamates is diethyldithiocarbamate (DDC), a dithio derivative of diethylcarbamic acid. DDC at millimolar concentrations (0,5-1 мМ) is used as a Cu,Zn-SOD inhibitor, which produces a prooxidant effect; however, under some conditions, it exhibits antioxidant and antiapoptotic properties [11,12]. Under in vivo conditions, DDC appears in the bloodstream as a result of the metabolism of the anti-alcohol drug disulfiram (DSF) and undergoes further transformations in the liver and kidneys [13–15]. There is substantial evidence in the literature indicating that, due to the binding of copper

Abbreviations: HOCbl, hydroxycobalamin (a form of vitamin B_{12}); Cbl, cobalamin; DDC, N,N- diethyldithiocarbamate; DTC, dithiocarbamates; DSF, disulfiram; SOD, superoxide dismutase; ROS, reactive oxygen species; BCS, bathocuproine disulfonate; NAC, N-acetylcysteine; GSH, glutathione; HBSS, Hank's solution; LC/MS, mass spectrometry; RT, retention time

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ions, DTC produce a pronounced cytotoxic effect [13,14,16–18]. DTC are also capable of forming complexes with zinc, cadmium, and gold; at present, their antineoplastic activity is extensively studied [19–23]. It was shown that the chelation of copper by DDC/DSF and its transport to the cell leads to the generation of intracellular ROS [24–28]. To date, attempts have been made to introduce DSF and its derivatives into antitumor therapy since it was found that they synergistically enhance the cytotoxic effect of antineoplastic drugs, such as cisplatin, gemcitabine, and paclitaxel and increase the efficacy of some therapeutic methods, e.g., radiotherapy [20,26,28–33]. It was found that the active metabolite of DSF when it is used in combination with copper is a dithiocarb-copper complex [17,34]. Because this complex can damage normal tissues, different ways to increase the DSF toxicity without the use of copper ions and to protect the adjacent tissues by encapsulating the components into micro- and nanoparticles are developed to date [15,35,36].

We have found earlier that HOCbl is capable of enhancing the cytotoxic effect of DDC. The mechanism of this effect is not yet clearly established; however it is known that cobalt ions do not significantly affect the toxicity of DDC and DSF [17,22]. Cobalamins form complexes with a variety of biologically active substances, participate as cofactors in many biological processes in the organism, and affect gene expression [37,38]. The enhancement of the cytotoxic effect of DDC by Cbl detected in the study may be taken as the basis in the design of novel antitumor drugs. The use of modern ways of isolating active components by incapsulation (incorporation into liposomes as well as micro- and nanoparticles) [15,36,39–41] will enable one to avoid a possible unfavorable effect of the combination of DDC with Cbl on normal tissues. The goal of this work was to establish the mechanism by which HOCbl enhances the cytotoxic effect of DDC. We examined the products obtained during the reaction in solutions (DMEM, HBSS) and the cytotoxic effect of DDC + HOCbl in a culture of human tumor cells. We found that HOCbl catalyzes the aerobic oxidation of SH groups followed by DSF precipitation. In the aqueous phase, the accumulation of oxidized DSF derivatives occurs, which just causes the cytotoxic effect. Thus, under in vitro conditions, DDC + HOCbl is a binary catalytic system with a prolonged toxic effect.

2. Materials and methods

2.1. Chemicals

DDC, GSH and NAC were purchased from MPbiomedicals (USA); fetal bovine serum was from Gibco (USA). Acetone d6 (99.96%) was from CIL (UK). Other chemicals were from Sigma (USA).

2.2. Cell culture

Human lung carcinoma A549, human epidermoid larynx carcinoma HEp-2, and human squamous carcinoma A431 cell lines were obtained from the Russian Cell Culture Collection (Institute of Cytology, Russian Academy of Sciences, St. Petersburg). Cells were grown in DMEM supplemented with 10% FBS, 80 mg/l of gentamycin, and 20 mM sodium bicarbonate at 37 °C in an atmosphere of 5% CO_2.

2.3. Cytotoxicity assay and treatment of cells

Cells were seeded in 96-well microplates or culture dishes (Corning, USA) at a concentration of 2×10^5 cells/ml (2×10^4 cells in 100 µl/well). DDC was added from freshly prepared stock solutions (10–100 mM) in deionized H_2O, PBS, and HBSS, and HOCbl was added from a 2.5 mM stock solution in deionized H_2O. All treatments were made 24 h after cell seeding. DSF was added from the stock solution (200 mM) in DMSO under continuous stirring to a concentration of 200 µM and below. Cytotoxicity was determined using the crystal violet assay as described earlier [8]. Cell viability was also estimated by the trypan blue exclusion assay.

2.4. Detection of hydrogen peroxide in culture medium

Oxygen reduction by DDC and HOCbl in PBS was estimated in a 1-ml chamber with an O_2-electrode at 25 °C by polarography [8].

2.5. Estimation of DSF solubility

From a solution of DSF in DMSO, calibrating solutions in PBS, DMEM, and DMEM + serum were prepared through a series of intermediate dilutions at a DMSO concentration of 1%. The solutions were allowed to stand for 24 h at 37 °C in an atmosphere of 5% CO_2. Then, the samples were centrifuged (5 min, 14,500 rpm), and UV spectra of supernatants were recorded on a Cary 100 Scan spectrophotometer (Varian, Australia). The limit of DSF solubility was determined by extrapolating the linear segment of the dependence D = f(C) onto the region of optical densities of saturated DSF solutions.

2.6. UV spectrophotometry

Absorption spectra were measured in PBS on a Cary 100 Scan spectrophotometer. If necessary, the reaction mixture was diluted with PBS (1:10).

2.7. NMR spectroscopy

The main product of the reaction of HOCbl with DDC was identified on an AVANCE-III 600 spectrophotometer (Bruker, Germany) with a working frequency of 600 MHz. The temperature of a sample was 298 K. The spectrum width was 23.4 ppm. The duration of an impulse was 10 µs. For a good signal/noise ratio, no more than 32 scans were required. The repetition time was 1.14 s. The relaxation delay between scans was 10 s in the mode of simple scanning, without the suppression of signals of the solvent. All samples were dissolved in acetone.

2.8. HPLC and mass spectrometry

LC/MS was performed using a Waters Aquity UPLC system connected in photodiode array detector followed by a LCQ Deca XP (Thermo Finnigan, USA) mass spectrometer operating in the electrospray ionization mode.

The substances were separated using the following conditions and parameters of mass spectrometry. For HOCbl complexes, gradient chromatography was performed at 25 °C using a Synergi Hydro-RP 80 A column (4 µm, 4.6 × 50 mm) at a flow rate of 1.0 ml/min using water (solvent A) and CH3CN (solvent B). Positive electrospray ionization was used. Other LC/MS parameters were as follows: capillary voltage 4.50 kV and cone voltage 80 V. The source temperature was 250 °C. For the separation and detection of low-molecular-weight compounds, chromatography was performed at 25 °C using a Symmetry C18 column (5 µm, 4.6 × 150 mm) at a flow rate of 1.0 ml/min using the isocratic elution mode water/methanol (20/80 by volume). Positive electrospray ionization was used. Other LC/MS parameters were as follows: capillary voltage 4.00 kV and cone voltage 80 V. The source temperature was 165 °C.

All data were collected and processed using the Xcalibur® software. Samples were prepared for analysis by adding HOCbl to a PBS containing DDC with gentle stirring followed by filtration of the sample and dilution with water (1: 20).

2.9. Statistical analysis

Each experiment was performed at least three times. All the values represent the means ± s.e.m. The statistical significance of the results was analyzed using the Student's *t*-test for paired experiments. The