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DNA binding and oxidative DNA damage induced by climacostol–copper(II) complexes: Implications for anticancer properties

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ABSTRACT

Climacostol is a natural toxin isolated from the freshwater ciliated protozoan *Climacostomum virens* and belongs to the group of resorcinolic lipids. Climacostol exerts a potent antimicrobial activity against a panel of bacterial and fungal pathogens. In addition it inhibits the growth of tumor cell lines in a dose-dependent manner by inducing programmed cell death via intrinsic pathway. In this work, we investigated the possibility that climacostol exerts a prooxidant effect, inducing plasmid DNA strand breakage and eukaryotic DNA damage in presence of Cu(II) ions. Inhibition of DNA breakage using SOD, catalase and neocuproine confirmed the involvement of reactive oxygen species and Cu(I) ions in the DNA damage. UV–visible absorption changes and mass spectrometric analysis identified a product of reaction as a deprotonated form of climacostol. Study of the interaction with DNA, using fluorescence spectroscopic techniques, showed that climacostol binds with DNA. Given the structure–activity relationship of this compound and the mechanism of its prooxidant effect, we propose that the Cu(II)-mediated oxidative DNA damage by climacostol could explain its antimicrobial and antiproliferative activity.

1. Introduction

Climacostol (1,3-dihydroxy-5-[(Z)-non-2′-enyl]benzene) is a toxin physiologically produced by the ciliated protozoan *Climacostomum virens* for chemical defense against unicellular and multicellular predators [1–3]. From a chemical point of view, the toxin belongs to the family of resorcinolic lipids (also called alkylresorcinols, or 5-alkylresorcinols), widely detected in prokaryotes as well as in single-celled and multicellular eukaryotes [4,5]. To date, climacostol has been chemically synthesized by three alternative pathways [6–9]. In the approach adopted by Fiorini et al. [9] and used in this study, climacostol was obtained as a substantially pure compound, avoiding contamination with the undesired (*E*)-isomer present in the natural toxin purified from cultures of *C. virens*.

The compounds of this group have attracted attention for potential use in therapy and/or prevention of specific classes of diseases [10–13] because of their antimicrobial, cytotoxic, anticancer and genotoxic activities. Recent studies performed to evaluate the effects of climacostol on bacteria, fungi, protozoa, human cancer cell lines, and isolated rat mitochondria have suggested that the protozoan toxin exhibits this spectrum of activities, and have indicated some peculiar structural and functional traits of the molecule. Petrelli et al. [14] have reported that climacostol can exert a potent antimicrobial activity against a panel of bacterial and fungal pathogens, including *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Candida*. Buonanno and Ortenzi [15] demonstrated that the cytotoxic potency of climacostol on free-living freshwater ciliates can be modulated by the substitution of the double bond in the aliphatic chain of the toxin with a single or a triple one. Muto et al. [16,17] found that climacostol specifically inhibits respiratory chain complex I in rat liver mitochondria, inducing a consequent generation of reactive oxygen species (ROS). Finally, Buonanno et al. [18] and Fiorini et al. [9] observed that climacostol can inhibit the growth of human cancer cell lines in a dose-dependent manner and induce apoptosis by triggering the mitochondrial (intrinsic) pathway.

The intrinsic pathway is the most common one for apoptosis in vertebrates and can be activated by a variety of cellular stressors, including ROS and DNA-damaging molecules. Of note are reports

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that some resorcinolic lipids are capable of interacting with the DNA double helix; they are incorporated into the helix interior by intercalation of chains [5,19]. Furthermore, it was observed that some members of the class of resorcinolic lipids possess the ability to induce Cu^{2+}-dependent DNA cleavage [20,21] associated with ROS formation and apoptosis induction [22–25].

On the basis of these considerations, the objective of this study was to assess the prooxidant activity of climacostol. The results demonstrate that climacostol causes the copper-dependent strand breaks of pBR322 plasmid DNA, indicating that this alkylresorcinol exerts a prooxidant effect. Our examination of the structure–activity relationship of this compound and the mechanism of its prooxidant effect enables us to propose that the Cu(II)-mediated oxidative DNA damage by climacostol could explain its antimicrobial and antiproliferative activity.

2. Materials and methods

2.1. Materials

Chemically synthesized climacostol was obtained as previously reported [9]. Supercoiled plasmid pBR322 DNA, calf thymus DNA (ct-DNA), catalase, neocuproine, ethidium bromide (EB), diethylenetriaminepentaacetic acid (DTPA) and agarose were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Assay for oxidative DNA strand breakage

The induction of DNA strand breakage by climacostol was assessed by using agarose gel electrophoresis to measure the conversion of the supercoiled pBR322 plasmid DNA to open circular and linear forms [26]. pBR322 DNA (0.180 μg) was incubated with the indicated concentration of climacostol and/or Cu (II) in phosphate buffer saline (PBS) at pH 7.4 and 37 °C for 60 min. After incubation, the samples were mixed with 2 μl of a solution containing 50% glycerol (v/v), 40 mM EDTA and 0.05% bromophenol blue to stop the reaction, and the reaction mixture was immediately subjected to 1% agarose gel stained with ethidium bromide. The samples were electrophoresed in a horizontal slab gel apparatus in TBE buffer containing 40 mM Tris–HCl, 89 mM boric acid and 2 mM EDTA, pH 8.0, at 5 V/cm for 3 h. The gel was photographed using a UV transilluminator.

2.3. Detection of Cu(II) reduction

Copper reduction was tracked by determining the cuprous ion concentration with neocuproine [27]. The 1 ml samples contained PBS buffer, 500 μM of neocuproine, 50 μM $CuCl_2$, and various concentrations of climacostol. The neocuproine–Cu(I) complex was determined by measuring the absorbance at 450 nm immediately after mixing the reagents.

2.4. Detection of superoxide anion radical generation

The amount of $O_2^{\cdot-}$ generation was determined by measurement of cytochrome c reduction. A mixture containing 40 μM ferricytochrome c, 100 μM climacostol, 20 μM $CuCl_2$ and 2.5 μM DTPA in 1.5 ml of 10 mM sodium phosphate buffer (pH 7.8) was incubated at 37 °C. A maximum absorption at 550 nm due to ferricytochrome c formed by ferricytochrome reduction was measured with a UV–visible Shimadzu UV-2401PC spectrophotometer. The content of $O_2^{\cdot-}$ at a low estimate was calculated by subtracting absorbance with SOD from that without SOD at 550 nm ($\varepsilon = 21.1 \times 10^3\ M^{-1}\ cm^{-1}$) [28].

2.5. DNA Binding Study by Fluorescence Spectroscopy

A stock solution of ct-DNA was prepared by dissolving the solid material in 10 mM phosphate buffer (pH 7.0) containing 50 mM NaCl and stirred for 12 h at 4 °C in the dark. The concentration of DNA was determined by UV absorbance at 260 nm using the molar absorption coefficient $\varepsilon = 260$ (6600 $M^{-1}\ cm^{-1}$) [29]. The competitive binding experiment was carried out by maintaining the EB and ct-DNA concentration at 5 μM and 55.7 μM, respectively, while increasing the concentration of the different compounds. The fluorescence spectra of a series of solutions with various concentrations of the derivative and a constant EB–ct–DNA complex were measured. All the fluorescence data were corrected for absorption of exciting and emitted light according to the relationship Eq. (1) [30]:

$$Fc = Fm \times e^{(A1+A2)/2} \quad (1)$$

where Fc and Fm are the corrected and measured fluorescence, respectively. A1 and A2 are the values of absorbance of climacostol and analogues at the exciting and emission wavelengths. Fluorescence quenching spectra were recorded using an ISS-Greg 200 spectrofluorimeter with an excitation wavelength of 500 nm and emission spectrum at 520–700 nm. For fluorescence quenching experiments, the Stern_Volmer's Eq. (2) was used [31]:

$$F_0/F = 1 + K_Q \tau_0 1/2[\text{Ligand}] = 1 + K_{SV}[\text{Ligand}] \quad (2)$$

where F_0 and F represent the fluorescence intensity in the absence and in the presence of drug. [Ligand] is the concentration of the derivative and K_{SV} is the Stern_Volmer constant which is equal to $k_Q\ \tau_0$, where k_Q is the bimolecular quenching rate constant and τ_0 is the average fluorescence lifetime of the fluorophore in the absence of drug.

2.6. UV–visible spectra measurements

UV–visible spectra were measured at room temperature with a Shimadzu UV-2401PC spectrophotometer. A solution of 100 μM of climacostol in PBS buffer was prepared, with a final volume of 3 ml and the spectral tracing was started by addition of 200 μM $CuCl_2$. The spectra were recorded every 5 min after addition of $CuCl_2$.

2.7. MS detection of products for the reaction of climacostol

Analyses were carried out using liquid chromatography-electrospray ionization tandem mass spectrometry (LC–ESI–MS). ESI-API low resolution mass spectra were recorded using an Agilent 1100 MSD ion trap mass spectrometer equipped with a standard ESI-API source. Nitrogen served both as the nebulizer gas and the dry gas. The samples were prepared by mixing 100 μM of climacostol and 200 μM $CuCl_2$ in 3 ml of 10 mM ammonium acetate buffer, and introduced by direct infusion with a syringe pump. Ammonium acetate is employed commonly in LC–MS because the ammonium plays the role of a volatile MS-friendly co-ion, instead of sodium or potassium. Also, for optimum MS analysis, ammonium acetate works as ionization reagent.

2.8. Statistical analysis

Data represent the mean ± standard error (S.E.), unless otherwise indicated, of three independent experiments. The significance of the differences between the mean values was examined by Student's t-test. The minimum level of significance considered was $P < 0.05$.