Journal of Photochemistry and Photobiology B: Biology 121 (2013) 75–85

Contents lists available at SciVerse ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Synthesis, characterization and *in vitro* DNA binding and cleavage studies of Cu(II)/Zn(II) dipeptide complexes

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ARTICLE INFO

Article history:
Received 29 August 2012
Received in revised form 29 November 2012
Accepted 19 December 2012
Available online 26 February 2013

Keywords:
Dipeptide Cu(II) and Zn(II) complexes
DNA binding profile
pBR322 DNA cleavage
In vitro anticancer activity

ABSTRACT

Novel dipeptide complexes Cu(II)·Val-Pro (**1**), Zn(II)·Val-Pro (**2**), Cu(II)·Ala-Pro (**3**) and Zn(II)·Ala-Pro (**4**) were synthesized and thoroughly characterized using different spectroscopic techniques including elemental analyses, IR, NMR, ESI–MS and molar conductance measurements. The solution stability study carried out by UV–vis absorption titration over a broad range of pH proved the stability of the complexes in solution. *In vitro* DNA binding studies of complexes **1–4** carried out employing absorption, fluorescence, circular dichroism and viscometric studies revealed the binding of complexes to DNA via groove binding. UV–vis titrations of **1–4** with mononucleotides of interest viz., 5′-GMP and 5′-TMP were also carried out. The DNA cleavage activity of the complexes **1** and **2** were ascertained by gel electrophoresis assay which revealed that the complexes are good DNA cleavage agents and the cleavage mechanism involved a hydrolytic pathway. Furthermore, *in vitro* antitumor activity of complex **1** was screened against human cancer cell lines of different histological origin.

1. Introduction

Small molecules binding to specific sites along DNA molecule are considered as potential chemotherapeutic agents. Peptides are attracting much attention since Novartis launched a vasopressin drug analog Lypressin in 1970, presently ca. 30 peptides have been marketed as key classes of therapeutics. Their role as mediators of key biological functions and their unique intrinsic properties make them particularly attractive therapeutic agents. They can surmount the hurdles of present cancer chemotherapeutic drugs including, little unspecific binding to molecular structures other than the desired target, minimization of drug–drug interactions and less accumulation in tissues reducing risks of complications due to intermediate metabolites. The fact that peptides affect the tumor cells rapidly and that their secondary metabolites are free amino acids proves that the peptides have minimized side effects as compared to other chemotherapeutic agents that are available currently. Recently, it has been reported that some peptide derivatives show antitumor activity with little toxicity against non-malignant cells either by triggering apoptosis [1,2] or by forming ion channels/pores [3]. Furthermore, some peptides were found to be cytotoxic against MDR cancer cells [4,5].

Peptides are versatile and powerful ligands for a range of metal ions since they contain a variety of potential donor centers. Metallopeptide systems are unique and can affect the biological activity of peptides. Metals may be directly bound into peptides and have been used for DNA selective recognition and/or cleavage [6,7]. It was reported that peptide fictionalization of polypyridyl ruthenium(II) [8,9] or rhodium(III) [10,11] metallo-intercalators improved the selectivity of the parent intercalators, with similar effects observed by conjugating minor groove binders with short peptides that mimic natural protein motifs [12].

The rationale of our strategy is to design peptide–metal complexes which prove to possess pronounced biological and better pharmacological activity. Amongst the metal ions chosen, we have opted for biocompatible endogenous metal ions Cu and Zn, known for their essential role in biological living system. In particular, the complexes based on essential metals are less toxic than those with non-essential ones. Cu(II) complexes are known to play a significant role either in naturally occurring biological systems or as pharmacological agents [13,14] whereas the importance of zinc for stabilization of protein loops in enzymes, zinc fingers, etc., has generated new interest in the field of Zn coordination chemistry [15,16]. The discovery of the ‘zinc fingers’ has triggered intensive research on the interaction of proteins with Zn ions [17,18]. The investigation of Cu(II)/Zn(II)-peptide complexes is of scientific and technological importance, since such systems may be regarded as models for both protein–DNA and antitumor agent-DNA interactions.

Herein we report, the synthesis, spectroscopic characterization, DNA binding and cleavage studies of dipeptide complexes of Cu(II) and Zn(II). The synthetic strategy involves peptide moiety as a scaffold for our complexes thereby, these complexes would exhibit desirable therapeutic properties for more efficacious and safer drug

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http://dx.doi.org/10.1016/j.jphotobiol.2012.12.009

administration owing to (a) better cellular uptake, (b) improved aqueous solubility, (c) selectivity and specificity towards the target at the molecular level and (d) reduced toxicity due to biocompatible endogenously friendly ligand and metal ions. *In vitro* DNA binding studies revealed that these complexes are avid DNA binding agents; thereby they can act as potent chemotherapeutics. Furthermore, DNA cleavage activity of **1** and **2** with pBR322 DNA has been carried out, in addition *in vitro* cytotoxicity of complex **1** was tested against various human cancer cell lines.

2. Experimental

2.1. Materials

Alanine, Valine, Proline, HOBT (N-Hydroxybenzotriazole) (SRL), $Cu(NO_3)_2{\cdot}3H_2O$, $Zn(NO_3)_2{\cdot}6H_2O$ (Ranchem), EDCI (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide Hydrochloride), Boc anhydride, $SOCl_2$, TFA (trifluro acetic acid), calf thymus DNA (CT-DNA), ascorbic acid (Merck), H_2O_2, 3-mercaptopropionic acid, glutathione, methyl green, DAPI, tertiary butyl alcohol, sodium azide, superoxide dismutase (Sigma), guanosine-5′-monophosphate disodium salt (5′-GMP) and thymidine-5′-monophosphate disodium salt (5′-TMP) (Fluka) were used without further purification.

2.2. Methods and instrumentation

Elemental analyses were performed on Elementar Vario EL III. Fourier transform infrared spectra were recorded on Interspec 2020 FTIR spectrometer in Nujol. ^{1}H NMR spectra was obtained on a Bruker DRX-400 spectrometer. Electrospray mass spectra were recorded on Micromass Quattro II triple quadrupol mass spectrometer. EPR spectra of the copper complexes were recorded on a Varian E 112 spectrometer at the X-band frequency (9.1 GHz) at LNT. Molar conductance was measured at room temperature on a Digisun Electronic Conductivity Bridge. Electronic spectra were recorded on a UV-1700 PharmaSpec UV–visible Spectrophotometer. Fluorescence measurements were made on Hitachi F-2500 Fluorescence Spectrophotometer. CD spectra were recorded on Applied Photophysics Chirascan Circular Dichroism Spectrometer with Stop Flow. Viscosity measurements were carried out from observed flow time of CT-DNA containing solution ($t > 100$ s) corrected for the flow time of buffer alone (t_0), using Ostwald's Viscometer at 25 ± 0.01 °C. Flow time was measured with a digital stopwatch.

DNA binding experiments which include absorption spectral traces, luminescence, circular dichroism and viscosity experiments conformed to the standard methods [19,20] and practices previously adopted by our laboratory [21]. While measuring the absorption spectra an equal amount of DNA was added to both the compound solution and the reference solution to eliminate the absorbance of the CT-DNA itself, and the CD contribution by the CT-DNA and Tris buffer was subtracted through base line correction.

2.2.1. In vitro antitumor studies

The cell lines used for *in vitro* antitumor screening activity were A498 (Renal cell), A549 (Lung), Zr-75-1 (Breast), HT29 (Colon adenocarcinoma grade II cell line), A2780 (Ovary), SiHa (Uterine cervix) and MCF7 (Human breast). These human malignant cell lines were procured and grown in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics to study growth pattern of these cells. The proliferation of the cells upon treatment with chemotherapy was determined using the Sulforhodamine B (SRB) semi automated assay [22]. Cells were seeded in 96 well plates at an appropriate cell density to give optical density in the linear range (from 0.5 to 1.8) and were incubated at 37 °C in CO_2 incubator for 24 h. Stock solutions of the complexes were prepared as 100 mg/mL in DMSO and four dilutions i.e. 10 μL, 20 μL, 40 μL, 80 μL, in triplicates were tested, each well receiving 90 μL of cell suspension and 10 μL of the drug solution. Appropriate positive control (Adriamycin) and vehicle controls were also run. The plates with cells were incubated in CO_2 incubator with 5% CO_2 for 24 h followed by drug addition. The plates were incubated further for 48 h. Termination of experiment was done by gently layering the cells with 50 μL of chilled 30% TCA (in case of adherent cells) and 50% TCA (in case of suspension cell lines) for cell fixation and kept at 4 °C for 1 h. Plates were stained with 50 μL of 0.4% SRB for 20 min. The bound SRB was eluted by adding 100 μL 10 mM Tris (pH 10.5) to each of the wells. The absorbance was read at 540 nm with 690 nm as reference wave length. All the experiments were repeated three times.

2.3. Syntheses

2.3.1. Synthesis of dipeptides (L_1, L_2)

The dipeptides used in this study were synthesized by conventional solution phase methodology [23]. The Boc group was used for N-terminal protection and the C-terminus was protected as a methyl ester. Coupling reactions were mediated by EDCI-HOBT.

Boc-amino acid (10 mmol) was dissolved in dichloromethane (DCM, 20 mL). To this solution, EDCI (4.77 g, 25 mmol) was added at 0 °C and the reaction mixture was stirred for 15 min amino acid methylester (10 mmol) was added followed by HOBT (2.74 g, 20 mmol) to the above reaction mixture under stirring. After 24 h, the reaction mixture was filtered; the residue was washed with DCM (30 mL) and added to the filtrate. The filtrate was washed with 5% sodium bicarbonate and saturated sodium chloride solutions. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by column chromatography.

L_1 Semisolid mass; Yield 63.7%; IR (Nujol, cm^{-1}): 3315 (νNH); 1716 (νC═O); 1617 (ν_{as}O—C═O); 1455 (ν_sO—C═O). ^{1}H NMR ($CDCl_3$, ppm): 5.20 (1H, —NH, Boc), 4.50 (1H, m, —CH, Val), 4.25 (1H, t, —CH, Pro), 3.80 (2H, t, —CH_2, Pro), 3.71 (3H, s, —OCH_3), 2.00 (2H, m, —CH_2, Pro), 2.25 (2H, m, —CH_2, Pro), 1.00 (3H, d, —CH_3, Val), 1.45 (9H, s, *t*Butyl group). ESI–MS (*m*/*z*): 329 $[C_{16}H_{28}N_2O_5 + H]^+$.

L_2 Semisolid mass; Yield 67.2%; IR (Nujol, cm^{-1}): 3317 (νNH); 1714 (νC═O); 1650 (ν_{as}O—C═O); 1443 (ν_sO—C═O). ^{1}H NMR ($CDCl_3$, ppm): 5.35 (1H, —NH, Boc), 4.51 (1H, m, —CH, Ala), 4.45 (1H, t, —CH, Pro), 3.61 (2H, t, —CH_2, Pro), 3.70 (3H, s, —OCH_3), 2.00 (2H, m, —CH_2, Pro), 2.25 (2H, m, —CH_2, Pro), 1.35 (3H, d, —CH_3, Ala), 1.45 (9H, s, *t*Butyl group). ESI–MS (*m*/*z*): 301 $[C_{14}H_{24}N_2O_5 + H]^+$.

2.3.2. Synthesis of Cu(II)·Val-Pro (**1**)

The dipeptide, L_1 (0.43 g, 2 mmol) was dissolved in minimum amount (15 mL) of absolute methanol. To this a methanolic solution of $Cu(NO_3)_2{\cdot}3H_2O$ (0.24 g, 1 mmol) was added. The solution was stirred for 1 h. The resultant pale blue solution was left for slow evaporation at room temperature. The blue precipitate obtained was filtered, washed with petroleum ether and dried in vacuo (Scheme 1).

Yield, 66.7%. IR (Nujol, cm^{-1}): 3218 (νNH); 1674 (νC═O); 1674 (ν_{as}O—C═O); 1408 (ν_sO—C═O); 452 (Cu—N); 519 (Cu—O). ESI–MS (*m*/*z*): 491 $[C_{20}H_{34}N_4O_6Cu + H]^+$. Molar Conductance, Λ_M (1×10^{-3} M, DMSO): 32.7 Ω^{-1} cm^2 mol^{-1} (non-electrolyte). UV–vis (DMSO, nm): 257, 645 nm. Anal. Calc. (%) (for $C_{20}H_{34}N_4O_6$-Cu): C 49.02; H 6.99; N 11.43. Found (%): C 49.10; H 6.94; N 11.47.