2009-08

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Polyhedron,Oxford,v. 28, n. 12, p. 2329-2334, Aug. 2009
http://www.producao.usp.br/handle/BDPI/49335

Polyhedron 28 (2009) 2329–2334

Contents lists available at ScienceDirect

Polyhedron

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

Interaction of Cu-dipeptide complexes with Calf Thymus DNA and antiproliferative activity of [Cu(ala-phe)] in osteosarcoma-derived cells

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ARTICLE INFO

Article history:
Received 18 September 2008
Accepted 22 April 2009
Available online 3 May 2009

Keywords:
Cu(II)complexes
DNA-interaction
EPR
CD
Osteosarcoma

ABSTRACT

In this work the study of Calf Thymus DNA interaction with several Cu(L-dipeptide) complexes was reported. The binding stoichiometry (Cu(mmol)/DNAmol base) was determined and in an attempt to clarify the binding mode, EPR and CD experiments were performed. All the studied complexes interacted with DNA, in a more selective way than $[Cu(H_2O)_6]^{2+}$, being the [Cu(ala-phe)] the complex with the highest interaction. The EPR experiments suggested that the monomeric species formed in solution were coordinated through a nitrogen atom of the DNA bases (inner-sphere binding) and the CD studies showed structural changes upon the DNA–complex interaction. Besides, the ratio Cu(mmol)/DNAmol base obtained by the binding stoichiometry experiments was close to that found by EPR and CD determinations. The effect on cell proliferation determined by the crystal violet bioassay on UMR106 rat osteosarcoma-derived cells showed that the [Cu(ala-phe)] complex exerted an antiproliferative action against this tumor line.

1. Introduction

The interaction of metal complexes with nucleic acids and their constituents has been a subject of interest in bioinorganic chemistry, especially since the discovery of cisplatin and their analogues. These studies have been useful for the development and the comprehension of the activity of new chemotherapeutic agents designed for the treatment of numerous diseases [1–3]. Many of these chemotherapeutic agents act by inhibition of the synthesis of the deoxyribonucleic acid (DNA), a natural target due to its predominant role in cellular replication. One way to achieve this inhibition is by direct DNA binding in either a noncovalent interaction by intercalation, groove-face binding or external electrostatic binding or a covalent interaction due to an outer-sphere or inner-sphere binding and (or consecutive) strand breakage [4,5].

In particular, in the search for new drugs the proposal that complexes based on essential metals may be less toxic than those with non essential ones led to the study of copper based drugs, some of them having an important cytotoxic effect [6–8]. Consequently, active copper compounds and their interaction with DNA have attracted great interest [5,9–11]. These studies have mostly used methods that focus on changes in the DNA structure as electronic absorption titration, fluorescence spectroscopy, viscosity measurements and circular dichroism, among others. For instance, chemotherapeutic copper complexes with polypyridil ligands showed an important interaction with DNA as intercalators as determined by UV–Vis and circular dichroism (CD) spectroscopies and viscosity [3]. Moreover, Sarkar's group studied the DNA interaction of Cu(II) complexes with piroxicam and meloxicam (anti-inflammatory drugs) which exhibited anticancer activity by UV–Vis and circular dichroism (CD) spectroscopies. They showed that their complexes bind strongly to DNA-backbone possibly with intercalation [12].

On the other hand, there is much less work using methods based on properties related to the metal ion. In this area relevant studies were performed by Chikira's group that provided information on the binding of copper complexes with amino acids or peptides to DNA fibers by electronic paramagnetic resonance (EPR) [13–20].

As a part of our research on metal-based drugs we have studied several copper complexes with oligopeptides as potential antitumoral agents encouraged by the antecedents in the literature [21,22]. In particular, we have synthesized and structurally characterized in solid state and in water solution a series of copper complexes with dipeptides [23–30] and analysed their magnetic interactions [31,32].

Due to these previously reported antecedents and with the aim of obtaining structural information about the interaction of DNA with copper-dipeptide complexes we explored changes in the environment of the copper ion and in the DNA upon coordination.

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doi:10.1016/j.poly.2009.04.029

In particular, the study of the DNA interaction with several Cu(L-dipeptide) complexes previously characterized by our research group was performed. Several dipeptides commercially available were selected in an effort to include ligands with inverted sequences of aminoacids and to cover a series containing aminoacids with different sizes. The aim of this selection was to perform a screening that provides structural information useful for biggest systems. Firstly, the direct quantification of the amount of copper bound to the DNA (binding stoichiometry) was measured. Secondly, EPR experiments were made with the complex that presented the best binding stoichiometry and with two complexes that presented medium interaction, in order to test whether there was any change in the Cu(II) coordination sphere when binding to the DNA. CD technique was used to explore the distortion introduced in the DNA structure upon binding with complexes or the distortion in the Cu(dipeptide) complexes.

Besides, to evaluate potential pharmacological activity of the complexes, a cell proliferation assay on UMR106 rat osteosarcoma-derived cells was performed.

2. Materials and methods

2.1. Synthesis of the complexes and analytical characterization

The copper (II) complexes with L-dipetides (SIGMA) ala-ala, ala-val, ala-ile, ala-leu, ala-thr, ala-phe, ala-tyr, phe-ala, phe-leu, phe-phe, val-phe, val-gly were obtained and characterized as described previously [26,27,29,30].

Table 1 summarizes the stoichiometry and analytical data for the Cu(II) complexes. Elemental analysis was performed with a CARLO ERBA EA 1108 equipment.

2.2. Spectroscopic measurements

Electronic absorption spectra of aqueous solution samples were recorded on a Milton Roy Spectronic 3000 spectrophotometer, using 1-cm path length quartz cells.

The copper concentration was measured by atomic absorption spectroscopy using a Perkin–Elmer 5000 instrument, with a Photron lamp for copper analysis, at 325 nm with air/acetylene flame.

X-band (9.5 GHz) EPR measurements were carried out on frozen aqueous solutions using a Varian E109 spectrometer and cavity with 100 kHz field modulation. The measurements were performed at N_2 liquid temperature. The *g*-values were obtained from spectral simulations using the QPOW program [33].

The CD spectra were recorded using a Jasco 720 instrument in a 1-cm path length quartz cell, in the 200–300 nm range.

2.3. Determination of the stoichiometry of the interaction Cu(II)dipeptide–DNA

The technique used in order to determine the stoichiometry of the interaction of the complexes with DNA was adapted from Mahnken et al. [34]. An aqueous solution of Calf Thymus DNA (SIGMA) (c.a. 1.7 mg/mL, 1 mL) was allowed to react with an aqueous solution of Cu-dipeptide (c.a. 1 mM, 1 mL) for 24 h at 37 °C.

The DNA was precipitated afterwards by addition of absolute ethanol (4 mL) and an aqueous solution of NaCl (2 M, 0.2 mL), and it was centrifuged (30 min at 50 rpm). The supernatant was discarded. The precipitate was dissolved in 2 mL of H_2O. This process was repeated three times. DNA and copper concentrations were monitored in the final solution. DNA concentration was determined at 260 nm using $\varepsilon_M = 6000\ M^{-1}\ cm^{-1}$. Copper concentration was measured by atomic absorption spectroscopy as described in Section 2.1. The solubility of the complexes was checked in the conditions of the precipitation of the DNA. The interaction was expressed as the ratio: Cu(mmol)/DNA(mol base).

2.4. Cu(II)dipeptide–DNA binding: characterization by EPR measurements

The EPR spectra of an aqueous solution of Calf Thymus DNA (SIGMA, 2.45 mg/mL) in presence of increasing amounts of copper complexes were recorded. The dilution scheme for each Cu(II) complex covered a 10–500 μM range, while the concentration of the DNA solution varied from 500 to 700 μM in DNA bases (pH 7). After each addition of copper complex the mixture was allowed to mix for 15 min at room temperature and then an aliquot was frozen at N_2 liquid temperature in order to register the EPR spectrum.

2.5. Cu(II)dipeptide–DNA binding: characterization by CD measurements

The CD spectra of an aqueous solution of Calf Thymus DNA (SIGMA) in presence of increasing amounts of copper complexes were recorded. The dilution scheme for each Cu(II) complex covered a 0.5–5 μM range, while the concentration of the DNA solution (DNA concentration was determined at 260 nm using $\varepsilon_M = 6000$) remained approximately constant at 50 μM in DNA bases (pH 7). As the data were obtained in excess of DNA, the total molar concentration remained nearly constant in all the spectra of each complex. This enabled us to perform a Job analysis of the data at 218 nm in order to estimate a binding constant for DNA–[Cu-peptide] [35].

Table 1
Stoichiometry, code and analytical data for the Cu(II) complexes.

Complex code	Complex stoichiometry	%C		%N		%H	
		Calc.	Exp.	Calc.	Exp.	Calc.	Exp.
1	$[Cu(ala\text{-}ala)]\cdot 2H_2O$	27.93	27.57	10.86	10.41	5.40	4.89
2	[Cu(ala-val)]	38.44	38.40	11.21	11.15	5.60	5.66
3	[Cu(ala-ile)]	40.94	40.85	10.61	10.55	6.06	6.18
4	$[Cu_3(ala\text{-}leu)_3(H_2O)_3(CO_3)]\cdot PF_6\cdot H_2O$	31.40	32.23	7.85	8.17	5.54	6.09
5	$[Cu(ala\text{-}thr)]\cdot {}^1/_2H_2O$	32.22	32.30	10.74	10.66	4.98	5.05
6	$[Cu(ala\text{-}phe)]\cdot {}^1/_2H_2O$	48.35	48.15	9.40	9.30	4.70	4.81
7	$[Cu(ala\text{-}tyr)]\cdot H_2O$	43.40	43.33	8.44	8.54	4.82	4.98
8	$[Cu(phe\text{-}ala)]\cdot {}^1/_2H_2O$	46.93	47.12	9.13	9.19	4.89	4.23
9	[Cu(phe-leu)]	53.01	53.21	8.24	8.52	5.93	6.26
10	[Cu(phe-phe)]	57.82	57.42	7.49	7.57	4.85	4.76
11	[Cu(val-phe)]	51.60	51.96	8.60	8.71	5.57	6.01
12	[Cu(val-gly)]	35.67	35.92	11.88	11.96	5.13	5.58

2.6. Cell proliferation assay

Due to the fact that [Cu(ala-phe)] presented the higher interaction with DNA, it was selected to study the antiproliferative activity. The effect of the copper salt and [Cu(ala-phe)] complex over the cell proliferation was study in a model of osteoblastic tumoral UMR106 cells.

UMR106 rat osteosarcoma-derived cells were grown in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) serum bovine fetal (FBS) at 37 °C, 5% CO_2. When 70–80% confluence was reached, cells were subcultured using 0.1% trypsin 1 mM EDTA in Ca^{2+}–Mg^{2+} free phosphate buffered saline (PBS) (11 mM KH_2PO_4, 26 mM Na_2HPO_4, 115 mM NaCl, pH: 7.4). For experiments, cells were grown in 24-well plates. When cells reached 70% confluence, the monolayers were washed twice with DMEM and were incubated in different conditions according to the experiment.

A mitogenic bioassay was carried out as described by Okajima with some modifications [36].

Briefly, cells were grown in 48-well plates. When cells reached 60% confluence, the monolayers were washed twice with serum-free DMEM and were incubated with different concentrations of $CuSO_4$ or Cu-complex. The monolayers were washed with PBS buffer and fixed with 5% glutaraldehyde/PBS at room temperature for 10 min. Then, they were stained with 0.5% crystal violet/25% methanol for 10 min. After that, the dye solution was discarded and the plate was washed with water and dried. The dye taken up by the cells was extracted using 0.5 ml/well 0.1 M glycine/HCl buffer, pH 3.0/30% methanol and transferred to test tubes. Absorbance was read at 540 nm after a convenient sample dilution [37].

3. Results and discussion

3.1. Determination of the stoichiometry of the interaction Cu(II)dipeptide–DNA

The results of the binding stoichiometry of Cu(II)dipeptide–DNA are shown in Table 2. As shown in Table 2, the stoichiometry of binding depends on the complex, from what it is possible to assume that it is the Cu(II)dipeptide complex, and not only the free copper ion that interacts with DNA. The $[Cu(H_2O)_6]^{2+}$ complex interacts with DNA at a higher extent (>150 (mmol)/DNA(mol base)), probably in a less selective way interacting also with phosphate groups due to the positive charge.

All the studied complexes showed interaction, in a lower, and possibly more selective way than Cu(II) aqua ion. The extent of the interaction depended on the dipeptide ligand. The [Cu(ala-phe)] complex presented the highest binding stoichiometry while the rest of the series [Cu(ala-X)] (X = different aminoacids) showed significantly lower values. Besides, the interaction of [Cu(phe-ala)] was almost four times smaller than that of the [Cu(ala-phe)]. It might be possible that the phenyl ring (without substituents) in the second aminoacid is important in the interaction when the first aminoacid is alanine. Another observation is that in the complexes with a voluminous first aminoacid in the dipeptide ligand the interaction with DNA seems to be disfavoured. This is the case of [Cu(phe-ala)], [Cu(phe-leu)], [Cu(phe-phe)], [Cu(val-phe)] and Cu(-val-gly)].

Based on these results, [Cu(ala-phe)] that presented the best binding stoichiometry and [Cu(ala-val)] and [Cu(val-phe)] that presented medium interaction, were chosen for carrying out EPR and CD experiments with the aim to obtain structural information of the interaction.

Table 2
Binding stoichiometry expressed as Cu(mmol)/DNA(mol base).

Complex number	Complex stoichiometry	Cu(mmol)/ DNA(mol base)
1	$[Cu(ala\text{-}ala)]\cdot 2H_2O$	11
2[a]	[Cu(ala-val)][a]	9
3[a]	[Cu(ala-ile)][a]	14
4	$[Cu_3(ala\text{-}leu)_3(H_2O)_3(CO_3)]\cdot PF_6\cdot H_2O$	20
5	$[Cu(ala\text{-}thr)]\cdot {}^1/_2H_2O$	14
6[a]	[Cu(ala-phe)][a]	42
7	$[Cu(ala\text{-}tyr)]\cdot H_2O$	10
8	$[[Cu(phe\text{-}ala)]\cdot {}^1/_2H_2O$	11
9[a]	[Cu(phe-leu)][a]	13
10[a]	[Cu(phe-phe)][a]	2
11	[Cu(val-phe)]	8
12	[Cu(val-gly)]	10

[a] Previously reported [38].

3.2. Cu(II)dipeptide–DNA binding: EPR measurements

EPR experiments were carried out with solutions of the complexes [Cu(ala-phe)], [Cu(ala-val)], and [Cu(val-phe)]. Moreover, EPR spectra of DNA solution in presence of increasing concentrations of the Cu-dipeptide complexes were also recorded (see Section 2.3). The Fig. 1 shows the EPR spectra of a solution of free [Cu(ala-phe)] and solutions of this complex in presence of DNA at various mmol Cu(ala-phe)/mol bases DNA ratios. Besides, the spectral simulations are included in Fig. 1. Similar results were obtained studying the spectra of [Cu(ala-val)], and [Cu(val-phe)] in presence of DNA.

[Cu(ala-phe)] (Fig. 1A) in frozen solution presented an EPR spectrum characteristic of coupled Cu(II) ions with a single resonance around $g \sim 2$ ($H \sim 310$ mT) [30,39,40] whereas the spectrum of the Cu(ala-phe) complex with DNA at low mmol Cu(ala-phe)/DNA mol bases ratios (Fig. 1B) is the typical spectrum of

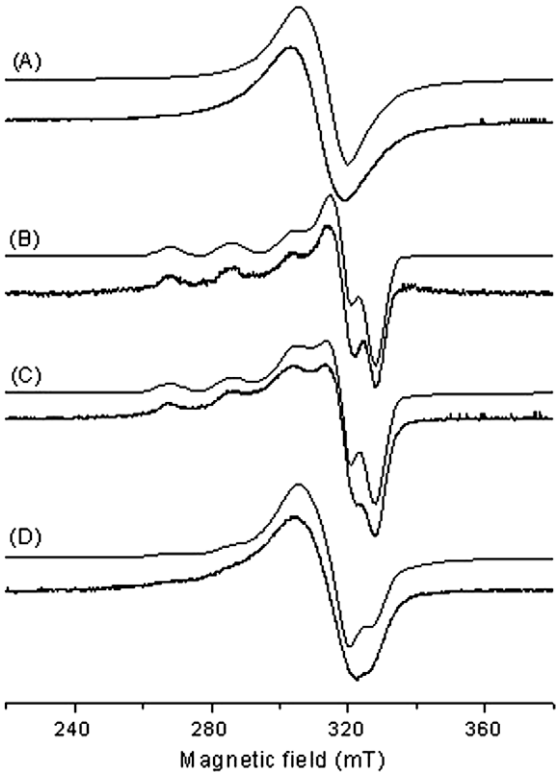

Fig. 1. Experimental (lower trace) and simulated (upper trace) X-band EPR spectra of a frozen solution of: (A) [Cu(ala-phe)] and [Cu(ala-phe)] in presence of DNA at (B) 30, (C) 110, and (D) 500 mmol Cu(ala-phe)/DNA mol bases.

monomeric Cu(II) complexes where the four hyperfine lines arising from the interaction of the $S = 1/2$ electron spin with the $I = 3/2$ copper nucleus are observed. This result showed that, at low Cu-dipeptide per DNA ratios (low copper complex levels), the magnetically coupled structure of the Cu-dipeptide complex is lost due to the coordination of the Cu-dipeptide monomer with the DNA molecule. The g values obtained from simulations of the spectrum at low mmol Cu(ala-phe)/mol bases DNA are: $g_{//} = 2.217$, $g_{\perp} = 2.048$, $A_{//} = 550$ MHz and $A_{\perp} = 35$ MHz.

With the aim to obtain structural information Peisach's proposal was used [19,41]. The magnitude of $A_{//}$ and $g_{//}$ was interpolated in the Peisach graph and the nature of the coordinating atoms in the equatorial plane was assigned. Our $A_{//}$ and $g_{//}$ values are similar to those reported for Cu(II) coordinated through three N atoms and one O atom (N3O).

Fig. 2 shows the Cu(II) environment in solution as presented in our previous work [38].

As shown in Fig. 2, in the free complex Cu(II) is coordinated through two N atoms and two O atoms in the equatorial plane. After the interaction with DNA, the EPR spectra suggest that the copper ion is equatorially coordinated through three N atoms and one O atom. This behaviour would show that one O atom from the copper environment is replaced by one N from a DNA base (inner-sphere binding).

When the Cu-dipeptide per DNA ratios increases (Fig. 1C and D) a spectrum of coupled Cu(II) ions appears superposed to the monomeric signal. The spectra in Figs. 1C and D could only be satisfactorily simulated when two spectral components were included in the fitting protocol using the QPOW program: the spectrum of the polymeric species (Cu(ala-phe) alone) and the spectrum of the monomeric specie (Cu(ala-phe) bonded to DNA), described above.

The existence of two spectral components supports the hypothesis that the DNA is able to bind only a small amount of Cu(dipeptide), and the remaining Cu(dipeptide) exists as free complex in solution (not coordinated to the DNA).

3.3. Cu(II)dipeptide–DNA binding: characterization by CD measurements

Circular dichroism experiments were carried out with [Cu(ala-phe)], [Cu(ala-val)] and [Cu(val-phe)] in (a) copper complex solutions and (b) copper complex solutions in presence of DNA. The aim of these experiments was to confirm the interaction and determine whether the DNA or the Cu(dipeptide) complex experimented structural changes due to the Cu(dipeptide)–DNA interaction.

(a) The spectra of aqueous solutions of the Cu(dipeptide) complexes were registered at increasing concentrations. As an example, the inset of Fig. 3 shows the spectra of Cu(ala-phe) complex. As expected, the ellipticity at 218 nm (maximum wavelength) linearly increases with the concentration, as shown Fig. 3, with a molar ellipticity of 765 M^{-1} cm^{-1}. Similar results were obtained for the complex [Cu(val-phe)] (molar ellipticity at 218 nm: 1190 M^{-1} cm^{-1}) while complex [Cu(ala-val)] does not show any significant ellipticity at 218 nm (15 M^{-1} cm^{-1}).

(b) The spectra of a DNA solution in presence of increasing concentrations of Cu(dipeptide) complexes were registered. Fig. 4 shows the spectra obtained for the [Cu(ala-phe)]–DNA system. Similar results were obtained with the [Cu(val-phe)] complex.

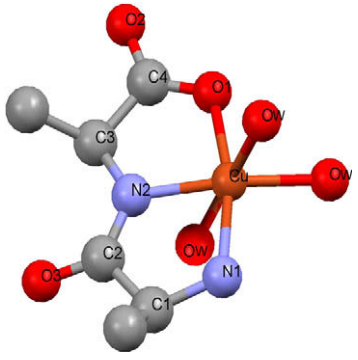

Fig. 2. Scheme of the Cu(II) environment of the Cu-dipeptide complexes in solution.

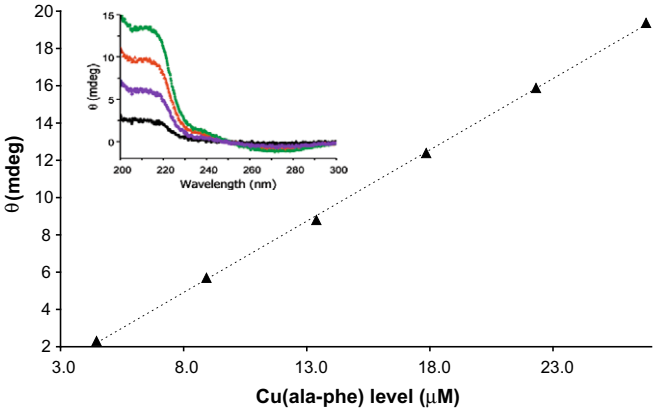

Fig. 3. Ellipticity of [Cu(ala-phe)] at 218 nm *vs.* concentration. Inset: CD spectra of [Cu(ala-phe)].

Fig. 4 presents the ellipticity of the system at 218 nm *versus* [Cu(ala-phe)] concentration.

It can be observed that the ellipticity at 218 nm does not increase linearly with the [Cu(ala-phe)] concentration, as would be expected if there were no interaction between the [Cu(ala-phe)] complex and the DNA. At low Cu(dipeptide) concentration (that is, low mmol [Cu(ala-phe)]/mol bases DNA ratio) the ellipticity almost does not increase with the increment of the Cu(dipeptide) concentration. As the ratio is increased, the ellipticity increases, showing a molar ellipticity similar to that of the free Cu(dipeptide) complex. This may be explained considering that at low mmol

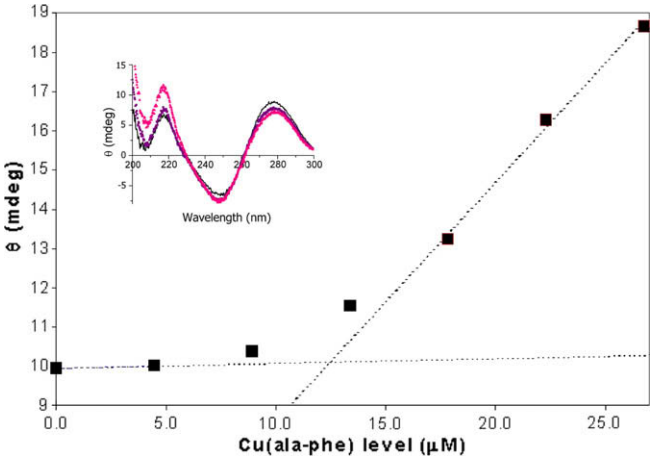

Fig. 4. Ellipticity of the [Cu(ala-phe)]-DNA system at 218 nm *vs.* [Cu(ala-phe)] concentration. Inset: selected CD spectra of DNA and DNA in presence of [Cu(ala-phe)] (with mmol [Cu(ala-phe)]/mol bases DNA ratios from 5 to 55).

[Cu(ala-phe)]/mol bases DNA ratio all the Cu(dipeptide) complex added is bonded to the DNA. The coordination with DNA induces structural changes with the consequently quenching of the complex ellipticity. As the mmol Cu(dipeptide)/mol bases DNA ratio is increased, the DNA is not more able to bind the Cu(dipeptide) complex, therefore the system behaves as if there were no interaction between the excess of Cu(dipeptide) complex and the DNA. In the Cu(dipeptide) concentration range of 8.9–13.4 μM (where we observed the slope change), the Cu(mmol)/DNA(mol base) ratio is 18–32 mmol Cu/DNA (mol base). Although the binding stoichiometry presented in Section 3.1 (42 Cu(mmol)/DNA(mol base)) is not included in this range it is still comparable and supports the proposal of the DNA saturation.

In the case of the [Cu(ala-val)] complex the results are different from the previous ones, as shown in Fig. 5.

As the complex has a very low molar ellipticity at 218 nm, the ellipticity of a solution of DNA with increasing concentrations of [Cu(ala-val)] should slightly increase at this wavelength. On the contrary, a decrease in the signal at 218 nm was observed. This result shows that the molar ellipticity of the DNA decreases in presence of [Cu(ala-val)], as previously reported for metallic complexes which bind to DNA [41]. This behaviour suggests a structural change in the DNA. It is possible that in the case of the complex [Cu(ala-phe)] there is also a decrease in the molar ellipticity of the DNA, which is masked by the decrease in the ellipticity of the complex bounded to the DNA.

In relation to the binding constant, the obtained values for [Cu(ala-phe)] and [Cu(val-phe)] were 1.2×10^4 and $5.9 \times 10^3\ M^{-1}$, respectively. These results are of the same order that the corresponding ones for complexes with biological activity reported in the bibliography [42,43].

The spectra of [Cu(ala-val)] does not show enough differences to estimate a binding constant.

3.4. Cell proliferation assay

The obtained results are shown in Fig. 6. As can be seen from the figure, at concentrations lower than 50 μM, the complex do not exert any inhibition on cell proliferation since no differences *versus* basal results could be observed. At higher doses, the complexes and the copper salt caused different inhibition of cell growth in a dose response manner. [Cu(ala-phe)] showed the greatest cell proliferation inhibition at the highest concentration (500 μM) where 45% cell survival could be detected when the osteosarcoma cells were incubated with this complex.

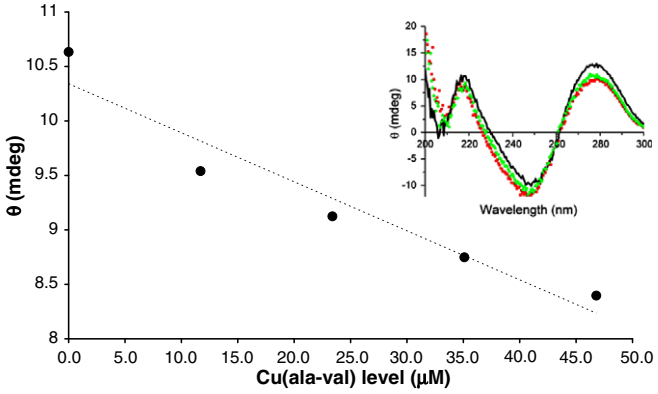

Fig. 5. Ellipticity of the [Cu(ala-val)]/DNA system at 218 nm *vs.* [Cu(ala-val)] concentration. Inset: selected CD spectra of DNA and DNA in presence of [Cu(ala-val)] (with mmol Cu(ala-val)/mol bases DNA ratios from 20 to 100).

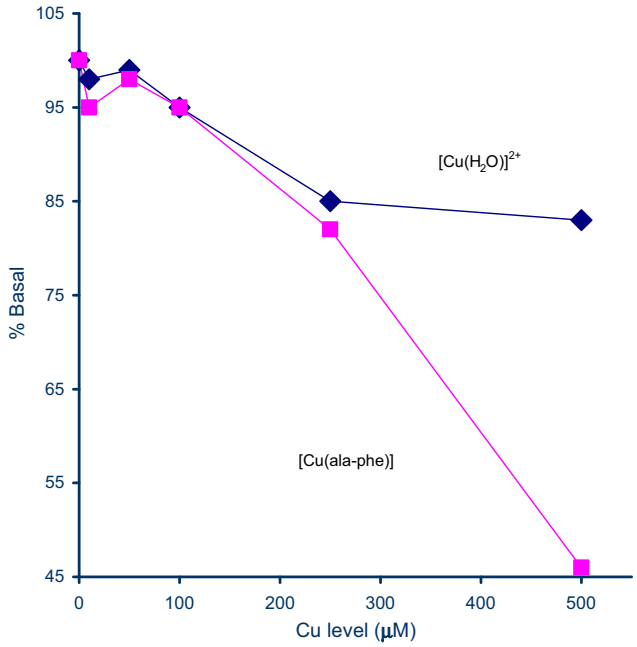

Fig. 6. Cell proliferation experiments with UMR106 after the treatment with [Cu(ala-phe)] and copper salt.

4. Conclusions

This work provided information about the interaction of a series of Cu(dipeptide) complexes with Calf Thymus DNA using different methodologies. All the studied complexes showed interaction, in a lower, and possibly more selective way than Cu(II) aqua ion. The extent of the interaction depended on the dipeptide ligand.

Interestingly, the EPR and CD studies suggested that the DNA binds a fixed amount of the Cu(dipetide) complex (which is coordinated to the bases of the DNA) up to saturation. Moreover, the systems start to show saturation at a mmol Cu(dipeptide)/mol bases DNA ratio close to that obtained in the binding stoichiometry values.

The inhibitory effect caused by [Cu(ala-phe)] on cell proliferation in the cultures of UMR106 osteoblasts is encouraging for future studies on the bioactivity of this complex with other tumoral cell lines since it may have potential application as antitumoral agent.

Acknowledgements

The authors thank CSIC, PEDECIBA Química (Uruguayan organisations), AMSUD-Pasteur and CNPq for financial support. SBE and DAB are members of the Carrera del Investigador, CONICET, Argentina and they are indebted with CONICET and UNLP for the financial support for the biological studies.

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