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Original

Cytotoxic activity of copper(ii), nickel(ii) and platinum(ii) thiosemicarbazone derivatives: Interaction with DNA and the H2A histone peptide / Bisceglie, F.; Orsoni, N.; Pioli, M.; Bonati, B.; Tarasconi, P.; Rivetti, C.; Amidani, D.; Montalbano, S.; Buschini, A.; Pelosi, G.. - In: METALLOMICS. - ISSN 1756-5901. - 11:10(2019), pp. 1729-1742. [10.1039/c9mt00166b]

Availability:

This version is available at: 11381/2867239 since: 2019-11-28T16:48:40Z

Publisher:

Royal Society of Chemistry

Published

DOI:10.1039/c9mt00166b

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note finali coverpage

(Article begins on next page)

20 July 2024

ARTICLE

When the metal makes the difference: an insight into the genomic targets of copper(II), nickel(II), and platinum(II) thiosemicarbazone analogues

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Franco Bisceglie^{a,b,c}, Nicolò Orsoni^a, Marianna Pioli^a, Beatrice Bonati^a, Pieralberto Tarasconi^{a,c}, Claudio Rivetti^a, Davide Amidani^a, Serena Montalbano^a, Annamaria Buschini^{†a,b,c}, Giorgio Pelosi^{†a,b,c}

Metal complexes still represent a promising pharmacological tool in the development of new anticancer drugs. Bis(citronellalthiosemicarbazone)nickel(II) is a metal compound extremely effective against leukemic and NCS cancer cell lines. Preliminary experiments performed with this compound and with its Cu(II) and Pt(II) analogues, have evidenced alterations, detectable by Comet assay, in the DNA of treated U937 cells. In addition, [Cu(tcitr)₂] and [Pt(tcitr)₂] were also able to induce gene mutations and to produce frameshift events. To get further insights into the mechanism of action of these metal compounds, we have carried out a multidisciplinary study to investigate whether their biological activity can be ascribed to a direct interaction with DNA or with chromatin. DNA interaction was investigated by means of CD and UV-Vis spectroscopic techniques and by AFM, whereas chromatin interaction was studied by analyzing the effect of the compounds on the structure of a peptide that mimicks the potential metal binding site in the “C-Tail” region of histone H2A by means of NMR, CD, UV-Vis and MS. The entity of the effects induced by the metal compounds on the peptide follows the order [Ni(tcitr)₂] > [Pt(tcitr)₂] >> [Cu(tcitr)₂]. From the AFM data, a remarkable DNA compaction was observed in the presence of [Pt(tcitr)₂], while [Ni(tcitr)₂] causes the formation of large interlaced DNA aggregates.

Introduction

In the latest years, research of new anticancer therapies has been oriented towards the use of monoclonal antibodies, however, the development of metal complexes still represent a promising pharmacological tool for the treatment of cancer. For instance, cis-diamminedichloridoplatinum(II) (cisplatin) and its analogues are still widely and effectively used anticancer drugs, regardless of their elevated level of toxicity and the emergence of intrinsic or acquired resistance in some cancer types. In a search aimed to overcome these drawbacks, many efforts have been made to develop new metal based anticancer drugs that do not present such side effects¹.

The activity of the cis-platin derivatives seems to be due mainly to their direct interaction with DNA. It is therefore of interest to investigate coordination compounds containing different metal ions and the same ligands², active against the proliferation of cancerous

cells, in order to understand the role of the metal in their biological activity³.

Novel cisplatin analogues are synthesized with the aim to exploit the different modes of covalent and noncovalent interactions that the drug can make with DNA. Namely, major and minor grooves binding, intercalation, cross-linking, and DNA strand-scission⁴. An alternative strategy for the development of anticancer therapies focuses on targeting different DNA morphologies including the stabilization of higher order DNA structures such as the G-quadruplex DNA⁵⁻⁷. Even if metal ions and complexes often exert an effect on DNA structure or metabolism, it is still not clear if this arises from direct or indirect actions⁸⁻¹².

Following our interest in the biological activity of thiosemicarbazone metal complexes¹³⁻¹⁸, we recently made some interesting observations regarding the effect of long chain aliphatic thiosemicarbazone metal complexes, especially the citronellal thiosemicarbazone derivatives¹⁸⁻²⁶, on histiocytic lymphoma cell line U937. In particular, we found that a Ni(II) metal complex, bis(citronellalthiosemicarbazone)nickel(II), was extremely effective against a few tumour cell lines^{21, 26}.

In this paper we describe the synthesis and the characterization of a new Pt(II) thiosemicarbazone derivative, together with an in-depth study aimed to investigate its biological activity and its interaction with DNA or with a peptide that mimicks

^a Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parco Area delle Scienze 11/a, 43124 Parma, Italy

^b COMT - Centre for Molecular and Translational Oncology, Department of Chemical and Life Sciences and Environmental Sustainability, University of Parma, Parma, Italy

^c CIRCMSB - Inter-University Consortium for Research on Chemistry of Metals in Biological Systems, Parma Unit, Parco Area delle Scienze 17/A, 43124 Parma, Italy

† A.B. and G.P. are both corresponding authors.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

the potential metal binding site in the “C-Tail” region of Histone H2A. The study is corroborated by the comparison of results obtained with Ni(II) and Cu(II) thiosemicarbazone analogues^{20, 24}. In addition, we also report an *in vitro* study aimed to detect genotoxic and mutagenic activities of these compounds.

Materials and Methods

Materials

Thiosemicarbazide and platinum (II) chloride were purchased from Sigma-Aldrich; nickel(II) acetate tetrahydrated and copper(II) chloride dihydrated were obtained from Carlo Erba; (S)-citronellal was obtained from Alfa Aesar. All solvents for the syntheses were obtained from Sigma-Aldrich. TESHK was purchased from Genecust (Luxembourg) and used without further purification.

Instrumentation

IR spectra (4000-700 cm^{-1}) were recorded with a Nicolet 5PC FT-IR ATR spectrophotometer (Thermo Fisher Scientific Inc.). The relative intensities of reported FT IR signals are defined as s = strong, br = broad, m = medium, and w = weak. The $^1\text{H-NMR}$, $^1\text{H-}^1\text{H COSY}$ and $^1\text{H-}^1\text{H TOCSY}$ spectra were recorded on a Bruker Avance 400 spectrometer (Bruker Corporation). Chemical shifts (δ) are reported in parts per million (ppm) referenced to that of residual solvent protons. The splitting of proton resonances in the reported $^1\text{H NMR}$ spectra are defined as s = singlet, br s = broad singlet, d = doublet, t = triplet, and m = multiplet. UV-visible spectra were recorded with a Perkin Elmer Lambda 25 spectrophotometer equipped with a Peltier device, using matched quartz cells of 1 cm path length. ESI mass spectrometry fragmentation patterns were obtained, using samples dissolved in methanol, with a Finnegan 1020 6c mass spectrometer (Thermo Finnegan Co.) equipped with a quadrupole mass selector MATSSQ 710.

DNA binding studies

CD titrations. Circular dichroism (CD) spectra were recorded at 25 °C on a Jasco J-715 spectropolarimeter with buffer compensation; each spectrum is the average of three independent measurements. 1 cm path-length quartz cuvettes were used. Disodium salt of calf thymus DNA (CT DNA) (Sigma) was used as received and stored at 4 °C. Solutions of DNA in 10 mM of PBS (pH=7.4) 137 mM NaCl, 2.7 mM KCl gave a ratio of UV absorbance at 260 and 280 nm, A_{260}/A_{280} , of 1.9, indicating that the DNA was sufficiently free of protein. The concentration of stock solutions of DNA, expressed in moles of nucleotide phosphate [NP] was determined by UV absorbance at 260 nm. The extinction coefficient, ϵ_{260} , was taken as $6600 \text{ M}^{-1} \text{ cm}^{-1}$ ²⁷. Stock solutions were stored at 4 °C and used after no more than 4 days. Metal complexes were dissolved in DMSO. The final concentration of DMSO in the buffered solution never exceeds 5%.

The effect of the metal complexes on the DNA secondary structure was studied by keeping the concentration of CT-DNA at $5 \cdot 10^{-5} \text{ M}$ [bp] while varying the concentration of the complex in the above mentioned buffer solution ($r = [\text{complex}]/[\text{DNA}] = 0, 0.1, 0.2, 0.4, 0.6$). All CD spectra were recorded in the wavelength range 220 to 320 nm.

Atomic Force Microscopy. AFM experiments were conducted using a 965 bp linear DNA template obtained by PCR from plasmid pNEB193 and suitable primers under standard reaction conditions. The DNA fragment was gel purified by electroelution using an Elutrap apparatus (Schleicher & Schuell, Keene NH), phenol/chloroform extracted, ethanol precipitated and resuspended in 5 mM Tris-HCl pH 7.4. The DNA concentration was determined by absorbance at 260 nm. A solution of 40 nM DNA was incubated for one hour at room temperature in the presence of increasing concentrations, 1 μM , 10 μM , 100 μM and 1 mM, of the thiosemicarbazone compounds. The reaction was diluted 20-times in deposition buffer (4 mM HEPES pH 7.4, 10 mM NaCl, 2 mM MgCl_2) and immediately deposited onto freshly-cleaved. After 2 minutes of incubation the mica surface was rinsed with milliQ water and dried with nitrogen. AFM imaging was performed with the tapping mode in air using a Nanoscope IIIA microscope (Digital Instruments). Images were collected with a scan size of 2 μm at a scan rate of 2.5 lines per second. DNA contour length measurements were performed as previously described²⁸ using the following contour length estimator: $L = (0.963n_e + 1.362n_o) \times S/W$, where “ n_e ” and “ n_o ” are the number of even and odd chain codes respectively, S is the image scan size, W is the image width in pixels.

Histone binding studies

$^1\text{H NMR}$. To investigate the possible interaction between $[\text{Ni}(\text{tcitr})_2]$ and the peptide modeling the potential metal binding site in the “C-Tail” Region of histone H2A, a series of mono- and bi-dimensional $^1\text{H-NMR}$ spectra were recorded in DMSO- d_6 at 7 mM concentration with a preliminary incubation of 24h in solution at 37.5°C. $^1\text{H-NMR}$, $^1\text{H-}^1\text{H COSY}$ and $^1\text{H-}^1\text{H TOCSY}$ experiments **have** been used for the total characterization of TESHK and subsequently the same experiments were performed in presence of the complex (complex: peptide ratio = 1:1).

Biological assays

Cytotoxicity of metal complexes. The antiproliferative activity of $[\text{Ni}(\text{tcitr})_2]$ in histiocytic lymphoma cell line (U937) after 24h treatment was studied by Buschini et al.²². *In vitro* effects of $[\text{Pt}(\text{tcitr})_2]$ and $[\text{Cu}(\text{tcitr})_2]$ toward cell line U937 were examined using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison WI, USA). Cells were seeded at a density of 5×10^3 cells/well into 96-well plates. After 24h, cells were treated with metal complexes at various concentrations (0.5-1-5-10-50-100 μM) for 24 and 72h. Dimethyl sulfoxide (DMSO) was used as the experimental control. After treatment time, MTS reagent was

added to each well and absorbance was measured at 485 nm by Tecan SpectraFluor Plus plate reader.

The GI_{50} values were calculated as the drug concentration necessary to reduce the absorbance to 50% of the untreated control (DMSO).

Cells U937, a human histiocytic lymphoma cell line, were obtained from American Type Culture Collection (ATCC) and were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2 .

Alkaline Comet Assay. To identify DNA damage, we performed Alkaline Comet Assay in order to detect single and double DNA strand breaks and alkali-labile sites. The genotoxicity of $[Ni(tcitr)_2]$ was previously investigated²².

In this work, we used the Comet assay to determine the genotoxicity of copper $[Cu(tcitr)_2]$ and platinum $[Pt(tcitr)_2]$ complexes. As negative control was used the highest concentration of DMSO, as positive control was used ethyl methanesulfonate (EMS) (2 mM).

U937 cells were seeded at a concentration of 1×10^5 cell/mL in 1 mL wells and, after 24h, were treated with $[Cu(tcitr)_2]$ and $[Pt(tcitr)_2]$ (1-5-10-20-50 μ M) for 1 and 24h at 37 °C.

After treatment period at 37 °C, determination of cell numbers and viabilities was performed with the trypan blue exclusion method: cells were resuspended in complete medium and Trypan blue was added. 100 cells for each concentration were counted manually using a hemocytometer. Only the treatments that had a viability higher than 70% have been processed in the Comet Assay.

Cells were transferred onto degreased microscope slides previously dipped in 1% normal melting agarose (NMA) for the first layer. The agarose was allowed to set for 20 min at 4 °C before addition of a final layer of low melting agarose (LMA). Cells were lysed with a buffer containing 2.5 M NaCl, 10 mM Na_2EDTA , 10 mM Tris-HCl, 1% Triton X-100 and 10% DMSO, pH 10 at 4 °C overnight. The electrophoretic migration was performed under alkaline buffer condition (1 mM Na_2EDTA , 300 mM NaOH, 0 °C) at pH > 13 with 20 min of unwinding time and 20 min of electrophoresis (0.78 V/cm; 300 mA). Slides were then washed with a neutralization solution (0.4 M Tris-HCl, pH 7.5) and fixed in absolute ethanol for 5 min.

DNA was stained with 75 μ L ethidium bromide (10 μ g/mL) before the examination at 400 X magnification under a Leica DMLS fluorescence microscope (excitation filter BP 515–560 nm, barrier filter LP 580 nm), using an automatic image analysis system (Comet Assay IV – Perceptive Instruments Ltd).

DNA damage was quantified measuring the DNA percentage in the tail region (TI%). For each sample, coded and evaluated blind, 50 cells were analyzed.

The “IBM SPSS Statistics 24” software was used to analyze statistical differences between samples. The mean values from the repeated experiments were used in a one-way analysis of variance (ANOVA). If significant F-values ($p < 0.05$) were obtained, Student's t-test (Bonferroni's version) was performed.

Salmonella typhimurium reversion test (Ames test). Ames test was performed by the plate incorporation procedure, with and without rat liver S9 mix (exogenous metabolic activation system), as outlined by OECD guidelines (No. 471). Mutagenicity was determined with two different Salmonella typhimurium strains: TA98 and TA100 detecting frame-shifts and base substitutions respectively.

We choose five different concentrations of $[Pt(tcitr)_2]$ and $[Cu(tcitr)_2]$ (2.5-5-10-50-100 μ g per plate). Dimethyl sulfoxide (DMSO) was used as negative control; 2-nitrofluorene [20 μ g per plate] and sodium azide [15 μ g per plate] were used as positive control for TA98 and TA100 strains, respectively, in the assay without S9 mix; 2-aminoanthracene [10 μ g per plate] was used as positive control for both strains in the assay with S9 mix.

The colonies were counted and the data are reported as mean and standard deviation of revertant plate. In accordance with the guidelines, the results of the Ames test were considered positive if two consecutive dose levels or the highest non-toxic dose level produced a response at least twice that of the solvent control (DMSO), and at least two of these consecutive doses showed a dose-response relationship.

Results and Discussion

Synthetic procedures

Compounds $[Ni(tcitr)_2]$ and $[Cu(tcitr)_2]$ were synthesised using improved reaction conditions based on the procedures reported in²⁰ and properly characterized. Compound $[Pt(tcitr)_2]$ was synthesised anew and characterized through IR, HNMR, MS. In addition, single crystals of size suitable for an X-ray study were obtained by recrystallization in DMSO and the compound was also structurally characterized.

Crystallography

X-ray structure of compound $[Pt(tcitr)_2]$. The molecular structure of the platinum derivative, as determined by single-crystal X-ray crystallography, is reported in Fig. 1. The Pt(II) coordination geometry is square planar, as already observed for analogous copper

and nickel^{20, 22} compounds: the four donor atoms derive from two anionic bidentate ligands, each of which uses the iminic nitrogen and the thiolate sulfur as chelating atoms. The coordination plane is formed by two 5-membered (N,S) chelate rings. The platinum(II) ion lies on the average plane formed by the four donor atoms, but its geometry is slightly distorted from the ideal square planar being the bite angles N-Pt-S 85.5 (2)°. The Pt-S bond distances, 2.290(4) and 2.293(4) Å, and the Pt-N(iminic) distances, 2.039(12) and 2.010(12) Å, are similar to those observed in analogous systems²⁹. Thiosemicarbazones normally present a thione-thiol tautomerism and the deprotonation, concomitant to the coordination to the metal centre, brings to the stabilization of the thiolate form. In our case this is confirmed by the length of the C-S distances of 1.781(16) and 1.772(2) Å that suggests a marked single-bond thiol character, since it is much longer than the C=S bond length observed in free thiosemicarbazones (the average value of 511 non deprotonated structures found in the CSD²⁹ is C=S = 1.69(2) Å). The N(hydrazine)-C(thiol) bond distances of 1.31(2) Å and 1.29(2) Å, accordingly, is markedly shorter than the average 1.40(1) Å of the free thiosemicarbazones of the same set, suggesting a double bond-like character.

Fig 1

Fig. 1 ORTEP representation of the asymmetric unit of compound [Pt(tcitr)₂]₂DMSO

The structure is characterized by the presence of two DMSO molecules that play an important role in the packing by bridging two complex units according to the scheme reported in Fig S1 (Electronic Supplementary Information). This pattern creates ribbons of molecules which develop in the *a* axis direction of the unit cell. These ribbons present on the sides hydrophobic protrusions which insert in the corresponding groove of the adjacent ones forming a bidimensional structure running parallel to the (7 0 -14) plane family. The geometry is therefore comparable to those of the analogous copper and nickel compounds and this ensures that the biological effects observed are not assignable to differences in the shape of the compounds.

DNA Interaction Studies

CD studies. To verify whether the binding of the metal complexes induces any significant conformational change of the DNA double helix, CD spectra of CT-DNA were recorded at increasing complex/CT-DNA concentration ratios. The observed CD spectrum of natural calf-thymus DNA consists of a positive band at 280 nm due to base stacking and of a negative band at 250 nm due to helicity, features characteristic of the right-handed B form DNA. It is known that at wavelengths above 230 nm, the CD spectrum of B form DNA displays of a longwave positive and a shortwave negative band of nearly equal magnitude with the intersection point at the UV absorption maximum (λ max, 260 nm)³⁰. In the case of [Cu(tcitr)₂], the CD spectrum of CT-DNA exhibits a monotonous decrease of both band

intensities²⁴. This effect can be associated to an induced change of DNA conformation. The presence of an isodichroic point suggests that the metal complex binds DNA through one mode of interaction. In the case of [Ni(tcitr)₂], the CD spectrum of CT-DNA exhibits a monotonous decrease of the band at ca. 245 nm together with a redshift of ca. 3 nm²⁶. In addition, the positive band at ca. 285 nm undergoes a dramatic change, decreasing as the concentration of the compound increases. We hypothesize that since simple groove binding and electrostatic interactions with small molecules show little or no perturbation on the base stacking and helicity bands,²⁶ it is clear that [Ni(tcitr)₂] exerts a strong effect on the nucleic acid. We believe that this dichroic spectral behaviour is consistent with a possible B to C form transition of DNA,²⁶ where the positive band basically disappears. The absence of isodichroic points suggests that more than one DNA-complex species might be present at the same time. It should be noted that the observed CD spectral changes are also consistent with a condensed Ψ -DNA form²⁶. Studying the interaction of [Pt(tcitr)₂] with DNA by CD was more difficult due to solubility problems. In fact for *r* = 0.1 no significant DNA perturbations were observed, while for *r* > 0.25 the turbidity of the resultant solution did not allow a reliable spectra interpretation. For *r* = 0.25, the observed decrease of intensity of both positive and negative bands is compatible with a B → C form transition of DNA. (Fig. 2).

Fig. 2

Fig. 2. Circular dichroism spectrum of CT-DNA without and with [Pt(tcitr)₂] at *r* = 0.25 (*r* = [complex]/[DNA]).

In general, from the spectroscopic pattern, we can exclude an intercalation mechanism of binding of all metal complexes to DNA. Overall, the CD spectra suggest that [Cu(tcitr)₂] and [Pt(tcitr)₂] can induce a B to C form transition of DNA while [Ni(tcitr)₂] seems to favour the formation of condensed Ψ -DNA, even though the B to C transition cannot be excluded.

Atomic Force Microscopy. Thiosemicarbazones of divalent cations are particularly interesting molecules because they can bind DNA as already demonstrated for cisplatin. To get further insights into their interaction with DNA, we have employed the Atomic Force Microscopy (AFM) technique to visualize the effect of these metal compounds on DNA. Initially, linear DNA fragments 965 bp long were imaged in the absence of the compounds. Under these conditions, the DNA appears as a homogeneous filament with thickness of 20 ± 2 nm and height of 1 ± 0.3 nm (Fig. 3a). Along the entire DNA path, no particular feature such as knots or kinks were observed. Besides visual inspection, we also accurately measured the contour length of a large number of single DNA fragments obtaining a mean value of 308.8 ± 11.9 nm. This results in a rise/bp of 0.32 nm, in agreement

with literature data for B-form DNA deposited and imaged under similar conditions (data not shown)³¹.

Fig. 3

Fig. 3. Closed-up views of 965 bp DNA molecules deposited onto mica and imaged by AFM in the absence and in the presence of the metal compounds. The image profile taken along the direction indicated by white arrows is shown on top of each panel and spans a distance of 100 nm. (a) DNA fragments in the absence of compounds. (b) DNA fragments in the presence of 100 μ M [Cu(tcitr)₂]. (c) DNA fragments in the presence of 100 μ M [Pt(tcitr)₂]. (d) DNA fragments in the presence of 100 μ M [Ni(tcitr)₂]. Knot-like structures and hairpins are shown as white globular features along the DNA double helix. Scale bar 100 nm.

Subsequently, the 965 bp DNA fragment was treated for one hour with increasing concentrations (1, 10, 100 μ M and 1 mM) of the metal compounds under scrutiny, deposited onto mica and imaged in air by AFM. As shown in Fig. 3b-d, the treatment caused the formation of knot-like structures and hairpins located along the DNA path. These structural alterations of the DNA were observed at all concentrations, independently of the compound, however, the number of knots was proportional to the concentration of the compound, particularly for [Pt(tcitr)₂] and [Cu(tcitr)₂] (Table 1). Knots and hairpins have a thickness of about 2–4 nm and are preferentially found near the ends of the DNA fragments. Contour length measurements of these DNA fragments revealed a DNA shortening, relative to the untreated DNA, up to 50 nm (Table 1). The morphology of knots and hairpins and the shortening of the DNA suggest local overlapping or condensation of the double helix induced by the interaction with the metal compounds. Furthermore, a shortening of about 10–15 nm was also observed for DNA fragments that do not display knots or hairpins along the path (Table 1). Such phenomenon was particularly evident in the case of DNA fragments incubated with the platinum derivative, while for the copper or nickel derivatives no significant shortening of the DNA was observed (Table 1). The measured rise/bp of the DNA was of 0.31 and 0.30 nm in the presence of 1–10 μ M and 100 μ M [Pt(tcitr)₂], respectively. Treatment of the DNA fragments with a compound concentration of 1 mM gave different results. As shown in Fig. 4a,b, treatment with 1 mM [Cu(tcitr)₂] resulted in images very similar to those observed at lower concentration, i.e. DNA filaments decorated with some knots. Conversely, treatment with 1 mM [Pt(tcitr)₂] resulted in DNA molecules partially or completely condensed into a globular structure (Fig. 4c,e). In some cases, one end of the DNA was seen exiting from the blob. These DNA conformations were never observed at lower concentration or in the presence of other metal compounds. Treatment with 1 mM [Ni(tcitr)₂] gave rise to a completely different scenario in which the DNA fragments sticks together forming large interlaced DNA aggregates (Fig. 4d,f).

Fig. 4

Fig. 4. AFM images of 965 bp DNA fragments in the absence and in the presence of 1 mM metal compounds. (a) DNA fragments deposited onto mica in the absence of compounds. (b) DNA fragments in the presence of 1 mM [Cu(tcitr)₂]. (c) DNA fragments in the presence of 1 mM [Pt(tcitr)₂]. (d) DNA fragments in the presence of 1 mM [Ni(tcitr)₂]. (e) Closed-up views of [Pt(tcitr)₂] induced DNA condensation. (f) Closed-up views of [Ni(tcitr)₂] induced DNA aggregation. (a-d) Scale bar 500 nm. (e,f) Scale bar 100 nm.

It should be noted that the described effects of the metal compounds on the DNA structure and conformation have never been observed when the DNA was treated either with the metal cations Cu⁺⁺, Ni⁺⁺ and Pt⁺⁺ nor with the metal-free Htcitr compound (Table 1).

The DNA contour length reduction observed for DNA fragments treated with the metal compounds suggests a compound-DNA interactions different from a classical intercalation mechanism which would result in a general stretching of the DNA double helix. The shortening observed for DNA molecules with knots and hairpins is certainly due to the local compaction of the DNA within the knot or hairpin. However, the contour length reduction observed for DNA molecules treated with [Pt(tcitr)₂] but without visible knots or hairpins, suggests that this compound can also induce a DNA structural modification that leads to a more compact helical structure with a reduced rise/bp. Interestingly, the 0.30–0.31 nm rise/bp measured for these DNA molecules is compatible with a B \rightarrow C form transition of the DNA²⁶. Moreover, the different morphological and structural effects observed for DNA molecules treated with the different compounds might suggest different action modes of the compound-DNA interaction, depending on the coordinating metal ion. This hypothesis is further supported by the different DNA topologies observed in the presence of the metal compounds at a concentration of 1 mM. Under these conditions, [Cu(tcitr)₂] has no visible effect on the DNA topology with respect to lower concentrations, while this is not the case for the platinum and nickel derivatives. [Pt(tcitr)₂] causes a remarkable DNA compaction, thus favouring intrahelical interactions, while [Ni(tcitr)₂] causes the formation of large interlaced DNA aggregates, thus favouring interhelical interactions.

TABLE 1

Table 1: DNA contour length of DNA molecules imaged by AFM in the absence and in the presence of different concentration of the metal compounds. The values reported correspond to the average \pm standard deviation. In parenthesis is the number of DNA molecules analysed. The right-hand column reports the percentage of molecules with knots or hairpins with respect to the total number of DNA molecules in the images. The last three rows report the DNA contour length of DNA molecules in the presence of the metal cations and of the metal-free compound Htcitr.

Interactions with histone tail TESHHK

Complex [Ni(tcitr)₂]. Chemical shifts of ¹H of free and bound hexapeptide are presented in Table S1. The presence of [Ni(tcitr)₂] causes a remarkable alteration of TESHHK spectrum, principally in the 7.0 - 8.5 ppm zone (Fig. 5 a, green points).

Fig. 5

Fig. 5 High field (above) and low field (below) ¹H-NMR spectra of (a) histone tail, (b) histone tail + [Ni(tcitr)₂], (c) [Ni(tcitr)₂] and (d) Htcitr.

These signals are assigned to the amide groups of the backbone together with side chains of lysine and histidines. The addition of the complex to the peptide brings to a total disappearance of these signals, except for the lysine side chain (Fig. 5 b, green point). Another particularity of the resultant spectrum is the presence of a set of signals not associable to the peptide or to the complex but linked to the ligand, Htcitr (Fig. 5 b, red points, Fig. 5 b, orange point). This information suggests that [Ni(tcitr)₂] is able to lose a ligand and uses the metal centre to interact with the hexapeptide, affecting the amide groups of the backbone and the nitrogens of the imidazole rings. The coordination geometry of the metal centre in the adduct among [Ni(tcitr)₂] and the hexapeptide is not the same if the amide groups of the backbone or the side chains of the histidines are involved. In the first case, the plausible structure of the adduct is the square planar geometry, with a conservation of the diamagnetism (Scheme 1).

Scheme 1

Scheme 1 Proposed adduct between [Ni(tcitr)₂] and TESHHK, in which the metal centre interacts with the amide groups of the backbone.

It is suggested by the small variations of chemical shift of the α protons, that are very close to the amide groups. On the other hand, in the case of complexation through the imidazole rings of the histidines there is a probable change of geometry of the metal centre to an octahedral environment, with the formation of a paramagnetic adduct. This could explain the fact that C2 and C5 signals of imidazole rings disappear with the addition of [Ni(tcitr)₂] (Scheme 2).

Scheme 2

Scheme 2 Proposed adduct between [Ni(tcitr)₂] and TESHHK, in which the metal centre interacts with the imidazole rings. "X" stands for "solvent".

A further investigation of the interaction between [Ni(tcitr)₂] and the hexapeptide was carried out with UV-visible and CD spectroscopies. Both experiments were performed in methanol solutions at 50 μ M concentration. The CD and UV/visible spectra are presented in Fig. 6 a and Fig. 6 b, while the spectroscopic parameters are presented in Table 2. Also in this case, the study of the interaction between the

species is performed with a preliminary incubation of the peptide with the metal complex for 24h at 37.5°C.

Fig. 6

Fig. 6 (a) UV-visible spectra and (b) circular dichroism (CD) spectra of peptide (black line), [Ni(tcitr)₂] (red line), Htcitr (blue line), peptide + [Ni(tcitr)₂] (green line).

Table 2

Table 2. Spectroscopic parameters of peptide, [Ni(tcitr)₂], Htcitr and the adduct among peptide and [Ni(tcitr)₂].

These electronic spectra confirmed the interaction between the peptide and [Ni(tcitr)₂] complex. The UV-visible spectrum of the species incubated is remarkable different if compared to the spectra of the single species while in CD spectrum is possible to notice the presence of a band at 226 nm assignable to Htcitr. Also CD spectrum suggests that a ligand is displaced during the interaction among Ni complex and the peptide.

Complex [Pt(tcitr)₂]. The study of the interaction between [Pt(tcitr)₂] and TESHHK has been inquired in the same way as for [Ni(tcitr)₂]. Mono- and bi-dimensional ¹H-NMR were recorded in the same conditions, i. e. in DMSO-D₆ at 7 mM concentration with a preliminary incubation of 24h in solution at 37.5°C. Also in this case ¹H-NMR, ¹H-¹H COSY and ¹H-¹H TOCSY have been used for comparing the spectra of free and bound peptide. The chemical shifts are reported in Table S1 (ESI).

The addition of the platinum complex induces an alteration in TESHHK spectrum (Fig. 7 b) that differs from [Ni(tcitr)₂] case for two reasons.

Fig. 7

Fig. 7 ¹H-NMR spectra at low field of (a) peptide, (b) peptide + [Pt(tcitr)₂], (c) [Pt(tcitr)₂] and (d) Htcitr.

First, the signals of the amide groups of the backbone and the side chains of the histidines are still present; second, the interaction between [Pt(tcitr)₂] and the hexapeptide does not set a ligand free, Htcitr. To confirm the selective activity of the [Pt(tcitr)₂] respect to Htcitr we have recorded mono- and bi-dimensional ¹H-NMR with a 1:2 ratio peptide:Htcitr, in the same conditions of previous experiments. The resultant spectrum (Fig. 8 b) proves that the only presence of Htcitr does not induce any alteration of TESHHK signals.

The data we recorded suggest that the platinum complex interacts with TESHHK without a direct involvement of the metal center.

Fig. 8

Fig. 8 $^1\text{H-NMR}$ spectra at low field of (a) peptide, (b) peptide + Htcitr, (c) Htcitr.

Also in this case we obtained UV-visible and CD spectra for a further investigation of the interaction between $[\text{Pt}(\text{tcitr})_2]$ and the hexapeptide. The experiment was performed in methanol solutions at $50\ \mu\text{M}$ concentration. The CD and UV/visible spectra are presented in Fig. 9 a and b, while the spectroscopic parameters are presented in Table 2. We have performed the study of the interaction between the species with a preliminary incubation of 24h in solution at 37.5°C .

Fig. 9

Fig. 9 (a) UV-visible spectra and (b) circular dichroism (CD) spectra of peptide (black line), $[\text{Pt}(\text{tcitr})_2]$ (red line), Htcitr (blue line), peptide + $[\text{Pt}(\text{tcitr})_2]$ (green line).

TABLE 3

Table 3. Spectroscopic parameters of peptide, $[\text{Pt}(\text{tcitr})_2]$, Htcitr and the adduct among peptide and $[\text{Pt}(\text{tcitr})_2]$.

As in the case of $[\text{Ni}(\text{tcitr})_2]$, the UV-visible spectrum of the species incubated is remarkable different if compared to spectra of the single species. Instead, the CD spectrum of the species incubated is consistent with NMR data; in fact the interaction among the peptide and $[\text{Pt}(\text{tcitr})_2]$ produces a spectrum (Fig. 9 b, green line) remarkably different from the spectra of the single species without any band amenable to Htcitr.

Complex $[\text{Cu}(\text{tcitr})_2]$. $[\text{Cu}(\text{tcitr})_2]$ is a paramagnetic complex and the study of the interaction with TESHK therefore can not be performed by NMR. As in previous cases, we studied the interactions of the peptide with the metal complex with other spectroscopic techniques. We have performed CD and UV experiments in methanol solutions at $50\ \mu\text{M}$ concentration. The CD and UV/visible spectra are presented in Fig. 10 a and b, while the spectroscopic parameters are presented in Table 4. We have performed the study of the interaction between the species with a preliminary incubation of 24h in solution at 37.5°C . In this case, the coordinative behaviour of the $[\text{Cu}(\text{tcitr})_2]$ complex towards TESHK is extremely different if compared with the previous complexes. In UV-visible spectra the interaction among copper complex and the peptide (Fig. 10 a, green line) increases only the intensity of the $[\text{Cu}(\text{tcitr})_2]$ band, without wave length displacement. In CD spectra the interaction among copper complex and the peptide (Fig. 10 b, green line) brings to the formation of a spectrum comparable with the copper complex one (Fig. 10 b, red line). With this information we can conclude that $[\text{Cu}(\text{tcitr})_2]$ complex interacts only in a negligible way with the peptide TESHK.

Fig. 10

Fig. 10 (a) UV-visible spectra and (b) circular dichroism (CD) spectra of peptide (black line), $[\text{Cu}(\text{tcitr})_2]$ (red line), Htcitr (blue line), peptide + $[\text{Cu}(\text{tcitr})_2]$ (green line).

Table 4

Table 4 Spectroscopic parameters of peptide, $[\text{Cu}(\text{tcitr})_2]$, Htcitr and the adduct among peptide and $[\text{Cu}(\text{tcitr})_2]$.

Biological assays

Effects of the metal complexes on cell proliferation. Different studies indicate that the antiproliferative potential of many thiosemicarbazones is increased with metal ion coordination. Buschini et al.²¹ showed the antiproliferative activity of $[\text{Ni}(\text{tcitr})_2]$ against cell line U937 after 24h of treatment. Nickel complex exhibited a strong cytotoxic activity with $\text{GI}_{50}=10\ \mu\text{M}$ after 24h.

We determined dose-response curves and GI_{50} value (50% Growth Inhibition) was determined. After 24h treatment, the most antiproliferative compounds resulted platinum ($\text{GI}_{50}=7.0\ \mu\text{M}$) and nickel complexes ($\text{GI}_{50}=10.0\ \mu\text{M}$); after 72h treatment, nickel complex induced the strongest cytotoxic effect ($\text{GI}_{50} = 8.0\ \mu\text{M}$) (Table S2).

Genotoxic effects of the metal complexes. To understand if metal complexes were able to induce alterations on the DNA molecule, we performed Alkaline Comet Assay on U937cells. This test could identify different kinds of DNA damage, such as single and double strand breaks, incomplete excision repair sites and alkali-labile sites. Previous experimental data showed that $[\text{Ni}(\text{tcitr})_2]$ is able to induce a significant increase in DNA migration after 1h treatment²¹ In the Alkaline Comet Assay, exposure of cells to metal complexes resulted in a considerable DNA damage, as revealed by a high DNA migration. $[\text{Cu}(\text{tcitr})_2]$ was genotoxic at the highest concentrations ($20.0\text{-}50.0\ \mu\text{M}$) after 1h treatment, while at $10.0\ \mu\text{M}$ after 24h (Fig. 11A). As regards $[\text{Pt}(\text{tcitr})_2]$, the lowest effective doses were $5.0\ \mu\text{M}$ and $1.0\ \mu\text{M}$, respectively for 1h and 24h treatment (Fig. 11B). Meanwhile, the induction of cell death after treatment with copper and platinum complexes was quantified by trypan blue exclusion method. $[\text{Cu}(\text{tcitr})_2]$ and $[\text{Pt}(\text{tcitr})_2]$ showed an increase in trypan blue-positive cells (dead cells) at the highest concentrations (20.0 and $50.0\ \mu\text{M}$) mainly after 24h treatment (Fig. 11).

Fig. 11

Fig. 11. DNA damage detected by the Comet assay ($\text{pH} > 13$) in cells U937 treated with increasing dose of $[\text{Cu}(\text{tcitr})_2]$ (A) and $[\text{Pt}(\text{tcitr})_2]$ (B) for 1h and 24h. DNA damage is expressed as tail intensity (TI%). TOX: viability $< 70\%$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Effects of the metal complexes on Salmonella typhimurium strains

In order to understand if the DNA damage caused by $[\text{Ni}(\text{tcitr})_2]$ treatment at the subtoxic concentration ($1.0 \mu\text{M}$) could give rise to gene or chromosomal mutations, in a previous study Ames test and micronucleus assay were performed: $[\text{Ni}(\text{tcitr})_2]$ did not induce gene mutation or micronuclei induction²². In accordance with the guidelines (OECD, No 471), the results of the Ames test were considered positive if mutagenicity ratio (MR) >2 . Both the complexes induced a promutagen activity in the test with TA98 + S9. In the other tests, we observed a low toxicity. Results are expressed as revertants/plate (mean \pm standard deviation) and mutagenicity ratio (MR) in *S. typhimurium* TA98 and TA100 strains treated with $[\text{Cu}(\text{tcitr})_2]$ (Table S3) and $[\text{Pt}(\text{tcitr})_2]$ (Table S4), with and without S9 mix.

Conclusions

In this work, the interaction of bis(citronellalthiosemicarbazone) copper(II), nickel(II), and platinum(II) analogues with DNA and with a peptide mimicking the H2A histone tail, have been studied through a combination of CD, UV-Vis, NMR, MS and AFM. The three thiosemicarbazone complexes display very similar molecular structures as observed by a comparison of the X-ray results. The three complexes have significantly different behaviours towards both DNA and the C-tail of histone H2A. The platinum derivative, $[\text{Pt}(\text{tcitr})_2]$, induces a DNA structural modification compatible with a B \rightarrow C form transition but presents non covalent interaction with the histone fragment. A different behaviour is observed with $[\text{Ni}(\text{tcitr})_2]$ which shows the covalent interaction with the peptide and contemporarily causes the formation of large interlaced DNA aggregates. The copper complex is the less active on both sides since it shows no interactions with DNA and very weak interactions with the histone tail. All three compounds result genotoxic on the basis of the Comet assay but, whereas $[\text{Pt}(\text{tcitr})_2]$ and $[\text{Cu}(\text{tcitr})_2]$ show mutagenic activity and induce frameshift events, the nickel derivative $[\text{Ni}(\text{tcitr})_2]$ presents no such effects. This suggests that, notwithstanding the similarity of the three molecules under scrutiny, the metal must play a crucial role in determining the events that take place in cells and the biological outcomes cannot be ascribed solely to the interactions we have observed in our model systems.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work has been carried out in the frame of the activities of the 'COMP-HUB' initiative, funded by the 'Department of Excellence' Project of the Italian Ministry for Education, University and Research (MIUR). The authors wish also to thank for the facilities the Centro Interdipartimentale Misura (CIM) of the University of Parma.

Supplementary Materials

Supplementary material can be found ...

Author Contributions

Conceptualization, A.B., F.B., G.P., and C.R.; investigation, D.A., B.B., S.M., N.O. and M.P.; writing—original draft preparation, A.B., F.B., G.P., and C.R.; writing—review and editing, A.B., F.B., G.P., and C.R.; funding acquisition, A.B., F.B., C.R., G.P. and P.T.

Funding

This research was funded by the University of Parma (FL).

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