Synthesis and mass spectroscopy kinetics of a novel ternary copper(II) complex with cytotoxic activity against cancer cells

M. Barceló-Oliver a, Á. García-Raso a, Á. Terrón a,*, E. Molins b, M.J. Prieto c, V. Moreno c, J. Martínez d, V. Lladó e, I. López e, A. Gutiérrez d, P.V. Escrivá e,*

a Departament de Química, Grup de Química Bioinorgànica i Bioorgànica, IUNICS, Universitat de les Illes Balears, Campus UIB, Carretera Valldemossa km. 7.5, Palma de Mallorca E-07122, Spain
b Institut de Ciència dels Materials de Barcelona (CSIC), Campus de la Universitat Autònoma, Cerdanyola E-08193, Spain
c Departments of Inorganic Chemistry and Microbiology, Faculty of Chemistry, Universitat de Barcelona, Barcelona E-08028, Spain
d Servei d’Hematologia, Hospital Universitari de Son Dureta, Palma de Mallorca, Spain
e Department of Biology, Laboratory of Molecular and Cellular Biomedicine, IUNICS, (CSIC Associated Unit), Universitat de les Illes Balears, Campus UIB, Carretera Valldemossa km. 7.5, Palma de Mallorca E-07122, Spain

Received 6 June 2006; received in revised form 11 December 2006; accepted 14 December 2006
Available online 3 January 2007

Abstract

The X-ray structure of the [Cu(I-hip)(phen)2]⁺ Æ (I-hip/C0) Æ 7H2O complex (1) (where I-hipH is referred to 0-iodohippuric acid and phen is 1,10-phenanthroline) and its binary synthetic intermediate [Cu(I-hip)2(H2O)3] Æ (H2O)2 (2) have been solved and characterized by different techniques. This ternary [Cu(I-hip)(phen)2]⁺ Æ (I-hip/C0) Æ 7H2O complex generates the copper(I) complex [Cu(phen)2]⁺ in aqueous solution without the addition of any external reductant, possibly by an intramolecular red–ox process in the presence of oxygen; the ESI-HRMS spectra (electrospray ionization-high resolution mass spectroscopy) detect these species and 24 h after the solution, [Cu(phen)2]⁺ is the main product. The complex 1 is capable of cleaving DNA. To evaluate the biological properties, we carried out: cell culture, cell proliferation assays, cell cycle analysis, and electrophoresis (SDS–PAGE) and immunoblotting. Complex 1 induced apoptosis of A549 cells at low nanomolar and induced marked decreases of cancer cells at concentrations that did not change adipocyte survival. These data indicate that the parent complex is a potential anticancer drug.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Apoptosis; Anticancer drug; Cell cycle; Copper; Lung cancer; X-ray crystallography; Iodohippuric acid

1. Introduction

Cancer is currently the second cause of death in industrialized countries, accounting for about a quarter of all deaths. The increasing incidence and mortality of cancer and the decline in the mortality of other pathologies means that it is likely to become the first cause of death within the remainder of this century. Moreover, about one half of all cancers that are diagnosed result in the death of the patient, such that the development of new anticancer therapies is one of the fundamental goals in medicinal chemistry.

The chemical nuclease activity of the cuprous complex of 1,10-phenanthroline (abbreviated as phen) was demonstrated by Sigman, et al. [1] and reviewed recently [2]. The [Cu(phen)2]⁺ complex oxidatively degrades DNA and RNA by attacking the sugar group [2]. Complexes of 1,10-phenanthroline (phen) have been shown to exhibit interesting clinical activities including antitumoural, antimycobacterial, antifungal and antimicrobial activities [3–7]. Moreover, these compounds can be used as DNA intercalating agents [8] and metal synthetic nucleases [2,9–12]. On the other hand, the medical applications of copper–carboxylato complexes as anti-inflammatory drugs...
are also well known [13]. Some phenanthroline–amino acid or peptide complexes effectively cleave DNA, although under physiological conditions this activity requires, in general, the presence of: (i) thiols and hydrogen peroxide; (ii) reductants and air; or (iii) irradiation with UV or visible light [11,12]. These complexes exhibit significant differences in their nuclease activity depending on the nature of the amino acid or peptide moiety. For instance, the complex [Cu(gly-L-trp)(phen)]·2H2O, dissolved in ethanol, yields the complex [Cu(phen)2]2+ in 30 min detected by ESI-HRMS (electrospray ionization–high resolution mass spectrometry), in the absence of an external agent. The reduction of Cu(II) in this case could be explained by an active role of the peptidic moiety coming along with molecular oxygen [12]. Recently, specific apoptosis in liver carcinoma cell line induced by copper-1,10-phenanthroline was observed in an effort to elucidate the mechanistic links between reactive oxygen species and cell death [14]. Other authors like Meunier [15], knowing that the best nucleases activities of 1,10-phenanthroline-copper complexes have been observed for a chelate/metal stoichiometry of 2/1, has designed a new ligand “clip-phen” with two phenanthrolines linked via their C2-carbons by a short flexible arm in order to improve the association constant of Cu(I)-phenanthroline ligand by chelate effect that is for the second 1,10-phenanthroline only 105.5 M−1 [16]. The nuclease activity of the new Cu(II)-“clip-phen” complex is greater than those for the Cu(II)–phen system [15].

In the present study, we describe the synthesis, structural characterization and biological activity of a novel ternary copper(II) coordination complex with phenanthroline and an amino acid derivative [Cu(I-hip)(phen)2]2+·(I-hip−)·7H2O (1) (where I-hip is the abbreviation of ortho-iodophenylurate), which presents nucleases and anticancer activities acting in the absence of external agents for its activity in an oxygen atmosphere. We selected o-iodophenylhydrazide (I-hipH), a compound used for the detection of the adrenal gland in mammography analysis, as an ancillary ligand and as a lead molecule for further drug design [17,18]. The N–H peptide bond of this amino acid derivative is not prone to interact with metal ions by coordination in hippocrural derivatives [19–21]. In addition, the peptide bond of this N-substituted guanine derivative is perpendicular to the aromatic ring group because of steric hindrance of ortho-iodophenyl moiety [19]. The impossibility of any conjugation of the peptide group should facilitate the mero-stabilized radicals generation onto α-carbon, helped by its substituents (COO− and NH) which stabilize radicals for this ligand [22,23]. The presence of this secondary ligand may enhance the formation of active species such as [Cu(phen)2]2+. On the other hand, the role of the copper transport protein Ctr1p in the anticancer activity of cisplatin has been recently highlighted [24]. In the present study, we also defined the X-ray structure of the binary intermediate [Cu(I-hip)2(H2O)3]·2H2O (2) in order to assess carboxylate coordination. Finally, we determined that its anticancer potential is due to induction of apoptosis in cancer cells.

2. Experimental section

2.1. Synthesis of the coordination complexes

Complex 1 was prepared by refluxing basic copper carbonate [CuCO3·Cu(OH)2] (1 mmol) and I-hipH (3 mmol) in water (100 ml) for 2 h and then subsequently adding solid phen (3 mmol). This synthesis is similar to that reported previously to prepare acyclovir ternary compounds [3]. Before addition of the phen ligand, we left the solution for a few days so that the binary intermediate 2 could accumulate [3]. In both cases, crystals were obtained after 2–3 days from the parental solution.

2.2. Analysis and physical measurements

Elemental microanalyses were carried out using a Carlo Erba model 1108 microanalyser [complex 1, Found: C, 43.76; H, 3.48; N, 7.31. Calc. for C13H30CuI2N4O6·7H2O: C, 43.56; H, 3.83; N, 7.26. complex 2, Found: C, 28.28; H, 3.14; N, 3.66. Calc. for Cu13CuH8I2N4O6·2H2O: C, 28.38; H, 3.18; N, 3.68]. IR spectra in the solid state (KBr pellets) were measured on a Brucker IFS 66 spectrometer. The reagents were used as received from Sigma (o-iodophenylacetic acid), Merck (1,10-phenanthroline) and Aldrich (metallic salts).

High Resolution Mass Spectroscopy with Electro Spray Ionization (ESI-HRMS) of 1 was focused on an AUTO-SPEC 3000 with PEG-600 and PEG-900 as standards for exact mass determination. The kinetics of ESI-HRMS from the initial parenteral solution [complex 1 (2 mg) was dissolved in water (10 ml)] was obtained by recording the spectra at different times, obtaining different aliquots from the same water solution (pH 6.6). The amount of the different species in each spectrum vs. the time between the solution and recording spectrum is represented. The ESI-HRMS study of 1 confirmed the presence of the cationic molecular complex [Cu(I-hip)2(phen)3]2+ [Exact mass, 727.0142; Calc., 727.0142] and also the formation of [Cu(I-hip)(phen)]+ [Exact mass, 546.9457; Calc., 546.9454], [Cu(phen)2]+ [Exact mass, 423.0676; Calc., 423.0671], [Cu(COOCH2OH)(phen)(H+)]+ [Exact mass, 319.0268; Calc., 319.0244], [Cu(phen)2]+ [Exact mass, 211.5348; Calc., 211.5330] and [Cu(solvent)(phen)]+. The same results are obtained dissolving the sample in ethanol at the same concentration and the exact mass corresponding to [Cu(solvent)(phen)]+ was characterized only in ethanol, resulting as [Cu(EtOH)(phen)]+ [Exact mass, 289.0413; Calc., 289.0402]. Three of this spectra recorded at different solution times (0, 2.5 and 24 h after solution) are included in Supporting Information.

Electrochemical data were obtained by cyclic voltammetry under nitrogen at 20 °C using acetonitrile (HPLC grade) as solvent and tetrabutylammonium hexafluorophosphate (0.1 M) as supporting electrolyte. The measured potentials were referred to a Ag/AgNO3 (0.1 M in acetonitrile) electrode separated by a medium porosity fritted disc. A
platinum wire auxiliary electrode was used in conjunction with a platinum disc TACUSSEL-EDI rotatory electrode (3.14 mm²). Cyclic voltammograms of 10⁻³ M solutions, previously deoxygenated with gaseous N₂, of the samples in acetonitrile were run and the measured potentials were then referred to ferrocene, which was used as internal standard to facilitate the interpretation of the results. The experiments were carried out at scan rate v = 0.1 V · s⁻¹, from −1 to +1 V and following a reduction forward step and a subsequent oxidation reverse step to complete the cycle.

Cyclic voltammetry was performed with the compound I and the monomeric [Cu(phen)₂(acetato-O,O')][NO₃]·2H₂O which X-ray structure confirms that the metal ion is coordinated to the same donor atoms that the parent complex. This model compound was synthesized according to the literature procedures [25]. It is quite important to write down that the solution of compound I became colourless immediately after solution in deoxygenated acetonitrile, whereas, [Cu(phen)₂(acetato-O,O')][NO₃]·2H₂O did not lose colour after solution.

2.3. Crystallographic studies

Suitable crystals of 1 and 2 were selected for single X-ray crystal diffraction experiments and mounted at the tips of glass fibres on an Enraf–Nonius CAD4 diffractometer producing graphite monochromated Mo Kα radiation. After a random search of 25 reflections, the indexation procedure gave the cell parameters. The crystal data of both compounds are summarised in Table 1. Data were collected in the ω-2θ scan mode. Absorption correction was performed following the Y-scansemi-empirical (2) and the empirical DIFABS (1) methods. The structural resolution procedure was made using the WinGX package [26]. Resolution of structure factor phases were performed with SHELXS86 (2) and SIR2002 [27] (1), and the full-matrix refinement with SHELXL97 [28]. Non-H atoms were refined anisotropically, and H-atoms were introduced into the calculated positions and refined riding on their parent atoms. A summary of the refinement parameters can be also seen in Table 1.

### 2.4. Cell culture

A549 human lung adenocarcinoma and 3T3-L1 adipocyte cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained at 37 °C in a fully humidified atmosphere of 5% CO₂ in air. Monolayer cultures were maintained in exponential growth using glutamine (2 mM)-supplemented RPMI-1640 medium, containing 10% foetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericine B (0.25 μg/ml). Tissue culture medium and supplements were all purchased from Sigma.

### Table 1

<table>
<thead>
<tr>
<th>Identification code</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal data and structure refinement for both complexes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Empirical formula</td>
<td>C₈H₆Cu₂N₄O₁₃</td>
<td>C₁₀H₇Cu₁N₂O₁₂</td>
</tr>
<tr>
<td>Formula weight</td>
<td>1158.17</td>
<td>779.75</td>
</tr>
<tr>
<td>Temperature</td>
<td>293(2) K</td>
<td>294(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.71073 Å</td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Triclinic</td>
<td>Monoclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P – 1</td>
<td>P 21/a</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td>a = 12.845(8) Å</td>
<td>a = 16.547(6) Å</td>
</tr>
<tr>
<td></td>
<td>b = 14.598(7) Å</td>
<td>b = 8.102(10) Å</td>
</tr>
<tr>
<td></td>
<td>c = 15.253(11) Å</td>
<td>c = 20.724(5) Å</td>
</tr>
<tr>
<td></td>
<td>α = 83.31(3)°</td>
<td>α = 90°</td>
</tr>
<tr>
<td></td>
<td>β = 67.06(4)°</td>
<td>β = 97.94(3)°</td>
</tr>
<tr>
<td></td>
<td>γ = 68.12(4)°</td>
<td>γ = 90°</td>
</tr>
<tr>
<td>Volume</td>
<td>2289(2) Å³</td>
<td>2691.9(12) Å³</td>
</tr>
<tr>
<td>Z</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Density (calculated)</td>
<td>1.680 Mg/m³</td>
<td>1.924 Mg/m³</td>
</tr>
<tr>
<td>Absorption coefficient</td>
<td>1.895 mm⁻¹</td>
<td>3.164 mm⁻¹</td>
</tr>
<tr>
<td>R(000)</td>
<td>1154</td>
<td>1516</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.36 × 0.35 × 0.14 mm³</td>
<td>0.75 × 0.45 × 0.29 mm³</td>
</tr>
<tr>
<td>Theta range for data collection</td>
<td>1.55–24.99°</td>
<td>2.03–24.99°</td>
</tr>
<tr>
<td>Index ranges</td>
<td>−14 ≤ h ≤ 15, −15 ≤ k ≤ 17, 0 ≤ l ≤ 18</td>
<td>−19 ≤ h ≤ 19, 0 ≤ k ≤ 9, 0 ≤ l ≤ 24</td>
</tr>
<tr>
<td>Reflections collected</td>
<td>8354</td>
<td>4866</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>8021 [R(int) = 0.0393]</td>
<td>4724 [R(int) = 0.0271]</td>
</tr>
<tr>
<td>Completeness</td>
<td>99.5%</td>
<td>99.8%</td>
</tr>
<tr>
<td>Max. and min. transmission</td>
<td>0.7773 and 0.5487</td>
<td>0.4606 and 0.2000</td>
</tr>
<tr>
<td>Data/restraints/parameters</td>
<td>8021/0/577</td>
<td>4724/0/307</td>
</tr>
<tr>
<td>Goodness-of-fit on F²</td>
<td>1.062</td>
<td>1.01</td>
</tr>
<tr>
<td>Final R indices [I &gt; 2σ(I)]</td>
<td>R₁ = 0.0604, wR₂ = 0.1560</td>
<td>R₁ = 0.0446, wR₂ = 0.1170</td>
</tr>
<tr>
<td>R Indices (all data)</td>
<td>R₁ = 0.1104, wR₂ = 0.1806</td>
<td>R₁ = 0.0549, wR₂ = 0.1247</td>
</tr>
<tr>
<td>Extinction coefficient</td>
<td>−</td>
<td>0.0084(4)</td>
</tr>
<tr>
<td>Largest diff. peak and hole</td>
<td>0.747 and −1.447 e Å⁻³</td>
<td>1.294 and −1.389 e Å⁻³</td>
</tr>
</tbody>
</table>
2.5. Cell proliferation assays

A549 cells were plated at a density of 1.5 × 10⁵ cells in 24-well culture dishes with 0.5 ml of culture medium (see above) per well. After incubating the cells overnight to permit attachment, they were exposed to complex I (250 nM-2.5 μM) for 0–120 h. At the end of this period, unattached cells were recovered by centrifugation of the medium for 5 min at 600 g (room temperature). These cells were then combined with the cells that had been harvested after detaching them from the flask with 0.05% trypsin in sterile phosphate buffered saline (PBS buffer) [PBS: 137 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, at pH 7.3] for 5 min at 37 °C. The cells were then centrifuged at 12,000 g for 3 min. For immunoblotting, 30 μl of total protein from the cell lysates (5–60 μg from control samples for standard curves) was submitted to SDS–PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were incubated with blocking solution (PBS buffer containing 5% non-fat dried milk, 0.5% bovine serum albumin and 0.1% Tween 20) for 1 h at room temperature. The membranes were then incubated overnight at 4 °C in fresh blocking solution containing the specific primary antiseraum, polyclonal anti-poly ADP-ribose polymerase (anti-PARP, diluted 1:1000, Cell Signaling). Subsequently, the membrane was incubated with anti-rabbit IgG horseradish peroxidase-labeled antiseraum (diluted 1:2000 in blocking solution) for 1 h at room temperature. The immunoreactive protein bands were detected using the enhanced chemiluminescence (ECL) western blotting system detection system (Amersham).

2.8. Preparation of adduct DNA–metal complex

Fifteen ng of pBR322 DNA were incubated in an appropriate volume with the required concentration corresponding to the molar ratio ri = 0.5. Cu complexes (I) were dissolved in HEPES buffer (4 mM HEPES pH 7.4, 5 mM KCl and 2 mM MgCl₂). The two different solutions as well as Milli-Q water were passed through 0.2 nm filters (Scheicher & Schuell, Germany) and centrifuged at 4000 g for 30 min. The samples were then centrifuged at 12,000 g for 3 min. The culture was incubated overnight to permit attachment, they were exposed to complex I (250 nM-2.5 μM) for 0–120 h. At the end of this period, unattached cells were recovered by centrifugation of the medium for 5 min at 600 g (room temperature) and resuspended in PBS. Cellular RNA was digested with RNase A (100 μg/ml) in PBS. Finally, cells were stained with trypan blue exclusion method (0.2% trypan blue in PBS buffer). For comparison of the differential effects of complex I, tumour (A549) and non-tumour (3T3-L1) cells were incubated as indicated above in the absence or presence of this complex (75 nM) for 24 h.

2.6. Cell cycle analysis

Analysis of the cell cycle was performed by flow cytometry on cells treated in the presence or absence (control) of complex I for 24 h. For this purpose, the cultured cells were washed twice with PBS, detached with trypsin and fixed with 100% methanol for 2 h at 4 °C. The cells were then centrifuged for 5 min at 600 g (room temperature) and resuspended in PBS. Cellular RNA was digested with RNase A (100 μg/ml) in PBS. Finally, cells were stained with 30 min with ethidium bromide (100 μg/ml) and then analyzed on a Beckman Coulter Epics XL flow cytometer. Cell populations in each phase of cell cycle (G₀/G₁, S, G₂/M and the apoptotic sub-G₁) were determined based on their DNA content as described previously [29].

2.7. Electrophoresis (SDS–PAGE) and immunoblotting

Cells were incubated in 6-well culture plates containing 2 ml of culture medium and exposed to complex I as indicated above. When control cells reached ~70% confluence, they were washed twice with PBS and harvested with a rubber policeman in 300 μl of 10 mM Tris·HCl buffer, pH 7.4, containing 50 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 1% SDS, 5 mM iodoacetamide, 1 mM PMSF (phenylmethylsulfonyl fluoride). The cells were then homogenized by ultrason for 10 s at 50 W in a Braun Labsonic U sonicator (20% cycle), and aliquots of 30 μl were removed for total protein quantification. To the remaining homogenate, 30 μl of 10 × electrophoresis loading buffer (120 mM Tris·HCl buffer pH 6.8, 4% SDS, 10% β-mercaptoethanol, 50% glycerol, 0.1% bromophenol blue) was added, and the samples were boiled for 3 min. For immunoblotting, 30 μg of total protein from the cell lysates (5–60 μg from control samples for standard curves) was submitted to SDS–PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were incubated with blocking solution (PBS buffer containing 5% non-fat dried milk, 0.5% bovine serum albumin and 0.1% Tween 20) for 1 h at room temperature. The membranes were then incubated overnight at 4 °C in fresh blocking solution containing the specific primary antiseraum, polyclonal anti-poly ADP-ribose polymerase (anti-PARP, diluted 1:1000, Cell Signaling). Subsequently, the membrane was incubated with anti-rabbit IgG horseradish peroxidase-labeled antiseraum (diluted 1:2000 in blocking solution) for 1 h at room temperature. The immunoreactive protein bands were detected using the enhanced chemiluminescence (ECL) western blotting system detection system (Amersham).

2.9. Atomic force microscopy (AFM) (sample preparation and imaging)

Samples were prepared by placing a drop (3 μl) of DNA solution or DNA–metal complex solution onto peeled green mica (Ashville–Schoonmaker Mica, Newport New, VA, USA). After adsorption for 5 min at room temperature, the samples were rinsed for 10 s in a jet of deionised water of 18 MWcm⁻¹ from a Milli-Q water purification system directed onto the surface with a squeeze bottle. The samples were blow dried with compressed argon over silica gel and then imaged in the AFM.

The samples were imaged in a Nanoscope III Multi-mode AFM (Digital Instrumentals, Santa Barbara, CA, USA) operating in tapping mode in air at a scan rate of 1–3 Hz. The AFM probes were 125 nm-long monocrystal-line silicon cantilever with integrated conical-shaped Si tips (Nanosensors, Germany) with an average resonance frequency f₀ = 330 kHz and spring constant K = 50 N/m. The cantilever is rectangular and the tip radius given by the supplier is 10 nm, a cone angle of 35° and high aspect ratio. The images were obtained at room temperature (T = 23 ± 2 °C) and the relative humidity (RH) was <55%.

2.10. Gel electrophoresis of drug-pBR322 complex

pBR322 DNA aliquots (0.25 μg/ml) were incubated in the presence of the compounds in TE buffer (10 mM
Tris·HCl pH 7.5, 1 mM EDTA) at molar ratio \( r_i = 0.50 \) for electrophoresis study. Incubation was carried out in the dark at 37°C for 24 h; 24-ml aliquots of complex–DNA compound containing 0.5 µg/DNA underwent 1% agarose gel electrophoresis for 4 h at 2 V/cm in 0.5 TBE (45 mM Tris-borate, 1 mM EDTA pH 8.0) buffer. Gel was subsequently stained in the same buffer containing ethidium bromide (1 mg/ml). The gel was photographed with an Image Master VDS, Pharmacia Biotech.

3. Results and discussion

3.1. Crystal structures of \([\text{Cu}(\text{I-hip})(\text{phen})_2]^{+}\) (I-hip)·7H2O (1) and \([\text{Cu}(\text{I-hip})_2(\text{H}_2\text{O})_3]\cdot2\text{H}_2\text{O} (2)\)

The X-ray crystal structure of 1 (Fig. 1) presents a copper(II) ion with a distorted octahedral coordination. This copper(II) ion is linked to the chelating atoms O(1) and O(2) of the carboxylate anion of I-hip [Cu–O distances: O(1), 1.975(6) Å and O(2), 2.82 Å] and to the four N atoms from the two phen molecules [Cu–N dist: N(1'), 1.992(7) Å; N(10'), 2.206(7) Å; N(10''), 2.062(7) Å and N(10''), 1.991(7) Å]. This complex ion maintains a positive charge that is compensated by the presence of another I-hip anion. Seven water molecules per unit complete the structure and form a 3D network by means of hydrogen bonds. This 3D network is formed by alternative layers of positive and negative ions.

In this structure, the stacking interaction between phenanthroline rings is the first intermolecular interaction present [dist., 3.39 Å; dihedral angle, 177.5°], although the main recognition factors are induced by a very interesting C–I···C(ring) interaction, similarly to the C–H···π interactions, [dist., 3.60 Å and 3.55 Å] between I atom of an anionic o-iodohippurate and [C(metha)-C(para)] fragment of a coordinated o-iodohippuric moieties [20]; and completed by a stacking lock that locks the rings of two coordinated I-hip ligands [dist., 3.39 Å; dihedral angle, 179.8°]. We are in the presence of a new C–I···π···π···I–C cluster pattern (Fig. 2).

On the other hand, the binary derivative 2, displays a distorted square pyramidal coordination (Fig. 3). In this structure, the metal ion is linked to the O(1) of two iodohippurate molecules (I-hip) in a trans disposition [dist. Cu–O: 1.943(3) Å and 1.931(4) Å] and three water molecules [dist. Cu–O(water): 1.959(4) Å, 1.991(4) Å and 2.232(4) Å], the last one being in an apical position. The two carboxylates are fully bound in an eclipsed sin disposition, and they are directed towards the opposite side of the apical water. This crystal structure is formed through hydrophobic interactions between aromatic rings and due to intermolecular hydrogen bonds between peptide moieties and water molecules. Finally, iodine–iodine interactions are also implicated [dist. I···I, 3.78 Å; angle C–I···I, 150.4°]. In both 1 and 2, the length of the iodine

![Fig. 1. ORTEP representation, at 50% probability, of the X-ray structure of the ternary complex 1, \([\text{Cu}(\text{I-hip})(\text{phen})_2]\cdot(I-\text{hip}^-)\cdot7\text{H}_2\text{O}\).](image1)

![Fig. 2. X-ray structure of the ternary complex 1, \([\text{Cu}(\text{I-hip})(\text{phen})_2]^+\cdot(I-\text{hip}^-)\cdot7\text{H}_2\text{O}\).](image2)

![Fig. 3. ORTEP representation, at 50% probability, of the X-ray structure of the binary intermediate 2, \([\text{Cu}(\text{I-hip})(\text{H}_2\text{O})_3]\cdot2\text{H}_2\text{O}\).](image3)
interactions is clearly shorter than van der Waals distances determined by Bondi [30–34].

Lastly, in order to understand its biological activities and solution reactivity, is necessary to emphasize that the two compounds present a non-coplanarity between the aryl ring and the peptide bond [dihedral angle, \( \text{ca. } 50^\circ \text{ and } 40^\circ \), respectively in compounds 1 and 2], contrarily to the coplanar disposition in normal hippurate derivatives [19]. This aspect will be focused in the subsequent section.

3.2. High resolution mass spectroscopy of \([\text{Cu(I-hip)(phen)}_2]^+\) (I- hip/C0Æ7H2O (1))

The ESI-HRMS study of 1 confirmed the presence of the cationic molecular complex [Exact mass, 727.0167; Calc., 727.0142] and also the formation of [Cu(phen)2]+ [Exact mass, 423.0676; Calc., 423.0671]. This cationic [Cu(I-hip)(phen)2]+ structure is similar to other previously described Cu(peptide)(phen) [12] which seems to generate the copper(I) complex, known to be an efficient DNA cleavage agent, without the need to add either a reducing agent or any other water soluble agent [1,2]. ESI–HRMS spectra were recorded at different times from the initial parental solution (kinetic study, Fig. 4) and the amount of the peak assigned to the complex [Cu(phen)2]+ known as a cleavage species increased from 0 to 2.5 h. From 2.5 to 24 h, [Cu(phen)2]+ was the major species found in the medium. Indeed, the only cationic complexes that remained at significant concentrations were the [Cu(I-hip)-(phen)2]+ and [Cu(I-hip)(phen)2]+, confirming the chelating capacity of the bound carboxylate. The reduction of Cu(II) with the absence of an external reducing agent for the nuclease activity could be explained by an active role of the peptidic moiety (i.e., [Cu(gly-L-tyr)(phen)]·2H2O [12]) or the iodohippuric (in the present paper) ligands as intramolecular reductants. The rapid and effective production of [Cu(phen)2]+ could be related with the presence of indole [12] or iodo-benzoyl rings that could help the stabilization of radical species. It is known that \( \alpha \)-aminoacids yield \( \alpha \)-carbon stabilized radicals due to synergistic effects of capto-dative substituents [22,23] in the presence of smooth inorganic radicals as Fremy’s salt [23]. Later, a redox reaction between the radical and a near Cu(II) would yield Cu(I) species until the most stable [Cu(phen)2]+ is obtained which has been claimed to be an active species for DNA damage [1,2,12,14,15]. In the case of [Cu(I-hip)-(phen)2]+(I-hip−)·7H2O (1), the presence of two coordinated phenanthrolines enhances possibly the rapid formation of the tetrahedral complex [Cu(phen)2]+. The detection of the peak in different complexes with one or two phenanthroline molecules and different amino acid or peptide coordinated ligands that could act as internal reductants (L-ala-gly, L-val-gly, gly-L-trp) with one phen molecule [12] and o-iodohippuric acid with two phen molecules (this work) precludes in our opinion any doubt of the formation of the [Cu(phen)2]+ as the main product in the four cases with different kinetics depending on the peptidic or amino acid ligand. Thus, in this case it is not necessary to afford external tiols or other reductants [2] because the ternary system generates radicals (Scheme 1).

On the other hand, the peak at ca. 319 could be tentatively assigned as the new complex [Cu(phen)(glycolato)+H]+ which would be formed by means of a nucleophilic substitution on the amide bond. This \( S_N \)
**S_0** reaction is favoured by the special characteristics of the amide group which present an orthogonal situation with the aromatic ring, instead of the general planar disposition in a typical peptidic bond. This characteristic prevent the conjugation of the peptide group and facilitate the mero-stabilized radicals generation onto α-carbon.

Scheme 1. Solution reactivity scheme for complex 1 supported by the ESI-HRMS experiences.
would be favored for the better electrophilic characteristics of this C=O group because the α-iodohippuric ring moiety is orthogonal and the carboxamide group cannot conjugate with this aromatic ring. This peak disappears rapidly while increases the suspected product [Cu(phen)₂]⁺.

Although Scheme 1 is only a hypothesis (possible explanation of experimental results), it is in agreement with the initial detection and subsequent disappearance of ca. 319 peak as a possible reaction intermediate while increases the amount of the copper(I) product.

3.3. Cyclic voltammetry of [Cu(I-hip)(phen)₂]⁺ (I-hip⁻) · 7H₂O (1) and its model compound [Cu(acetato-O,O')(phen)₂]⁺(NO₃⁻) · 2H₂O

The cyclic voltammograms of [Cu(I-hip)(phen)₂]⁺(I-hip⁻) · 7H₂O (1) and [Cu(acetato-O,O')(phen)₂]⁺(NO₃⁻) · 2H₂O are depicted in Fig. 5. Although the voltammogram of the acetate product shows one cathodic peak (assigned to the Cu(II)/Cu(I) reduction) with a directly associated oxidation peak on the reverse scan (assigned to the Cu(I)/Cu(II) oxidation), the corresponding voltammogram of compound 1 does not show any reduction peak, only the anodic peak corresponding to the Cu(I)/Cu(II) oxidation step is observed. The irreversibility of compound 1 come along with the role of the α-iodohippurate ligand in the internal reduction of Cu(II) to Cu(I), in agreement with the ESI–HRMS data that detect, in solution, the peak corresponding to the Cu(I) compound [Cu(phen)₂]⁺.

3.4. Biological tests of [Cu(I-hip)(phen)₂]⁺(I-hip⁻) · 7H₂O (1)

We first studied the interaction of 1 with DNA using plasmidic pBR322 nucleic acid (Fig. 6). The gel electrophoretic mobility of the native pBR322 plasmid is modified by the interaction with our complex; the treated plasmid shows an increase in the presence of open circular (oc) forms and does not clearly show a related effect on the covalently closed circular (ccc) forms. The treated plasmid also shows an important presence of linear forms, confirming the nuclease effect of this metallic complex. This effect on pBR322 was also confirmed by the AFM pictures which show fragments of the three different forms. These experiments show that 1 interacts and cleaves DNA in vitro, an effect that most likely accounts for induction of apoptosis in vivo (see below).

In order to determine the anticancer activity of compound 1, we studied its effects on human non-small lung
adenocarcinoma cell growth (A549). This compound (1) induced a marked and significant decrease in cell viability between 24 and 120 h at all concentrations used (500 nM–10 μM) (Fig. 7). This concentration-dependent cancer-cell growth inhibition was both potent (growth inhibition and apoptosis induction were observed at nanomolar concentrations) and selective for cancer cells at certain concentrations (see below).

We studied the different phases of the cell cycle by flow cytometry to investigate the molecular mechanisms underlying the inhibitory effects of 1 on cell growth (Fig. 8). The presence of complex 1 (24 h, 250 nM–2.5 μM) induced marked decreases in the number of cells in phases G1, S and G2/M and an increase in the number of apoptotic cells, as measured by DNA labeling with ethidium bromide followed by cell cytometry (Fig. 8). The marked increase of the subG0/G1 peak corresponds to cells whose DNA has been cleaved by cellular nucleases activated by the apoptotic machinery. Microscopic analyses of cells treated with 1, further showed the decreases in the number of cells and the induction of morphological changes, with the typical fragmentation of apoptotic cells (Fig. 9). In a separate series of experiments, the effect of complex 1 on a non-tumour cell line was studied. For this purpose, we investigated the effects of 1 (24 h, 75 nM) on human lung adenocarcinoma (A549) and adipocyte (3T3-L1) cells (Fig. 9). Although complex 1 induced death of cancer cells, non-cancer cells were not affected by the compound at this concentration. These results indicate that the compound is a potent cytotoxic, which induces cell death at nanomolar concentrations, thus showing similar or even higher potency than other anticancer drugs [35]. Treatment of human cancers with cytotoxic chemotherapy agents is a difficult task. In

![Fig. 7. Viability (0–120 h) of A549 cells in the presence and absence of complex 1. This complex induced a time- and dose-dependent decrease in viability, measured by the number of live cells. Results are expressed as mean ± sem of five independent experiments.](image)

![Fig. 8. Effects of complex 1 on the distribution of A549 cells in cell cycle populations. The cell cycle phase was determined by the cellular DNA content measured by flow cytometry of ethidium bromide stained cells. G1, S and G2/M denote the corresponding phases of the cell cycle. Sub-G1 events correspond to cells or cell fragments with lower DNA content and are indicative of the apoptotic death of the cells. The upper panels show representative flow-cytometry experiments and the lower panel shows the number of cells (percent of total cell number) in each phase of the cell cycle (mean ± sem values of five experiments).](image)

![Fig. 9. The upper panel shows phase-contrast microscopy photographs of A549 cells at magnifications of 45 × (upper panels) and 180 × (lower panels). The pictures show confluent cells in the presence and absence (C) of different concentrations of compound 1 (indicated by the number inside each panel). The lower panel shows the number of tumour (A549) and non-tumour (3T3-L1) cells incubated for 24 h in the absence (Control) or presence (complex 1) of 1 (results are expressed as percent of control untreated cells; mean ± sem of three experiments).](image)
contrast with bacterial infections, cancer cells are eukaryotic cells. Moreover, they are almost identical to the other cells in the patient’s organism. One of the few differences of tumour cells with respect to the rest of the cells in the organism is the growth rate. In dividing cells, DNA alterations (e.g., DNA cleavage) often induces apoptosis, so that at sub-lethal concentrations, cancer cells can be selectively killed by anticancer compounds. In this study, we showed that there are drug concentrations that result in the death of cancer cells without affecting non-tumour cells. This result further supports the pharmacological potential of complex 1 against cancer.

The induction of apoptosis by means of 1 in A549 cells was further studied by determination of poly ADP-ribose polymerase (PARP) fragmentation through immunoblotting. Apoptosis induces a number of proteases that induce degradation of many important proteins, being PARP one of the first target of apoptosis-induced proteolysis. Treatments with 1 induced apoptosis as determined by the marked decrease of the 113 kDa peptide (whole protein) and the appearance of a faint band of 89 kDa, corresponding to a transient proteolytic fragment (Fig. 10). It is noteworthy that A549 cells are refractory to undergo apoptosis, since certain pro-apoptotic compounds (e.g., staurosporine, data not shown) and anti-tumour drugs (e.g., taxotere) since certain pro-apoptotic compounds (e.g., staurosporine, data not shown) and anti-tumour drugs (e.g., taxotere) have failed to induce programmed cell death [29,36].

4. Conclusions

In summary, [Cu(I-hip)(phen)2]2+ · (I-hip−) · (H2O)7 (1) can generate the copper(I) complex [Cu(phen)2]2+ in aqueous solution in the presence of oxygen via the formation of radicals stabilized by capto-dative factors. The complex 1 cleaves DNA, possibly by generation of this [Cu(phen)2]2+ compound [2,15]. Complex 1 also induced apoptosis in A549 cells at low concentrations. The complex 1 proves to impair the growth of human lung cancer cells with a high potency and selectivity against cancer cells. Therefore, the parent complex can be considered as a potential candidate to be developed as a future anticancer drug. For this reason, a Patent Application (P200601111) has been filed for future developments.

Acknowledgements

This work was supported by the COST D-20 WG-0006 and Grants DGICYT BQU2002-02546, CTQ2006-09339/ BQU and SAF2003-00232. The authors are also grateful to the “Conselleria d’Economia, Hisenda i Innovacio del Govern de les Illes Balears” for doctoral fellowship FPI05-34068589T (M. Barceló-Oliver) and financial support as Competitive Groups. The authors thank the “Vicerectorat de Investigació i Política Científica” of the Universitat de les Illes Balears for its financial support (Project number: UIB 2005/5).

Appendix A. Supplementary data

X-ray crystallographic data for compounds 1 and 2 (CIF files) have been deposited with the Cambridge Crystallographic Data Centre, CCDC numbers 285763 and 285764. Copies of this information may be obtained free of charge from the Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK, (e-mail: deposit@ccdc.cam.ac.uk or www: http://www.ccdc.cam.ac.uk).

The ESI-HRMS spectra of complex 1, obtained in water solution recorded at different times (0, 2.5 and 24 h after solution) from the parent solution were available as supplementary material. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinorgbio.2006.12.008.

References