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Insight into the cytotoxicity of polynuclear Cu(I) camphor complexes

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ABSTRACT

Three new polynuclear Cu(I) camphor complexes, $[(CuBr)_2\{(p\text{-}H_2NC_6H_4)NC_{10}H_{14}O\}]_n$ (**2d**), $[(CuCl)_4\{m\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]_n$ (**5f**) and $[(CuBr)_4\{p\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]_n$ (**6e**), were synthesized and their cytotoxicity, as well as that of the former reported compounds $[(CuX)_2(YNC_{10}H_{14}O)]_n$ (X = Cl; Y = NMe_2 (**1a**), NH_2 (**1b**), NHMe (**1c**), $(H_2NC_6H_4)NC_{10}H_{14}O$ (**1d**); X = Br; Y = NMe_2 (**2a**)), $[\{Cu(Me_2NNC_{10}H_{14}O)\}_2(\mu\text{-}X)_2]$ (X = Cl (**3a**), Br (**4a**)) and $[(CuCl)_4\{p\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]$ (**5f**), were evaluated against the human colon adenocarcinoma cancer cell line HT29 using the colorimetric method (MTT assay). The calculated IC_{50} values indicate that all the complexes have cytotoxic activity that ranges from high to moderate or low, depending on the characteristics of the camphor ligand and the halide co-ligand. The complexes $[(CuCl)_4\{m\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]$ (**5f**, IC_{50} = 32.0 + 1.1 μM) and $[(CuCl)_2(H_2NNC_{10}H_{14}O)]_n$ (**1b**, IC_{50} = 37.0 ± 1.1 μM) display the lowest IC_{50} values, while $[(CuBr)_2\{(p\text{-}H_2NC_6H_4)NC_{10}H_{14}O\}]_n$ (**2d**, IC_{50} = 119.9 ± 1.1 μM) displays the highest one. The IC_{50} value for **5f** approaches that of *cisplatin* (26.3 + 1.1 μM). No cytotoxic activity was detected for the camphor compounds $H_2NNC_{10}H_{14}O$ (**b**) and $m\text{-}C_6H_4(NC_{10}H_{14}O)_2$ (**f**), used as ligands.

In selected cases, the copper accumulation in the cells was evaluated. No direct relationship was found between the copper uptake and the cytotoxicity of the complexes.

1. Introduction

The search for biologically active compounds, either organic compounds or complexes, that perform better than existing drugs, are less toxic or foresee new treatments, is actually a topic for investigation in two complementary fields: the synthesis of new compounds and assessment of biological properties. From the synthetic point of view, focus has been made on a number of organic molecules from which hydrazones by themselves [1–5] or as ligands in complexes reveal biological activity. A review on the biological applications of hydrazones and their complexes (Cu(II), Ni(II), Co(II), Mn(II), Fe(II) and Cd(II)) was recently published [6].

Among the metals studied, Cu(II) complexes evidenced relevant antimicrobial or cytotoxic activities [7–12], some behaving as potentials drugs due to their cytotoxicity through cell apoptosis (e.g. through binding to DNA) or enzyme inhibition [13–15]. A review on Cu(I) and Cu(II) complexes with anticancer activities was recently published [16].

Existing publications on hydrazone-type compounds and/or hydrazone complexes miss addressing camphor hydrazone compounds by themselves or as ligands in complexes. This is a gap worth filling, since camphor has recognized antimicrobial properties which may be enhanced and/or modified by the hydrazone function. Having this guiding goal, a survey on the biological activity of a selection of camphor hydrazone Cu(I) complexes, either new or previously described, was undertaken based on the existing skills for the synthesis of camphor derivatives or complexes [17,18], as well as to probe their biological activity [19].

The choice of copper was due to its low price, much lower than platinum, and conceivably lower toxicity (an endogenous metal) than *cisplatin*, which is the drug currently under use. The choice of a Cu(I) site, instead of Cu(II), aims at contributing to increase the number of Cu(I) complexes for which biological activity has been assessed, since a much lower number of Cu(I) than Cu(II) complexes have been reported to date.

As a target for cytotoxic studies, the human colon adenocarcinoma cell line HT29 was chosen due to the fact that some hydrazones and their derived copper complexes were reported to be active against HT29 [12,20].

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2. Experimental

2.1. Materials and methods

All the complexes were prepared under a nitrogen atmosphere using vacuum and Schlenk techniques. The complexes $[\{CuCl\}_2(YNC_{10}H_{14}O)]_n$ (Y = NMe_2, NHMe, NH_2 and $C_6H_4NH_2$) [21–23] and the camphor ligands were prepared by published methods [24–26]. CuCl was synthesized from $CuCl_2$ by reduction with sodium sulfite [27]. CuBr was purchased from Fluka and cisplatin from Sigma–Aldrich. The solvents were purchased from Sigma–Aldrich, further purified by conventional techniques and distilled before use. IR spectra were obtained from KBr pellets using a JASCO FT/IR 4100 spectrometer. UV–Vis spectra were obtained from 20 μM solutions of the complexes in the culture medium, using a JASCO V-650 spectrometer. NMR spectra (^{1}H, ^{13}C, DEPT, HSQC and HMBC) were obtained at 25 °C, from solutions in DMSO-d_6 or $CDCl_3$ and referenced to TMS (δ = 0 ppm) using Bruker Avance II$^+$ spectrometers (300 or 400 MHz).

The human colon adenocarcinoma cancer cell line HT29 was grown in McCoy's culture medium (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 95% of air and 5% CO_2 (Heraeus, Germany).

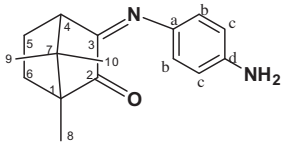
2.2. Synthesis of the complexes

2.2.1. General aspects

The complexes were typically obtained from the reaction of the copper halide (CuX) with the appropriate camphor ligand in THF (3 mL) upon stirring for *ca.* 18 h at room temperature. Filtration of the precipitate, washing with n-pentane (*ca.* 6 mL) and drying under vacuum affords the Cu(I) complex.

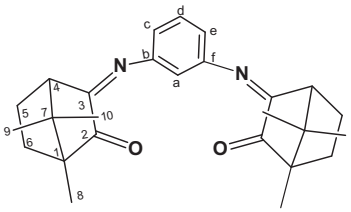
2.2.2. Synthesis of $[(CuBr)_2\{(p\text{-}H_2NC_6H_4)NC_{10}H_{14}O\}]_n$ (**2d**)

CuBr (0.11 g, 0.78 mmol) and 3-(4-aminophenylimino)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one (**d**, 0.10 g, 0.390 mmol) afford **2d** as a brown compound. Yield 84%. Elemental *Anal.* Calc. for $Cu_2Br_2C_{16}H_{20}N_2O{\cdot}3/2THF$: C, 39.5; N, 4.2; H, 5.1. Found: C, 39.9; N, 4.5; H, 4.9%. IR (cm^{-1}): 3435, 3333 (ν_{NH}), 1728 (ν_{CO}), 1580, 1503 (ν_{CN}). ^{1}H NMR (DMSO-d_6, δ ppm): 6.55 (s, 4H, C_6H_4), 5.72 (sl, 2H, NH_2), 2.21 (sl, 1H, *H*4), 1.85–1.47 (m, 4H, *H*5, *H*6), 0.96, 0.95 (2s, 3+3H, *H*9,10), 0.70 (s, 3H, *H*8). ^{13}C NMR (DMSO-d_6, δ ppm): 206.6 (*C*2), 166.6 (*C*3), 148.7 (*C*d), 135.7 (*C*a), 125.2 (2C, *C*b), 113.9 (2C, *C*c), 57.2 (*C*1), 50.6 (*C*7), 45.2 (*C*4), 30.1 (*C*6), 23.4 (*C*5), 20.4, 17.3 (*C*9,10), 9.0 (*C*8).



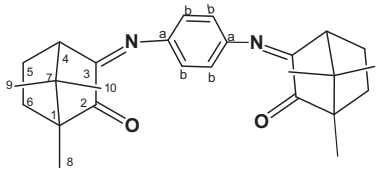
2.2.3. Synthesis of $[(CuCl)_4\{m\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]_n$ (**5f**)

3,3'-(*m*-Phenylenebis(azan-1-yl-1-ylidene))bis(1,7,7-trimethylbicyclo[2.2.1]heptan-2-one) (**f**, 0.055 g, 0.136 mmol) and CuCl (0.050 g, 0.50 mmol) afford **5f** as an orange compound. Yield 60%. Elemental *Anal.* Calc. for $Cu_4Cl_4C_{26}H_{32}N_2O_2$: C, 39.0; N, 3.5; H, 4.0. Found: C, 38.9; N, 3.1; H, 4.0%. IR (cm^{-1}): 1744 (ν_{CO}), 1596 (ν_{CN}). ^{1}H NMR (DMSO-d_6, δ ppm): 7.45 (t, J_{HH} = 7.7, 1H, Hd), 6.78 (d, J_{HH} = 7.7, 2H, Hc, e), 6.64 (sbr, 1H, Ha), 2.72 (d, J_{HH} = 4.2 Hz, 2H, *H*4), 2.1–1.7 (m, 8H, *H*5,*H*6), 1.14 (s, 6H, *H*8), 0.94, 0.80 (2s, 6+6H, *H*9, 10). ^{13}C NMR ($CDCl_3$, δ ppm): 207.3 (*C*2), 174.5 (*C*3), 151.8 (*C*b, f), 132.0 (*C*d), 118.9 (*C*c, e), 112.7 (*C*a), 59.5 (*C*1), 51.7 (*C*7), 46.0 (*C*4), 26.9 (*C*6), 25.3 (*C*5), 22.3, 18.8 (*C*9, 10), 10.7 (*C*8).



2.2.4. Synthesis of $[(CuBr)_4\{p\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]_n$ (**6e**)

3,3'-(1,4-Phenylenebis(azan-1-yl-1-ylidene))bis(1,7,7-trimethylbicyclo[2.2.1]heptan-2-one) (**e**, 0.101 g, 0.25 mmol) and CuBr (0.143 g, 1.00 mmol) afford **6e** as an orange compound. Yield 42%. Elemental *Anal.* Calc. for $Cu_4Br_4C_{26}H_{32}N_2O_2{\cdot}¼THF$: C, 32.5; N, 2.8; H, 3.4. Found: C, 32.7; N, 3.0; H, 3.2%. IR (cm^{-1}): 1741 (ν_{CO}), 1632 (ν_{CN}). ^{1}H NMR ($CDCl_3$, δ ppm): 7.01 (s, 4H, C_6H_4), 2.90 (d, J_{HH} = 4.0, 1H, *H*4), 2.15–1.65 (m, 8H, *H*5, *H*6), 1.12 (s, 6H, *H*8), 1.00, 0.90 (2s, 6+6H, *H*9, 10). ^{13}C NMR ($CDCl_3$, δ ppm): 206.3 (*C*2), 172.1 (*C*3), 146.8 (*C*a), 122.1 (*C*b), 58.2 (*C*1), 50.5 (*C*7), 45.0 (*C*4), 30.3 (*C*5), 24.5 (*C*6), 21.2, 17.7 (*C*9, 10), 9.2 (*C*8).



2.3. Cytotoxicity evaluation

The cytotoxicity of the complexes against HT29 cells was evaluated using a colorimetric method based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is reduced by viable cells to yield purple formazan crystals. The cells were seeded in 96-well plates at a density of 1×10^4 to 1.5×10^4 cells *per* well in 200 μL of culture medium and left to incubate overnight for optimal adherence. After careful removal of the medium, 200 μL of a dilution series of the compounds (stock solutions prepared fresh with DMSO) in the medium were added and incubation was performed at 37 °C/5% CO_2 for 48 h. The percentage of DMSO in the cell culture medium did not exceed 1%. At the end of the incubation period, the compounds were removed and the cells were incubated with 200 μL of MTT solution (500 μg/mL). After 3–4 h at 37 °C/5% CO_2, the medium was removed and the purple formazan crystals were dissolved in 200 μL of DMSO by shaking. The cell viability was evaluated by measurement of the absorbance at 570 nm using a plate spectrophotometer (Power Wave Xs, Bio-Tek) and calculated by dividing the absorbance of each well by that of the control wells. Each experiment was repeated at least two times and each point was determined in at least 4 replicates.

2.4. Intracellular distribution of the Cu complexes

The cellular uptake of copper by HT29 cells (*ca.* 1×10^6 cells in 5 mL medium) was assessed by exposure to a 50 μM solution of the complexes for 24 h at 37 °C. The cells were then washed with cold PBS and centrifuged to obtain a cellular pellet, following a previously described procedure [28]. Briefly, the samples were digested with ultrapure HNO_3, H_2O_2 and HCl in a closed pressurized microwave digestion unit (Mars5, CEM) with medium-pressure HP500 vessels and then diluted in ultrapure water to obtain a 2.0% (v/v) acid solution. The Cu content in each sample was measured by a Thermo XSERIES quadrupole ICP-MS instrument (Thermo Scientific). The instrument was tuned using a multi-element ICP-MS

71 C standard solution (Inorganic Venture). Indium-115 (10 μg/L) was used as an internal standard.

3. Results and discussion

3.1.1. Synthesis and characterization

Cu(I) polynuclear complexes of the general formula $[(CuX)_2L)]_n$ (X = Cl, **1**, X = Br, **2**), $[(CuL)_2(\mu\text{-}X)_2]$ (X = Cl, **3**, X = Br, **4**), $[(CuX)_4L]_n$ (X = Cl, **5**; X = Br **6**) (Fig. 1) were obtained from the corresponding copper halides (CuX) by reaction with the appropriate camphor compound (Fig. 2).

The complexes $[(CuBr)_2\{H_2NC_6H_4(NC_{10}H_{14}O)\}]_n$ (**2d**), $[(CuCl)_4\{m\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]$ (**5f**) and $[(CuBr)_4\{p\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]$ (**6e**) are new, while the Cu(I) camphor hydrazone complexes (**1a**, **1b**, **1c**), camphor imine (**1d**) and bicamphor (**5e**) complexes were reported previously [21–23,29].

Complexes of type **1** $[(CuCl)_2L)]_n$ and of type **2** $[(CuBr)_2L]_n$ are arranged as 1-D polymers [22], formed by sequential linear CuX and tetrahedral CuL units bound by the halide, while complexes **3** and **4** are dimers bridged by the halide, as structurally confirmed by X-ray diffraction analysis in a few cases [21,23]. In both types of complexes the camphor ligands chelate the copper atom through the nitrogen atom of the imine group and the oxygen atom of the camphor skeleton. The structural arrangements of the coordination polymers $[(CuX)_2L]_n$ and dimers $[(CuL)_2(\mu\text{-}X)_2]$ are schematically depicted in Fig. 1(a) and (b), respectively.

Although no crystals suitable for X-ray diffraction analysis were obtained, the formulation of $[(CuBr)_2\{H_2NC_6H_4(NC_{10}H_{14}O)\}]_n$ (**2d**), based on analytical and spectroscopic data, is akin to the former reported $[(CuX)_2L]_n$ coordination polymers, pointing to a similar structure. Formulation of **2d** closely resembles that of $[(CuCl)_2(Me_2NNC_{10}H_{14}O)]_n$ (**1a**), previously characterized by X-ray diffraction analysis [21]. With regards to complexes **5f** and **6e**, formulated

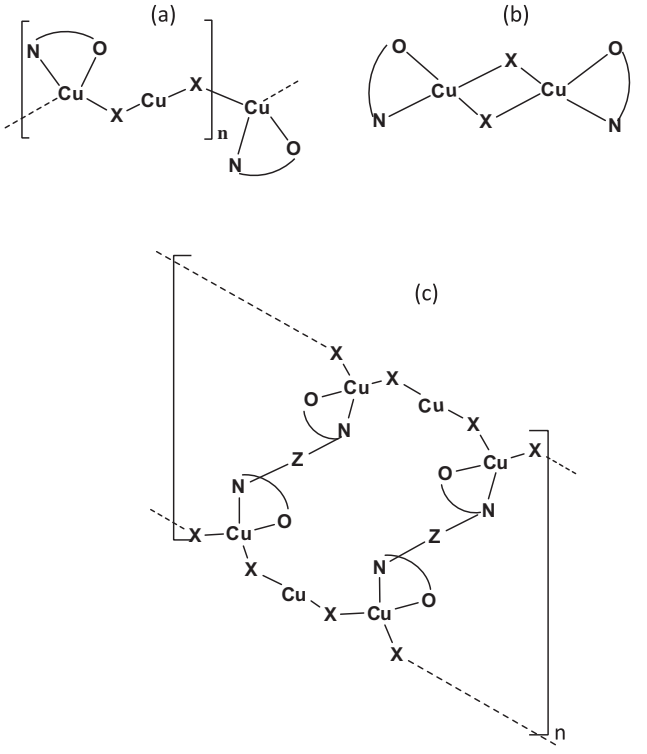


Fig. 1. Structural arrangement of the Cu(I) camphor complexes: (a) 1-D polymer (**1**, **2**), (b) dimer (**3**, **4**), (c) Structure proposed for complexes **5** and **6**. The ligand is displayed as N,O.

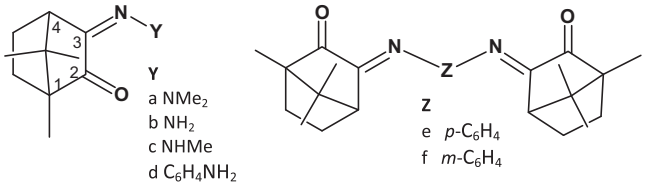


Fig. 2. Camphor compounds used as ligands (L) in this work.

as $[(CuX)_4L]_n$, the metal to ligand ratio (4:1) is double that found in **2d** (2:1), such as in the case of complex $[(CuCl)_4\{p\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]_n$ (**5e**), [29] formerly reported. The bi-camphor character of the **e**, **f** ligands (Fig. 2) with two N,O binding groups each, enables coordination to two metal sites *per* ligand, forming double chain coordination polymers (Fig. 1c). The spectroscopic characteristics of **5f** and **6e** are fine-tuned by the electron density (basicity) of the imine nitrogen atom. Extended electron delocalization in the *para* bicamphor ligand (**e**, Fig. 1) accounts for the lower electron density in the imine nitrogen atom and the strengthening of the C=N bond is responsible for a shift in the stretching frequency to higher values (ν_{CN} 1632 cm^{-1}, **6e**; 1641 cm^{-1}, **5e** [29]) compared to **5f** (1596 cm^{-1}) or **1**, **2** (1580, 1540 cm^{-1}) [21,23], where the effect of electron delocalization is less pronounced. For the C=O stretching frequencies, just small differences exist (*ca.* 10 cm^{-1}) between complexes **1** or **2** and the bicamphor complexes (**5f**, **6e** or **5e**), the values being slightly higher than those in the camphor hydrazone complexes **1** and **2** (1709 and 1740 cm^{-1}). The same trend was found in the NMR data, where the chemical shifts of the ketone carbon atoms do not differ appreciably between the bicamphor (δ_{CO}, 207.3 ppm, **5f**; 206.3 ppm, **6e**) and camphor hydrazone (δ_{CO}, 204 ppm, **1**; 202 ppm, **2**) complexes; while the chemical shifts of the imine carbon atoms differ considerably in the two sets of compounds, i.e. the values are higher for the bicamphor complexes (δ_{CN} 174.5 ppm, **5f**, 172.1 ppm, **6e**) than for the camphor hydrazone complexes **1** or **2** (δ_{CN}, 140–150 ppm, [21,23]), regardless of the stereochemistry of the bicamphor ligand. In contrast with the characteristics of the camphor ligand, the halide co-ligand has a small effect on the spectroscopic characteristics of complexes **5e** (X = Cl) and **6e** (X = Br).

3.2. Cytotoxicity activity

A selection of camphor polynuclear Cu(I) complexes was used to evaluate the cytotoxic activity based on the copper to ligand ratio [1:1 (dimers), 2:1 (1-D polymers) and 4:1 (2-D polymers)]. Two dimers (**3a**, **4a**, that differ just in the halide), four 1-D polymers (**1**), one of them having the ligand $Me_2NNC_{10}H_{14}O$ as the dimer (**1a**) and two other 1-D polymers (**1b**, **1c**) having Y groups (Fig. 2) that essentially differ from **1a** in the number of hydrogen atoms (considered as relevant for interactions with the cell) were considered. **1d** was selected to compare the activity of the imine (**1d**) with the hydrazone (**1b**) complexes, and **5** and **6** were chosen to compare the effects (if any) of a double CuX chain (compared to complexes **1** and **2**). Since **5** and **6** differ in the halide their choice was expected to clarify the beneficial effect of chloride compared to bromide.

The antiproliferative properties of the polynuclear Cu(I) camphor complexes **1**, **2**, **3**, **4**, **5** and **6** were thus assessed by monitoring their ability to inhibit cell growth on the human colon adenocarcinoma cell line HT29, using a colorimetric method (MTT assay). Dose-response curves were obtained after long-term exposure (48 h) using an appropriate range of concentrations of the complexes, and the IC_{50} values were calculated (Table 1). For comparative purposes, the cytotoxicity of *cisplatin* and that of the

camphor hydrazone ($H_2NNC_{10}H_{14}O$, **b**) and bi-camphor (*m*-C_6H_4(-$NC_{10}H_{14}O)_2$, **f**) ligands were evaluated under the same experimental conditions as for the Cu(I) complexes.

The camphor ligands (**b** and **f**) display no cytotoxic activity against the human colon adenocarcinoma cell line HT29. Their lack of activity contrasts with that of α-(*N*)-heterocyclic hydrazones, reported as active against the same cell line [20].

The analysis of the data in Table 1 shows that the IC_{50} values calculated for the Cu(I) complexes spread over a range of values from low (32.0 + 1.1 μM, **5f**) to quite high (119.9 μM, **2d**). It is noteworthy that the value of **5f** is not much higher than that of *cisplatin* (26.3 + 1.1 μM), in agreement with the high cytotoxic activity of **5f**. Another observation is that the IC_{50} values of the Cu(I) complexes are tuned by the characteristics of the camphor ligand (hydrazone (**a**, **b**, **c**); imine (**d**); bi-camphor(**e**, **f**)) and the halide co-ligand. The bromide compounds systematically display higher IC_{50} values than the related chloride ones, e.g. complexes **1d** (IC_{50}, 56.8 ± 1.2 μM) and **2d** (IC_{50}, 119.9 ± 1.1 μM) with the same camphor ligand (**d**) display IC_{50} values that differ by a factor of *ca.* 2. A similar trend is observed for complexes **3a** versus **4a** and **5e** versus **6e**. Another observation is that complex **1b** (Y = NH_2) displays the lowest IC_{50} value (37 ± 1.1 μM) among the hydrazone complexes (**1a**, **1b**, **1c**, **3a**, **4a**) under study. Such behaviour is attributed to hydrogen bonding enabled by the amine group (Y = NH_2) which in the imine camphor complex **1d** (Y = $C_6H_4NH_2$) is somehow deactivated due to electron delocalization through the aromatic ring, this conceivably affecting the cytotoxic activity (56.8 ± 1.2 μM, **1d**) that becomes lower than that of **1b**. Such an effect, reinforced by the deactivation effect of the bromide co-ligand, is considered as being responsible for the high IC_{50} value (119.9 ± 1.1 μM) displayed by **2d**.

The IC_{50} values calculated for two complexes with the same camphor ligand (**a**, Fig. 1) and halide co-ligand (X = Cl) that differ in the polymeric (**1a**, 64.2 ± 1.2 μM) or dimeric (**3a**, 55.1 ± 1.0 μM) structure show that the dimer displays a higher cytotoxic activity. The IC_{50} values calculated for the bicamphor complex **5e** (42.1 ± 1.1 μM) and the camphor hydrazone complex **1d** (56.8 ± 1.2 μM), both having the *p*-phenylenediamine moiety, show that the cytotoxicity is higher for the bicamphor complex (**5e**). The IC_{50} values calculated for the complexes (**5e** and **5f**) with two bicamphor ligands that differ in the relative position of the camphor groups bound to the aromatic ring show that the IC_{50} value is lower for the complex with the camphor groups in mutually *meta* positions (**5f**, 32.0 ± 1.08 μM) than for *para* positions (**5e**, 42.1 ± 1.07 μM). As mentioned above, the electronic properties of complexes **5e** and **5f** are considerably different, due to electron delocalization through the aromatic ring, which is responsible for a lower basicity of the nitrogen atoms in **5e** than in **5f**. Electron delocalization also supports the differences found in the cytotoxic activities of complexes **1b** and **1d**.

Table 1
IC_{50}[a] values calculated for Cu(I) camphor complexes.

Complex		IC_{50} μM
$[(CuCl)_2(Me_2NNC_{10}H_{14}O)]_n$	**1a**	64.2 ± 1.2
$[(CuCl)_2(H_2NNC_{10}H_{14}O)]_n$	**1b**	37.0 ± 1.1
$[(CuCl)_2(MeHNNC_{10}H_{14}O)]_n$	**1c**	68.9 ± 1.0
$[(CuCl)_2\{H_2NC_6H_4(NC_{10}H_{14}O)\}]_n$	**1d**	56.8 ± 1.2
$[(CuBr)_2\{(p\text{-}H_2NC_6H_4)NC_{10}H_{14}O\}]_n$	**2d**	119.9 ± 1.1
$[\{CuCl(Me_2NNC_{10}H_{14}O)\}_2]$	**3a**	55.1 ± 1.0
$[\{CuBr(Me_2NNC_{10}H_{14}O)\}_2]$	**4a**	89.2 ± 1.0
$[(CuCl)_4\{p\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]$	**5e**	42.1 ± 1.1
$[(CuCl)_4\{m\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]$	**5f**	32.0 ± 1.1
$[(CuBr)_4\{p\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]$	**6e**	62.1 ± 1.1
cis-Diamine dichloroplatinum(II)		26.3 + 1.1

[a] Values measured after 48 h incubation.

From the data in Table 1, it is worth highlighting that the IC_{50} value displayed by $[(CuCl)_4\{m\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]$ (32.1 ± 1.1 μM, **5f**) approaches that of *cisplatin* (26.1 ± 1.1 μM), which is the metallodrug clinically used for the treatment of colon adenocarcinoma cancer. These results challenge efforts to redesign and synthesize Cu(I) camphor complexes that are even more active.

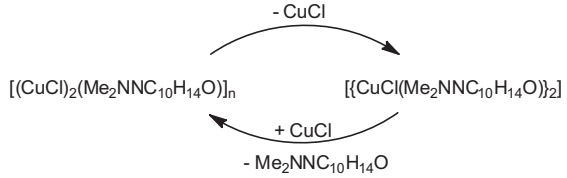
At this stage, no information exists concerning the intracellular fate of Cu(I) camphor complexes or their potential interaction with biomolecules. Nevertheless, it is relevant to strengthen that the camphor ligands (**b** and **f**) do not display cytotoxic activity by themselves. Thus, if dissociation occurs, the camphor ligands do not contribute to the observed biological activity, corroborating that the observed cytotoxicity is based on the complexes.

Both the new and former reported camphor Cu(I) complexes are stable in DMSO under an inert atmosphere for several days, as confirmed by ^{1}H NMR spectroscopy. To ascertain the stability of solutions of **1b** and **5f** in the cell medium, they were monitored by UV–Vis for 48 h. No evident changes were found. However, the results were not fully satisfactory because the concentration of the complexes was low (200 μM) and the spectra were very weak. Therefore, CuCl release from the polymeric complexes (**1**, **2**, **5**, **6**) or oxidation to Cu(II) in the cell culture cannot be completely excluded. If oxidation occurs, the Cu(II) species are not expected to enable cytotoxicity, since data show the biological activities of hydrazone Cu(II) polymer complexes are slightly lower [12] than those of the camphor polymer Cu(I) complexes in this study. However, if CuCl release occurs, as in the polymer to dimer conversion [23], this would contribute to increase cytotoxicity, since a comparison of the activities of **3a** and **1a** (see above) shows the dimer is more active than the related polymer. Polymer to dimer conversion is a peculiar characteristic of complexes **1a** and **3a** (Scheme 1), which is unlike that in the cell medium.

3.3. Copper uptake by cells

The bi-camphor $[(CuCl)_4\{m\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]_n$ (**5f**), hydrazone $[(CuCl)_2\{H_2NNC_{10}H_{14}O)\}]_n$ (**1b**) and imine $[(CuCl)_2\{(H_2NC_6H_4)NC_{10}H_{14}O\}]_n$ (**1d**) complexes were chosen as representative examples of complexes with high (**5f**, **1b**) or moderate-low (**1d**) cytotoxic activities for the study of copper uptake by HT29 cells. The Cu uptake was measured by ICP-MS, as copper accumulation upon exposure of the cells to equimolar solutions (50 μM) of the compounds (**1b**, **1d** and **5f**). The copper content was determined in extracts isolated from HT29 cells after 24 h of exposure. The results are displayed in Table 2.

Analysis of the data shows that the total cellular copper content spans from 31 to 41 nmol. Since in compounds **1b**, **1d** and **5f** the number of copper atoms *per* molecule is different, normalization is necessary. Calculations show that compound **5f** (4 Cu atoms *per* molecule) displays the lowest uptake (3.45%) *per* metal atom, despite having the highest cytotoxic activity. Further comparison shows that the copper uptake from **1b** is almost twofold that of **5f**, in spite of the similar cytotoxic activities of the two complexes (Table 2). Additionally, the imine complex **1d**, which is less cytotoxic than the hydrazone **1b**, causes a Cu accumulation *ca.* 1.3 times higher than **1b**. These data shows that copper uptake *per*

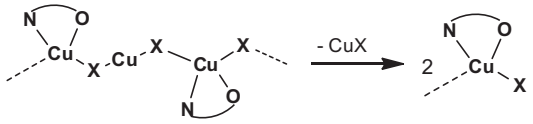


Scheme 1. Polymer to dimer conversion in camphor hydrazone complexes. [23].

Table 2
Cu uptake[a] by HT 29 cells upon exposure to Cu camphor complexes.

Complex		Cu/L	Cu uptake (nmol)	Cu uptake %[b]	IC_{50} μM
$[(CuCl)_2(H_2NNC_{10}H_{14}O)]_n$	**1b**	2:1	31	6.2	37.0 ± 1.1
$[(CuCl)_2\{H_2NC_6H_4(NC_{10}H_{14}O)\}]_n$	**1d**	2:1	41	8.2	56.8 ± 1.2
$[(CuCl)_4\{m\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]_n$	**5f**	4:1	34.5	3.45	32.0 + 1.1

[a] Values measured after 24 h.
[b] (%) of total Cu in complexes.



Scheme 2. Outline for the formation of unsaturated intermediates from complexes **1** or **2**.

se does not reflect the cytotoxic activity of the complexes, which is not surprising since the antiproliferative activity depends on the persistence of the reactive compounds in the cells, which results from the equilibrium between influx and efflux, as well as interactions with potential targets [28]. The influx/efflux equilibrium can be shifted by reducing the uptake by cells and facilitating cellular detoxification (in conjugation with glutathione (GHS) and metallothioneins (MTs)), leading to intracellular modified inactive Cu complexes.

Release of CuCl (such as in the polymer-dimer conversion, Scheme 1) in the cell cannot be completely excluded (see above). In that case, formation of coordinative unsaturated sites (Scheme 2) with a lower copper content and improved ability to interact with receptors would enhance the biological interactions (eventually promoting the reaction with S-donor molecules) and the activity. Under this perspective the polymer complexes would act as carriers to take the active camphor compounds to the cells.

4. Conclusions

The number of existing polynuclear Cu(I) camphor complexes was extended by the synthesis and characterization of $[(CuBr)_2\{(p\text{-}H_2NC_6H_4)NC_{10}H_{14}O\}]_n$ (**2d**), $[(CuCl)_4\{m\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]_n$ (**5f**) and $[(CuBr)_4\{o\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]_n$ (**6e**). Analysis of the biological activity of the new Cu(I) camphor complexes as well as a selection of previously reported camphor hydrazone polynuclear Cu(I) complexes showed that all of the complexes display cytotoxic activity against the human colon adenocarcinoma cancer cell line HT29. The IC_{50} values span from low values in $[(CuCl)_4\{m\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]$ (**5f**) (close to that of *cisplatin*) to rather high values in the case of $[(CuBr)_2\{(p\text{-}H_2NC_6H_4)NC_{10}H_{14}O\}]_n$ (**2d**).

The cytotoxic activity of the Cu(I) camphor complexes depends on their structure (polymer or dimer), the characteristics of the camphor ($YNC_{10}H_{14}O$) or bicamphor ($Z(NC_{10}H_{14}O)_2$) ligands and on the halide co-ligand (Cl or Br). The activities of the chloride (**1d**) and bromide (**2d**) complexes with the same ligand (**d**) clearly evidence that chloride enhances the biological activity. In complexes with the same ligand and co-ligand and different structural arrangements, the dimer (**3a**) displays higher activity than the polymer (**1a**).

The dissimilar cytotoxicities of complexes **5e** (*para*) and **5f** (*meta*), that differ just in the position of the camphor units bound to the aromatic ring, point to the high relevance of the basicity of the imine nitrogen in the biological properties. Electron delocalization in **5e** (*para*) lowers the basicity and conceivably the ability of the complexes to interact with receptors in the cells, which is conceivably responsible for the lower activity compared with **5f** (*meta*) where electron delocalization through the aromatic ring is inhibited. A related effect is also found for **1b** and **1d**.

No direct relationship between bioaccumulation and cytotoxicity was found, since the most cytotoxic complex (**5f**) displays a lower Cu uptake than complexes **1b** and **1d**. The results point to the number of copper atoms *per* polymer unit as being relevant for their activity, although, not all the copper content is found in the cell. Further studies are necessary to ascertain whether the high copper content polymer complexes $[\{CuX\}_4L]_n$ act as carriers to the cells of active species with lower Cu content.

The hydrazone camphor (**b**) and the bicamphor (**f**) compounds used as ligands display no cytotoxicity by themselves, emphasizing that the cytotoxic activity is based on the complexes.

The most significant outcome of this study is that the IC_{50} value of $[(CuCl)_4\{m\text{-}C_6H_4(NC_{10}H_{14}O)_2\}]$ is marginally higher than that of *cisplatin*, thus encouraging further efforts to redesign the compounds and reach even lower IC_{50} values.

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