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Molecular mechanisms of the biological activity of the anticancer drug elesclomol and its complexes with Cu(II), Ni(II) and Pt(II)

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ABSTRACT

The bis(thiohydrazide) amide elesclomol has extremely potent antiproliferative activity and is currently in clinical trials as an anticancer agent. Elesclomol strongly binds copper and may be exerting its cell growth inhibitory effects by generating copper-mediated oxidative stress. Nickel(II) and platinum(II) complexes of elesclomol were synthesized and characterized in order to investigate if these biologically redox inactive metal complexes could also inhibit cell growth. The nickel(II)-elesclomol and platinum(II) elesclomol complexes were 34- and 1040-fold less potent than the copper(II)-elesclomol complex towards human leukemia K562 cells. These results support the conclusion that a redox active metal is required for elesclomol to exert its cell growth inhibitory activity. Copper(II)-elesclomol was also shown to efficiently oxidize ascorbic acid at physiological ascorbic acid concentrations. Reoxidation of the copper(I) thus produced would lead to production of damaging reactive oxygen species. An X-ray crystallographic structure determination of copper(II)-elesclomol showed that it formed a 1:1 neutral complex with a distorted square planar structure. The kinetics and equilibria of the competition reaction of the strong copper(II) chelator TRIEN with copper(II)-elesclomol were studied spectrophotometrically under physiological conditions. These results showed elesclomol bound copper(II) with a conditional stability constant 24-fold larger than TRIEN. A log stability constant of 24.2 was thus indirectly determined for the copper(II)-elesclomol complex.

1. Introduction

The anticancer drug elesclomol (**4**) (Fig. 1) has undergone randomized Phase 2 and 3 clinical trials for the treatment of a variety of cancers [1–3]. Currently it is in phase 2 and 3 clinical trials for the treatment of acute myeloid leukemia, gynecological and other types of cancers (http://www.clinicaltrials.gov). In addition a study with elesclomol plus paclitaxel versus paclitaxel alone has just been published [4]. This study showed that patients on the elesclomol plus paclitaxel arm with normal serum lactate dehydrogenase levels had improved outcomes versus patients with high lactate dehydrogenase levels [4]. It was speculated that high lactate dehydrogenase levels may be a reflection of the state of tumor hypoxia. Because elesclomol may target metabolically active mitochondria it may be most active in well-oxygenated non-hypoxic cells dependent on oxidative phosphorylation rather than on glycolysis [4]. Elesclomol is an extremely potent inhibitor of the growth of cancer cells in culture and typically inhibits cell growth at low nanomolar concentrations [1,2,5]. Like the structurally related thiosemicarbazones [6,7], elesclomol is a strong copper(II) chelator [1].

It has been proposed that elesclomol induces cancer cell apoptosis through the induction of oxidative stress [5,8,9]. In support of this conclusion breast cancer MCF7 cells with a compromised ability to repair oxidative DNA damage displayed increased sensitivity to elesclomol [5]. It has been shown [1,2] that elesclomol scavenges copper from the culture medium and selectively transports copper to the mitochondria which induces oxidative stress. Thus, elesclomol may be inhibiting cell growth by specifically targeting the mitochondria [1,2]. Cu(II)-bis(thiosemicarbazone) complexes can be reduced by biological reductants and have been shown to transfer the Cu^{+} produced to the copper-binding proteins Atx1 and Ctr1c, thus suggesting a mechanism by which these complexes cause cellular retention of copper [10]. It has also been shown that the Cu(II)-bis(thiosemicarbazone) complexes are readily taken up by cells and that this uptake is driven by intracellular aggregation of the highly insoluble complex [11]. The sensitivity of yeast with homozygous gene deletions to treatment with elesclomol suggests that it exerts its cytotoxicity by a distinct mechanism of action which is unlike that of any other anticancer drug [2]. Thus given that elesclomol may represent a whole new class of anticancer drugs whose activity may be mediated through complex formation with Cu^{2+}, it is important that its mechanism of action be elucidated.

In order to test whether elesclomol exerted its growth inhibitory effects by inducing oxidative stress mediated through its Cu^{2+} complex we decided to prepare and characterize biologically redox inactive Ni^{2+} and Pt^{2+} complexes of elesclomol in order to compare their cell growth inhibitory activity with that of Cu(II)-elesclomol. Additionally,

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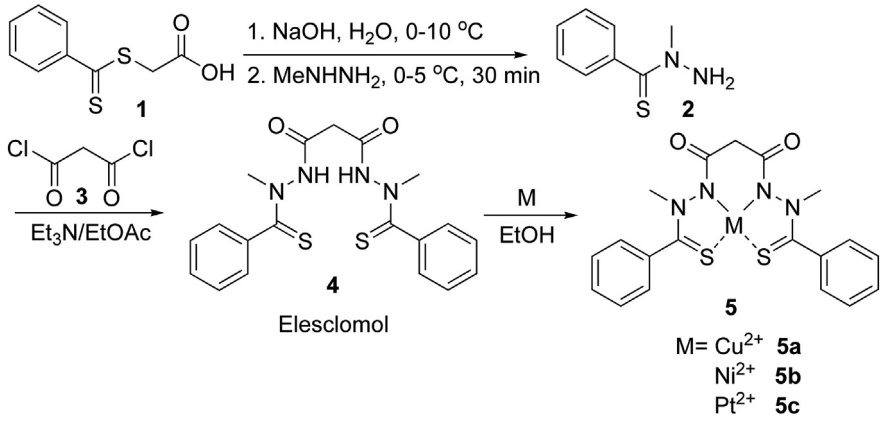

Fig. 1. Structure of elesclomol (**4**) and the reaction scheme for its synthesis and intermediates for the preparation of its complexes with Cu^{2+}, Ni^{2+} and Pt^{2+} (**5a**, **5b** and **5c**, respectively).

the synthesis and characterization of elesclomol (**4**) and the Cu(II)-elesclomol (**5a**) complex have only been incompletely described in the patent literature [12]. Thus, elesclomol (**4**), Cu(II)-elesclomol (**5a**), Ni(II)-elesclomol (**5b**), and Pt(II)-elesclomol (**5c**) (Fig. 1) were synthesized and fully characterized by NMR, electrospray ionization high resolution mass spectrometry HRMS (ESI) and for Cu(II)-elesclomol by X-ray structural analysis. The results are discussed in light of mechanisms demonstrated for structurally related copper thiosemicarbazone complexes and are put into perspective as putative inducers of oxidative stress.

2. Experimental

2.1. Materials, spectrophotometry, cell culture and growth inhibition assays

Unless specified, all reagents were obtained from Sigma-Aldrich (Oakville, Canada). The spectrophotometric experiments were carried out in 1 cm or 0.1 cm quartz cells on a Cary 300 double beam spectrophotometer with a thermostated cell holder. Human leukemia K562 cells, obtained from the American Type Culture Collection, were maintained as suspension cultures in αMEM (minimal essential medium alpha) (Invitrogen, Burlington, Canada) containing 10% fetal calf serum. The spectrophotometric 96-well plate cell (5×10^4 cell/ml, 0.1 ml/well) growth inhibition 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) CellTiter 96 AQueous One Solution Cell Proliferation assay (Promega, Madison, WI), which measures the ability of the cells to enzymatically reduce MTS after drug treatment, has been described previously [13]. The compounds tested were dissolved in DMSO and the final concentration of DMSO did not exceed an amount (typically 0.5% or less) that had any detectable effect on cell growth. The cells were incubated with the drugs for 72 h and then assayed with MTS. The IC_{50} values for cell growth inhibition were measured by fitting the absorbance-drug concentration data to a four-parameter logistic equation as we described [14]. The errors quoted are the S.E.M.s.

2.2. Chemistry

2.2.1. General

^1H and ^{13}C NMR spectra were recorded on a Bruker Avance NMR spectrometer operating at 300 MHz for ^1H NMR and 75 MHz for ^{13}C NMR, respectively, in $CDCl_3$ or DMSO-d_6. Melting points were uncorrected. The high resolution mass spectra were run on a Bruker microTOF Focus mass spectrometer (Fremont, CA) using electrospray ionization. TLC was performed on plastic-backed plates bearing 200 μm silica gel 60 F_{254} (Silicycle, Quebec City, Canada). Where applicable compounds were visualized by quenching of fluorescence by UV light (254 nm).

*2.2.2. Synthesis of N-methylbenzothiohydrazide (**2**)*

NaOH (0.58 g, 14.7 mmol) was dissolved in H_2O (5 ml) and then cooled to 0 °C. *S*-(thiobenzoyl)thioglycolic acid **1** (1.2 g, 5.65 mmol) was added and the mixture was stirred until all solids dissolved (Fig. 1). The temperature was maintained between 0 and 10 °C. In a separate flask, the methylhydrazine trifluoroacetic acid salt (1.12 g, 7.35 mmol) was dissolved in H_2O (5 ml). The solution was cooled to 0–5 °C and then the solution containing **1** was slowly added. The temperature of the reaction mixture was maintained between 0–10 °C. After 30 min the reaction was monitored by TLC to determine completeness. Upon completion, dichloromethane (100 ml) was added to the reaction mixture. The organic layer was separated from the aqueous layer and then washed with 0.1 M aqueous NaOH. The organic extract was concentrated by half. Heptane was then added to precipitate out the desired product which was then filtered and dried to constant weight to give the title compound **2** (0.8 g, 86%). ^1H NMR ($CDCl_3$, 300 MHz): δ 7.33–7.37(m, 5H), 5.99 (s, 2H), 3.33 (s, 3H); ^{13}C NMR ($CDCl_3$): δ 189.9, 141.2, 129.0, 128.4, 126.2, 42.8.

*2.2.3. Synthesis of elesclomol (**4**)*

N-methylbenzothiohydrazide (**2**) (0.8 g, 4.82 mmol) was dissolved in ethyl acetate (5 ml) and triethylamine (1.34 ml, 9.64 mmol). The solution was cooled to maintain a temperature of 0–10 °C. Malonyl chloride (0.34 g, 2.41 mmol) was dissolved in ethyl acetate (1 ml) and was added to the solution containing the hydrazide at a rate that maintained the temperature between 0 and 10 °C. The reaction mixture was stirred for about 30 min and then quenched with H_2O. The organic layer was separated and concentrated under vacuum to give the crude product which was purified by column chromatography on silica gel to afford the title compound **4** (1.25 g, 65%); mp: 192–195 °C. ^1H NMR ($CDCl_3$): δ 11.03 (s, 2H), 7.21–7.43 (m, 10H), 3.63 (s, 6H), 2.51 (s, 2H); ^{13}C NMR ($CDCl_3$): δ 202.5, 163.1, 141.7, 128.7, 128.2, 127.3, 125.9, 125.8, 42.9, 38.7.

*2.2.4. Synthesis of the Cu(II)-elesclomol complex (**5a**)*

To a stirred solution of elesclomol (**4**) (200 mg, 0.5 mmol) in ethanol (5.0 ml), $CuCl_2 \cdot 2H_2O$ was added in one portion. The mixture was stirred at room temperature for 20 min. Water was then added, and the precipitated solid was collected by filtration. The solid was taken up in methylene chloride. The resulting solution was washed with water (2×), dried (Na_2SO_4), filtered and concentrated to give the crude solid. The solid was washed with acetone to give the dark red title compound **5a**; (185 mg, 90%); mp: 198–202 °C (decomp); lit. 198–202 °C [15]. Single crystals for X-ray analysis

were obtained by recrystallization from acetonitrile at room temperature overnight. HRMS (ESI) $C_{19}H_{18}CuN_4O_2S_2$ *m/z* $(M)^+$: calcd 461.0162, obsd 461.0141.

*2.2.5. Synthesis of the Ni(II)-elesclomol complex (**5b**)*

Compound **5b** was prepared as described for compound **5a** by reaction with **4** and $NiCl_2 \cdot 6H_2O$ and afforded a green solid; mp: 321–325 °C. ^{1}H NMR ($CDCl_3$): δ 7.59–7.44 (m, 10H), 3.61 (s, 6H), 3.60 (s, 2H); ^{13}C NMR ($CDCl_3$): δ 183.9, 172.6, 133.1, 131.9, 128.9, 128.5, 47.2, 44.6; HRMS (ESI) for $C_{19}H_{18}N_4NaNiO_2S_2$ *m/z* $(M + Na)^+$: calcd 479.0117, obsd 479.0117.

*2.2.6. Synthesis of the Pt(II)-elesclomol complex (**5c**)*

Compound **5c** was prepared as described for compound **5a** by reaction with **4** and potassium tetrachloroplatinate(II) using ethanol:H_2O (1:1) as solvent and afforded a yellow-orange solid; mp: 314–317 °C. ^{1}H NMR ($CDCl_3$): δ 7.62–7.48 (m, 10H), 3.83 (s, 2H), 3.70 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 180.6, 174.4, 134.0, 131.7, 129.0, 128.3, 47.9, 46.9; HRMS (ESI) for $C_{19}H_{18}N_4NaO_2PtS_2$ *m/z* $(M + Na)^+$: calcd 616.0411, obsd 616.0440.

2.2.7. X-ray structure determination of the Cu(II)-elesclomol complex (5a)

X-ray data and crystal structure analysis for the Cu(II)-elesclomol complex were performed by the X-ray Laboratory, Department of Chemistry, University of Toronto. Crystal data were collected on a Nonius Kappa-CCD diffractometer using monochromated Mo Kα radiation. Crystallographic data for the Cu(II)-elesclomol complex has been deposited in the Cambridge Crystallographic Data Centre as supplementary publication CCDC-915646. A copy of the structure can be obtained free of charge by application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK.

3. Results

3.1. Synthesis of elesclomol and its complexes with Cu^{2+}, Ni^{2+} and Pt^{2+}

Elesclomol was synthesized in two steps (Fig. 1) using commercially available *S*-(thiobenzoyl)thioglycolic acid **1** as a starting material. Because methylhydrazine was not commercially available in Canada it was prepared from the acid hydrolysis of 1-boc-1-methylhydrazine using trifluoroacetic acid. Condensation of **1** with methylhydrazine under basic conditions smoothly produced the advanced intermediate *N*-methylbenzothiohydrazide **2**. The hydrazide was then reacted with malonyl chloride **3** to give elesclomol **4**. The transition metal complexes were formed by reacting equimolar amounts of the divalent metal salts with **4** to give **5a**, **5b** and **5c** (Fig. 1). While **5c** has not been described before, the synthesis of compounds **4**, **5a** and **5b** have been briefly described in the patent literature, albeit with only minimal characterization [12]. Elesclomol and its metal complexes **5a**, **5b** and **5c** were fully characterized by NMR, HRMS (ESI) and for **5a** by X-ray structural analysis.

3.2. X-ray structure analysis of Cu(II)-elesclomol

The ORTEP structure of Cu(II)-elesclomol is shown in Fig. 2. Coordination of the nitrogen and sulfur atoms about the Cu^{2+} atom gave a structure that was only approximately square planar. For example, the S–Cu–N and S–Cu–S bond angles were 86.66(6) and 97.10(3)°, respectively. Similarly, the S–Cu–N and S–Cu–N bond angles were 160.47(6) and 164.93(6)°, respectively. The Cu–S and Cu–N bond distances of 2.248(7) and 1.9548(19) Å were similar to those observed for [Cu(ATSM)], a Cu(II)-bis(thiosemicarbazone) complex (2.237 and 1.959 Å, respectively) [16]. These values can also be compared to [Cu(AATSM)], the acetylacetonate bis(thiosemicarbazone) complex of Cu^{2+}, with a Cu–S and Cu–N bond distances of 2.2469(5) and 1.9737(16), respectively, and with S–Cu–N and S–Cu–S bond angles of 86.79(5) and 91.728(19)°, respectively [17].

3.3. Spectrophotometric titration of elesclomol with Cu^{2+}

Elesclomol was titrated with $CuCl_2$ and the titration monitored by spectrophotometry in Tris buffer (10 mM, pH 7.4, 37 °C) that contained 25% (v/v) DMSO in order to characterize complex formation under conditions that approximated those found biologically. It was found necessary to carry out the titration in 25% (v/v) DMSO due to the low aqueous solubility of elesclomol and Cu(II)-elesclomol. As shown in Fig. 3A the absorbance at the 355 nm shoulder systematically increased with the addition of Cu^{2+}. A tight isosbestic point was seen at 285 nm, at least until Cu^{2+} was in molar excess. These results were consistent with only two absorbing species in solution, elesclomol and Cu(II)-elesclomol. The least square fits of the two linear segments of the plot of absorbance vs. Cu^{2+} concentration (Fig. 3B) intersected at 21 μM or at a Cu^{2+}:elesclomol ratio of 1:1.05. The fact that the plot had no significant curvature in the equivalence region was consistent with the formation of a very strong complex. Also, in accord with the X-ray structure (Fig. 2) these results were consistent with the formation of a 1:1 complex.

A spectrophotometric experiment to test whether Cu^{2+} could displace Ni^{2+} from the Ni(II)-elesclomol complex was also carried out by recording UV–vis spectra for 20 h of a 20 μM solution of Ni(II)-elesclomol to which 200 μM $CuCl_2$ had been added. The lack of any measurable increase in absorbance at 355 nm corresponding

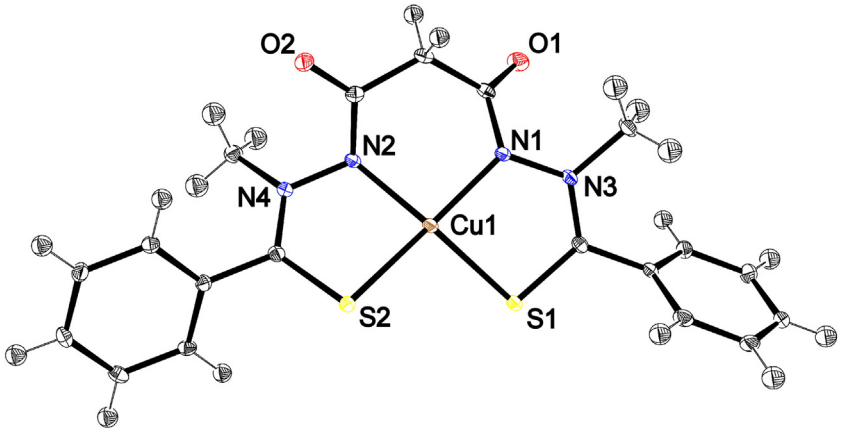

Fig. 2. ORTEP view of the molecular structure of the Cu(II)-elesclomol complex (ellipsoids at 30%) obtained from an X-ray crystallographic determination. No solvent molecules were detected in the structure. Selected bond lengths (Å): $Cu_1–S_1$ 2.248(7), $Cu_1–N_1$ 1.9548(19). Selected bond angles (°): $S_1–Cu_1–N_1$ 86.66(6), $S_1–Cu_1–S_2$ 97.10(3), $S_1–Cu_1–N_2$ 160.47(6) and $S_2–Cu_1–N_1$ 164.93(6).

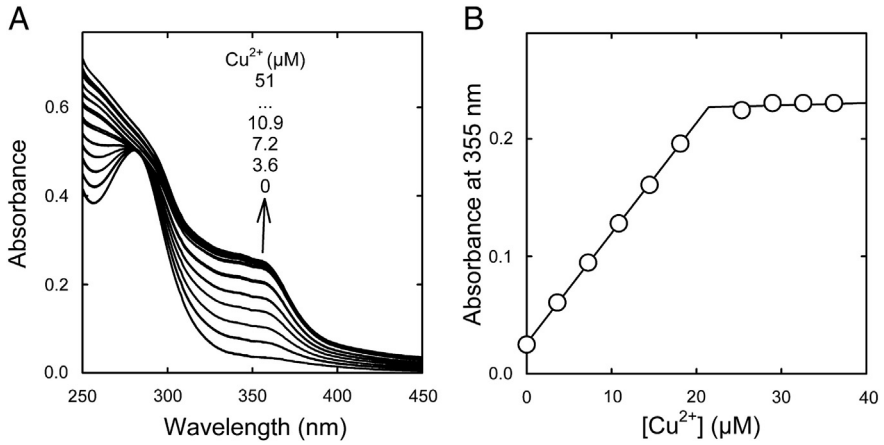

Fig. 3. Spectrophotometric titration of elesclomol by Cu^{2+}. A. UV–vis spectral changes observed when microliter amounts of $CuCl_2$ were added to 20 μM elesclomol in Tris buffer (10 mM, pH 7.4, 25% DMSO) at 37 °C in a 1 cm spectrophotometer cell. As indicated by the arrow, the lowest trace at the 355 nm shoulder increased in absorbance with the successive total concentrations of Cu^{2+} indicated. B. Spectrophotometric titration of elesclomol by Cu^{2+} at 355 nm. The intersection of the least squares calculated straight lines occurred at 21 μM which was consistent with the formation of a 1:1 complex between Cu^{2+} and elesclomol.

to that seen in Fig. 3A indicated that Cu^{2+} did not displace Ni^{2+} from Ni(II)-elesclomol to produce active Cu(II)-elesclomol.

Ligands that strongly bind Cu^{2+} often also bind Fe^{3+} or Fe^{2+}. It had been reported in an ESI mass spectrometry study that elesclomol may be binding to Fe^{2+} [18]. However, no evidence for binding in solution phase was seen during chromatographic separation [18]. Thus spectrophotometric experiments were carried out to determine if elesclomol bound to either Fe^{2+} or Fe^{3+}. The lack of any significant UV–vis spectral changes upon the addition of either ferrous ammonium sulfate or $FeCl_3$ in 3-fold excess over 10 μM elesclomol in Tris buffer (pH 7.4) was consistent with Fe^{3+} and Fe^{2+} having low affinity for elesclomol.

3.4. Kinetic and equilibrium competition reactions of TRIEN with Cu(II)-elesclomol

The competition reaction of TRIEN (triethylenetetramine) with Cu(II)-elesclomol (20 μM) was followed spectrophotometrically in Tris buffer (10 mM, pH 7.4, 37 °C, 25% (v/v) DMSO) both in order to examine the lability of the Cu(II)-elesclomol complex in competition with this nitrogen-containing ligand, and to obtain a measure of the conditional stability constant for Cu(II)-elesclomol, relative to that of TRIEN. TRIEN binds Cu^{2+} very strongly with a stability constant $K_{Cu\text{-}TRIEN}$ (ß) value of $10^{20.0}$ M^{-1} [19]. As shown in Fig. 4 the reaction of

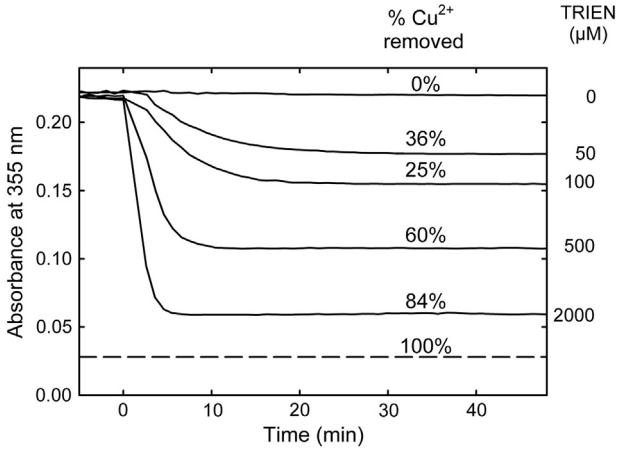

Fig. 4. Kinetics of the reaction of various concentrations of TRIEN with Cu(II)-elesclomol (20 μM) in Tris buffer (10 mM, pH 7.4, 25% DMSO) at 37 °C in a 1 cm spectrophotometer cell followed at 355 nm. Increasing concentrations of TRIEN displaced an increasing amount of Cu^{2+} from the Cu(II)-elesclomol complex at equilibrium. The broken dashed line is the absorbance that would be achieved for 100% removal of Cu^{2+} from Cu(II)-elesclomol.

TRIEN with Cu(II)-elesclomol proceeded relatively quickly, (e.g. $t_{1/2}$ of 3.3 min at 100 μM TRIEN). Even when TRIEN was in 100-fold excess (2000 μM), it still only partially displaced Cu^{2+} from Cu(II)-elesclomol. The stability of the Cu(II)-elesclomol complex relative to that of Cu(II)-TRIEN can be obtained from the concentrations of the various species at equilibrium using a spectrophotometric method that has been described for a copper binding peptide [20]. The small contributions to the absorbance of free elesclomol and Cu(II)-TRIEN are indicated by the broken horizontal line on Fig. 4.

In the expressions below Cu–E and E are Cu(II)-elesclomol and elesclomol, respectively, and Cu-TRIEN and TRIEN are Cu(II)-TRIEN and TRIEN, respectively. $K'_{Cu\text{-}E}$ and $K'_{Cu\text{-}TRIEN}$ are the pH-dependent conditional stability constants in equilibrium expressions (3) and (4), respectively. In the equilibrium expressions the concentrations refer to the total concentration of all protonated and non-protonated form of each species. The expressions for the competing equilibria (1) and (2) and the conditional stability constant expressions (3) and (4) are:

$$Cu{-}E \rightleftharpoons Cu + E \tag{1}$$

$$Cu{-}TRIEN \rightleftharpoons Cu + TRIEN \tag{2}$$

$$K'_{Cu-E} = [Cu{-}E]/[Cu][E] \tag{3}$$

$$K'_{Cu-TRIEN} = [Cu{-}TRIEN]/[Cu][TRIEN]. \tag{4}$$

Because both TRIEN and elesclomol bind Cu^{2+} strongly, at equilibrium the free Cu^{2+} concentration is very low relative to the other equilibrium concentrations. Thus the ratio of the two conditional stability constants from Eqs. (3) and (4) gives:

$$K'_{Cu-E}/K'_{Cu-TRIEN} = [Cu{-}E][TRIEN]/[Cu{-}TRIEN][E]. \tag{5}$$

From the stoichiometry of reactions (1) and (2) and the starting and equilibrium concentrations calculated from the data in Fig. 4 it was possible to calculate values for the ratio $K'_{Cu\text{-}E}/K'_{Cu\text{-}TRIEN}$. The average $K'_{Cu\text{-}E}/K'_{Cu\text{-}TRIEN}$ ratio calculated from the four TRIEN concentrations was 24 ± 1. Thus, at pH 7.4 elesclomol binds Cu^{2+} 24-fold more strongly than does TRIEN.

The pH-dependent conditional stability constant $K'_{Cu\text{-}TRIEN}$ for reaction (2) at pH 7.4 can be calculated from the successive pK_a's of protonated TRIEN of 3.59, 6.77, 9.22 and 9.81 [19] and the fraction α_{TRIEN} of TRIEN present in its neutral bound form. The fraction α_{TRIEN} at pH 7.4 is 4.7×10^{-5} which yielded a conditional stability constant

$K'_{Cu\text{-}TRIEN} = \alpha_{TRIEN} \times K_{Cu\text{-}TRIEN} = 10^{15.7}\ M^{-1}$. The conditional stability constant for $K'_{Cu\text{-}E}$ for Cu(II)-elesclomol was 24-fold higher, and thus it was calculated from the average $K'_{Cu\text{-}E}/K'_{Cu\text{-}TRIEN}$ ratio to have a value of $K'_{Cu\text{-}E} = 24 \times K'_{Cu\text{-}TRIEN} = 10^{17.1}\ M^{-1}$. This value compares to a conditional stability constant of $10^{18.8}\ M^{-1}$ at pH 7.4 determined for a 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazato) copper(II) complex [21].

From a distribution of the protonated species for elesclomol it is possible to calculate a stability constant $K_{Cu\text{-}E}$ (ß) for reaction (1). The pK_a's for elesclomol have not been measured experimentally and likely would be difficult to measure given its low micromolar solubility in aqueous media. Thus MarvinSketch and its associated calculator plugins were used to calculate the elesclomol pK_a values and the major protonated microspecies present at pH 7.4 (Marvin version 5.4, 2010, ChemAxon, http://www.chemaxon.com). The pK_a values for elesclomol were thus calculated to be 10.45 and 11.41. From these values the fraction α_E of the dianionic elesclomol species present at pH 7.4 was calculated to be 7.8×10^{-8}. Therefore the stability constant $K_{Cu\text{-}E}$ can be calculated from the conditional stability constant and α_E to yield a value of $K_{Cu\text{-}E} = K'_{Cu\text{-}E}/\alpha_E = 10^{17.1}\ M^{-1}/7.8 \times 10^{-8} = 10^{24.2}\ M^{-1}$.

3.5. Comparison of the cell growth inhibitory properties of elesclomol and its complexes with Cu^{2+}, Ni^{2+} and Pt^{2+}

Cu^{2+} complexes may be reduced under biological conditions both enzymatically and by non-enzymatic reductants such as ascorbic acid, glutathione or NADH. However, complexes of Ni^{2+} or Pt^{2+} would typically not be expected to be reduced under biological conditions. Thus, a comparison of the K562 cell growth inhibitory properties of elesclomol and its complexes with Cu^{2+}, Ni^{2+} and Pt^{2+} should shed light on whether a biologically reducible redox active metal ion is required for the cell growth inhibitory effects of elesclomol and its complexes. The cell growth inhibitory IC_{50} values for elesclomol, Cu(II)-elesclomol, Ni(II)-elesclomol and Pt(II)-elesclomol were determined from the data shown in Fig. 5. K562 cell growth was inhibited with IC_{50} values of 8.2, 5.4, 185 and 5600 nM for elesclomol, Cu(II)-elesclomol, Ni(II)-elesclomol and Pt(II)-elesclomol, respectively. Both elesclomol and Cu(II)-elesclomol inhibited K562 cell growth with similar IC_{50} values in the low nM range. However, in contrast, both Ni(II)-elesclomol and Pt(II)-elesclomol inhibited cell growth much less potently with IC_{50} values that were 34- and 1040-fold larger than for Cu(II)-elesclomol. For comparison a previous study showed that a 48 h treatment with Cu(II)-elesclomol inhibited breast cancer MDA-MB345 cell growth with an IC_{50} of 30 nM [1]. The lack of activity of the Ni(II)-elesclomol complex was also noted in an abstract [22].

3.6. Comparison of the oxidation of ascorbic acid by Cu(II)-elesclomol and Cu^{2+}

Cu^{2+} and Cu^{2+} chelates are well known to be able to efficiently catalyze the oxidation of ascorbic acid [23]. The first step in the copper-catalyzed oxidation would initially yield the ascorbate radical which would in turn react with O_2 to produce dehydroascorbic acid. At longer times dehydroascorbic acid is oxidized further to other more highly oxidized species [24]. In this redox cycling system the reaction of O_2 with Cu^+ would directly yield superoxide anion, which would in turn dismutate to H_2O_2. The kinetics of the oxidation of 1 mM ascorbic acid was followed by monitoring the first few percent of the reaction in Tris buffer (10 mM, pH 7.4) in 25% DMSO at 25 °C in a 0.1 cm spectrophotometer cell at the peak maximum of 268 nm. A 0.1 cm cell was used to reduce the background absorbance of the 25% DMSO present in the buffer. The initial velocities were calculated from the rate of decrease in absorbance and the change in extinction coefficient observed under our reaction conditions. A linear least squares fit of the initial velocity data of Fig. 6 showed that Cu^{2+} oxidized ascorbic acid 14.6-fold faster than did Cu(II)-elesclomol.

4. Discussion

The results of this study showed that elesclomol formed complexes with Cu^{2+}, Ni^{2+} and Pt^{2+}. The X-ray structure of Cu(II)-elesclomol (Fig. 2) showed that binding of Cu^{2+} occurred with the loss of two protons from the N1 and N2 atoms (Fig. 2) of elesclomol to produce a neutral Cu(II)-elesclomol complex. Cu(II)-elesclomol had bond lengths and bond angles similar to Cu(II) thiosemicarbazone complexes [7,16]. A spectrophotometric titration of elesclomol by Cu^{2+} at pH 7.4 also showed the formation of a strongly bound 1:1 complex in pH 7.4 Tris buffer.

The copper(II) binding ligand TRIEN was shown to be able to only partially displace Cu^{2+} from its complex with elesclomol even in large excess. This indicated that at pH 7.4 elesclomol bound Cu^{2+} much more strongly than did TRIEN. From the $K'_{Cu\text{-}E}/K'_{Cu\text{-}TRIEN}$ ratios calculated elesclomol bound Cu^{2+} 24-fold more strongly than did TRIEN at pH 7.4. The stability constant of Cu(II)-elesclomol was thus determined to be $10^{24.2}\ M^{-1}$ compared to $10^{20.0}\ M^{-1}$ for Cu(II)-TRIEN [19]. Because elesclomol bound Cu^{2+} so strongly it would be expected to dissociate extremely slowly from its complex. Thus, the fact that

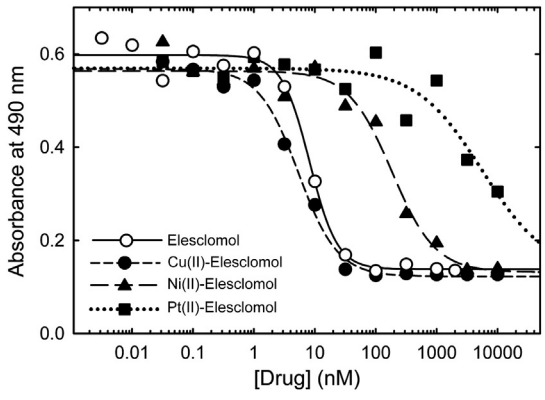

Fig. 5. Comparison of the growth inhibitory effects of elesclomol (○), solid line; Cu(II)-elesclomol (●), short dashed line; Ni(II)-elesclomol (△), long dashed line; and Pt(II)-elesclomol (■), dotted line on K562 cells. The cells were treated with the compounds for 72 h prior to the assessment of growth inhibition by an MTS assay. The curved lines were calculated from non-linear least squares fits to 4-parameter logistic equations and yielded IC_{50} values of 8.2 ± 0.9, 5.4 ± 0.6, 185 ± 48 and 5600 ± 1900 nM, for elesclomol, Cu(II)-elesclomol, Ni(II)-elesclomol and Pt(II)-elesclomol, respectively.

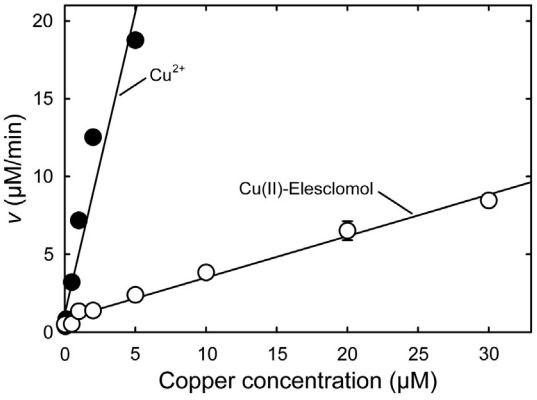

Fig. 6. Comparison of the initial velocities for the oxidation of 1 mM ascorbic acid by Cu(II)-elesclomol (○) and $CuCl_2$ (●). The initial velocities were measured by following the decrease in absorbance at 268 nm in Tris buffer (10 mM, pH 7.4, 25% DMSO at 25 °C) in a 0.1 cm spectrophotometer cell. The straight lines were linear least squares calculated. A comparison of the slopes indicated that Cu^{2+} oxidized ascorbic acid at a rate that was 14.6-fold faster than did Cu(II)-elesclomol.

the reaction with 100 μM TRIEN occurred with a $t_{1/2}$ of 3.3 min suggests that TRIEN may have displaced Cu^{2+} from Cu(II)-elesclomol through an associative displacement mechanism. An associative displacement reaction could also provide a mechanism by which copper binding proteins could remove copper from Cu(II)-elesclomol.

It has been proposed [1,2] that elesclomol exerts its in vitro cell growth inhibitory activity by scavenging copper from the culture medium. The fact that elesclomol and Cu(II)-elesclomol potently inhibited the growth of K562 cells in the low nanomolar range to about the same extent is consistent with this proposal. This result also indicated that our culture medium contained sufficient adventitious copper in the medium and/or copper binding proteins in the added serum for elesclomol to exert nearly its full potency by complexing with this available Cu^{2+}. Because Ni(II)-elesclomol and Pt(II)-elesclomol were 34- and 1040-fold less potent at inhibiting K562 cell growth, respectively, than Cu(II)-elesclomol, these results suggest that the redox inactive Ni^{2+} and Pt^{2+} complexes of elesclomol were almost inactive compared to Cu(II)-elesclomol. Given the fact that elesclomol itself displays cell growth inhibitory activity it was possible that what activity the Ni^{2+} and Pt^{2+} complexes did possess was due to partial dissociation of these complexes to produce free elesclomol. This free elesclomol could then bind Cu^{2+} from the media and exert its cell growth inhibitory activity through its copper complex. In a liquid chromatography–mass spectrometry study elesclomol was shown to bind Cu^{2+} much more strongly than Ni^{2+} [18] consistent with the Irving–Williams series [25] for ligand binding to divalent metals. This would be consistent with the Ni(II)-elesclomol complex exerting its cell growth inhibitory effects through partial dissociation to produce elesclomol. No spectrophotometric evidence was seen for Cu^{2+} being able to directly replace Ni^{2+} from its complex with elesclomol.

Elesclomol has been proposed to exert its anticancer activity by selectively transporting copper to the mitochondria and inducing oxidative stress [1,2]. It has been shown that elesclomol chelates Cu^{2+} outside of the cell and transports it in as the neutral Cu(II)-elesclomol complex [1]. Enzymatic or non-enzymatic reduction of the Cu^{2+} accumulated in the mitochondria would yield Cu^{+} species, which would be readily oxidized by O_2. It has also been proposed that after reduction to Cu^{+} and dissociation of the complex, the elesclomol is effluxed from the cell and continues to shuttle more Cu^{2+} into the cell [1]. Elesclomol may be exerting its cell growth inhibitory and anticancer activity by utilizing the copper present in blood. The major sources of copper in blood are from the plasma copper protein carriers albumin, transcuprein, and ceruloplasmin [26].

This study has also shown that Cu(II)-elesclomol can efficiently oxidize ascorbic acid at physiological ascorbic acid concentrations [27]. Reaction of the Cu^{+} produced with O_2 would yield superoxide which would dismutate to produce damaging H_2O_2 [28]. The reaction of H_2O_2 with Cu^{+} could also produce the even more damaging and highly reactive hydroxyl radical [28].

In conclusion, the results of this study have shown that Cu^{2+} binds elesclomol to form a 1:1 neutral complex that has a distorted square planar structure. The Ni(II)-elesclomol and Pt(II)-elesclomol complexes that were synthesized were much less potent than Cu(II)-elesclomol in inhibiting K562 cell growth. This result suggests that the inhibition of cell growth and the anticancer activity of elesclomol are dependent upon formation of its complex with Cu^{2+}, and possibly through the formation of damaging reactive oxygen species mediated through the reduction of Cu^{2+}. The Cu(II)-elesclomol complex was reduced by physiological concentrations of ascorbic acid. Thus, our results using a variety of assays, indicate that coordination with redox active Cu^{2+} was likely necessary for elesclomol to exert its activity. Our subsequent investigations of elesclomol will proceed with a focus on other specific potential targets such as DNA damage and inhibition/inactivation of redox sensitive enzymes critical for cell growth.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2013.04.013.

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