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Title: First-row transition-metal norharmane compounds as possible cytotoxic agents: new insights based on a coordination chemistry perspective

Article Type: SI: Cancer Metallodrugs

Keywords: copper; cobalt; nickel; zinc; 9H-Pyrido[3,4-b]indole; antiproliferative properties

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Abstract: New first-row transition-metal compounds with the ligand norharmane (9H-Pyrido[3,4-b]indole; Hnor) are reported. The compounds have the general formula [M(LL)(Hnor)(NO3)2](MeOH)0-1 (M=Co, Ni, Cu, Zn; LL = 2,2'-bipyridyl (bpy), 1,10-phenanthroline (phen)) and have been characterized by physical and analytical methods. X-ray structural analysis revealed that the compound of formula [Cu(phen)(Hnor)(NO3)2], (1) has a distorted 6-coordinated octahedrally-based geometry, with a planar-based [CuN3O] core, where Cu-L varies between 1.99-2.04 Å and two weak axial Cu-O contacts (2.209 and 2.644 Å) from two different nitrates. Based on spectroscopic similarities, the other compounds appear to have the same or very similar coordination geometries. The compounds showed clear cell growth inhibitory effects in two different cancer cell lines in vitro, with the copper and zinc complexes being the most toxic and in fact almost comparable to cisplatin. Flow-cytometry analysis confirmed induction of apoptosis in cancer cells treated with the compounds. Interestingly, co-incubation of the cells with metal complexes and CuCl2 induced an increase in the cytotoxic effects, most likely due to the conversion of the metal compounds in the corresponding, and most active, copper analogues.

Cover Letter

COVERLETTER JIB Special Issue Cancer & Metallodrugs, CEMM; February 29

Dear Editors:

It is our pleasure to submit to JIB the manuscript:

First-row transition-metal norharmane compounds as possible cytotoxic agents: new insights based on a coordination chemistry perspective

Authors: Rais Ahmad Khan,[1#] Andreia de Almeida,[2#] Khalid Al-Farhan,[1] Ali Alsalme,[1] Angela Casini,[2,3*] Mohamed Ghazzali,[1] and Jan Reedijk[1,4,*]

Corresponding authors: email: CasiniA@cardiff.ac.uk and Reedijk@chem.leidenuniv.nl;

The manuscript is accompanied by supporting information of routine spectral data to characterize the compounds. All files are uploaded as requested. The manuscript is meant for the CEMM symposium special issue.

We have also suggested names for potential reviewers.

All co-authors have given their consent for co-authorship and agreement with the final version

Looking forward to hear the outcome of the referee process.

Kindest regards

Angela Casini and Jan Reedijk.

***Response to Reviewers**

Replies to Referees; manuscript: JIB-16-0252R June 2016

Dear John:

Thanks for the message about the provisional acceptance of the manuscript with the number given above. (Rais et al.)

We have now prepared a revised manuscript and as requested. Below we address all referee comments in detail, and indicate how we changed the manuscript, also according to your own wishes. All changes in the revised ms file are marked in yellow highlights, to allow easy comparison.

W also have followed the list of guidelines given by your editor mail for the technical instructions as close as possible.

We hope we have sufficiently dealt with all comments and suggestions and look forward to your final decision.

Kindest regards

Jan

Reviewer #1:

Reviewer #1: Casini, Reedijk and co-worker have improved their submission entitled "First-row transition-metal norharmane compounds as possible cytotoxic agents: new insights based on a coordination chemistry perspective". Ignoring the different view of autoplagiarism (see below), the authors must fix the few errors in the references.
>It is now generally accepted that experimental details from one paper can be repeated in a later paper to assist the reader. This type of autoplagiarism is acceptable; but to please the referee we have now paraphrased the text.
There is NO development to accept self-plagiarism! In the ACS "Ethical Guidelines to Publication of Chemical Research" (http://pubs.acs.org/userimages/ContentEditor/1218054468605/ethics.pdf) we can read:
"Authors should not engage in self-plagiarism (also known as duplicate publication) - unacceptably close replication of the author's own previously published text or results without acknowledgement of the source."
In my opinion, by copying the experimental procedure from an earlier own work and especially without citing the paper in the section, the authors gave the wrong impression that this is novel experimental work.
Indeed, as we had phrased it, it could still be misleading as we had not yellow highlighted ref 55; we now have explicitly added more details inn this paragraph.
Author names and pages numbers of ref. 36 contains errors Indeed, we have now corrected this reference.
Is ref. 60 still in press? This publication cannot be found. This reference has indeed appeared and has been updated.
No volume numbers exist for ref. 67 and 71 These Volume numbers were added by our Endnote software, but have been removed now.

Reviewer #2: The authors addressed all the many concerns raised by the reviewers and have revised the English grammar mistakes and typos in the revised version of the manuscript. I believe that this work should be accepted at this point without further corrections. *No reply comment needed*

Reviewer #3: This manuscript has been revised carefully and I feel that it could be accepted for publication at present case. *No reply comment needed.*

Editor comments:

<Stylistic Changes and Other Issues> (From the Editorial Office) *Replies are in red italics.*

1. Six Keywords are allowed. Please reduce the current number of Keywords from seven to six.
In fact we had already 6 keywords (not 7) in the ms; now we have also 6 in the web space,.
2. We try to avoid having undefined non-standard abbreviations in the self-standing sections of the manuscript, which includes the Abstract, the Synopsis for the Graphical Abstract, the Highlights and the text. Please bear in mind that the readership of JIB is quite broad and abbreviations that are common in one area of bioinorganic research may not be standard in other areas.

a. In the Synopsis for the Graphical Abstract, bpy and phen are used without definition. Please define each (or just spell them out since each is only used once). In making these changes, be sure to keep the final Synopsis to 50 words or less. *OK, we have done so (and have 41 words)*.

b. In the text, please carefully go through the entire manuscript to be sure all abbreviations are defined when first used. On page 2, Hnor is used without definition. Since you have a Table of Abbreviations, please include all abbreviations in the Table. *OK, we have done so*

3. Highlights 2 and 3 are too long. Each Highlight must be 90 characters or less including spaces (The number "90" is already higher than the official number, but we find that highlights of up to 90 characters including spaces are usually accepted by the production office). In shortening Highlights 2 and 3, you could split one of these Highlights into two highlights since five Highlights are allowed and you currently have four.
OK, we have done so; each highlight is below 90 characters now.
2. Figures can be printed in color in the print version of the journal only when the color is essential to the message of the figure. I will approve Figures 1 and 3 to be printed in color in both the print and online versions of the journal (at no cost).*Thanks for this kind offer which we gladly accept.*
Scheme 1 can appear in black and white in the print version but in color (at no charge) *OK* in the online version of the journal. Figures 2 and 4 are already in black and white.

==========end of replies ==========

*Pictogram for the Graphical Abstract

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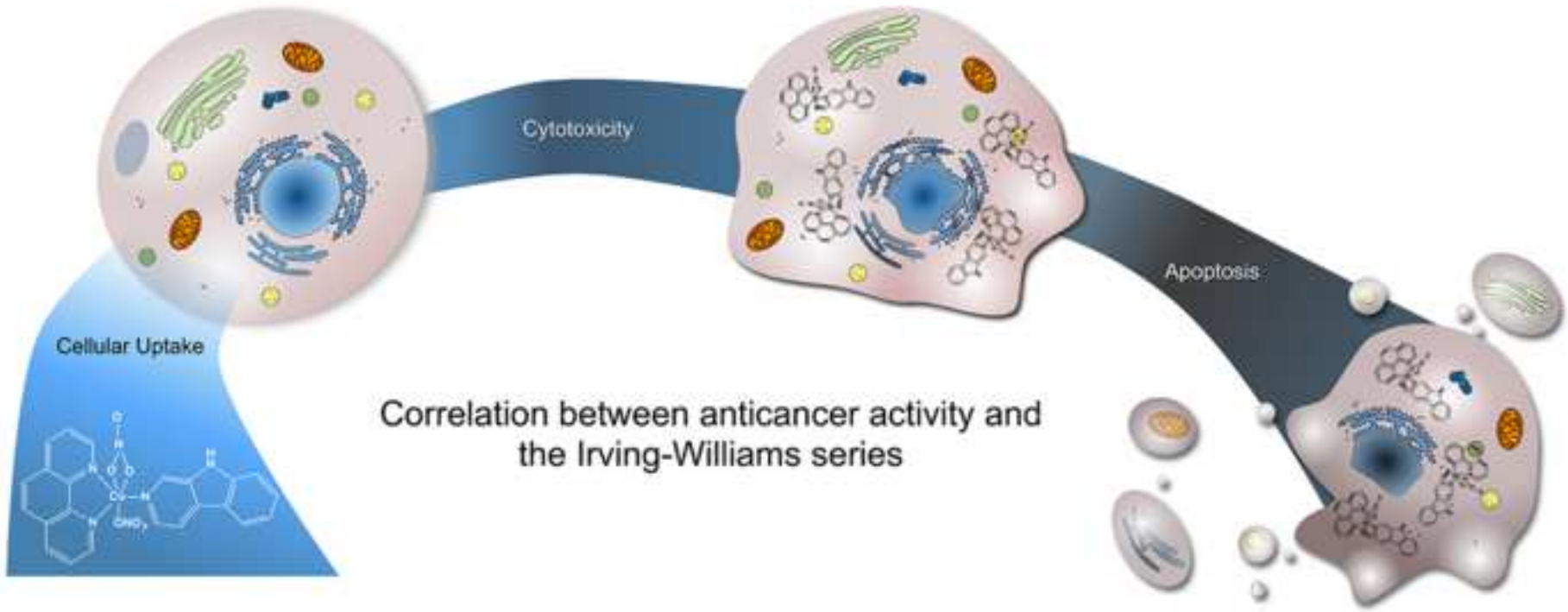
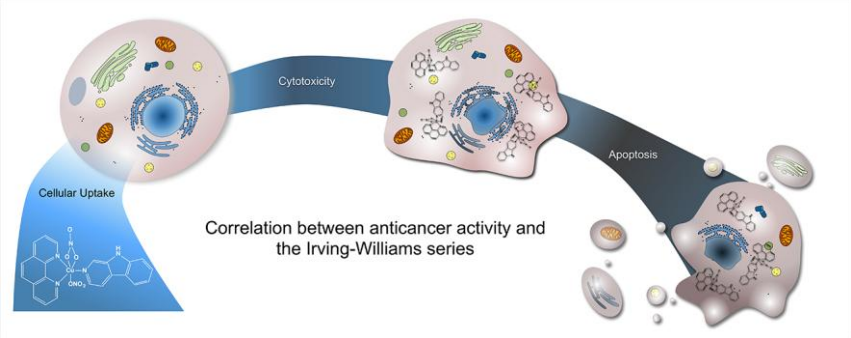


***Synopsis for the Graphical abstract**

graphabs + added text for the paper: JIB-16-0252

Transition-metal norharmane compounds as possible cytotoxic agents: new insights based on a coordination chemistry perspective

Rais Ahmad Khan, Andreia de Almeida, Khalid Al-Farhan, Ali Alsalme, Angela Casini, Mohamed Ghazzali, and Jan Reedijk'



Copper, Nickel, Cobalt and Zinc compounds with the ligand norharmane and with 2,2'-bipyridyl (bpy) and 1,10-phenanthroline (phen) as co-ligands are presented, including a 3D structure. Evidence for cell growth inhibitory effects in two different cancer cell lines *in vitro*, are presented.

Highlights for the JIB paper: JIB-16-0252

First-row transition-metal norharmane compounds as possible cytotoxic agents: new insights based on a coordination chemistry perspective

Rais Ahmad Khan, Andreia de Almeida, Khalid Al-Farhan, Ali Alsalme, Angela Casini, Mohamed Ghazzali, and Jan Reedijk'

Highlights:

- New compounds with Co, Ni, Cu and Zn containing norharmane have been prepared
- The structure of a Cu(II) compound with norharmane and phenanthroline has been determined
- The Cu and Zn compounds show cytotoxic properties in different cancer cell lines
- The cytotoxic properties are comparable to cisplatin
- The activity of the reported coordination complexes follows the Irving-Williams series

Submitted to JIB (Special Issue CEMM) 2016; Revised-II, JUne 2016

Transition-metal norharmane compounds as possible cytotoxic agents: new insights based on a coordination chemistry perspective

Rais Ahmad Khan,[1#] Andreia de Almeida,[2#] Khalid Al-Farhan,[1] Ali Alsalme,[1] Angela Casini,[2,3*] Mohamed Ghazzali,[1] and Jan Reedijk[1,4,*]

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Abstract:

New first-row transition-metal compounds with the ligand norharmane (*9H*-Pyrido[3,4-*b*]indole; Hnor) are reported. The compounds have the general formula $[M(LL)(Hnor)(NO_3)_2](MeOH)_{0\text{-}1}$ (M=Co, Ni, Cu, Zn; LL = 2,2'-bipyridyl (bpy), 1,10-phenanthroline (phen)) and have been characterized by physical and analytical methods. X-ray structural analysis revealed that the compound of formula $[Cu(phen)(Hnor)(NO_3)_2]$, (**1**) has a distorted 6-coordinated octahedrally-based geometry, with a planar-based $[CuN_3O]$ core, where Cu-L varies between 1.99-2.04 Å and two weak axial Cu-O contacts (2.209 and 2.644 Å) from two different nitrates. Based on spectroscopic similarities, the other compounds appear to have the same or very similar coordination geometries. The compounds showed clear cell growth inhibitory effects in two different cancer cell lines *in vitro*, with the copper and zinc complexes being the most toxic and in fact almost comparable to cisplatin. Flow-cytometry analysis confirmed induction of apoptosis in cancer cells treated with the compounds. Interestingly, co-incubation of the cells with metal complexes and $CuCl_2$ induced an increase in the cytotoxic effects, most likely due to the conversion of the metal compounds in the corresponding, and most active, copper analogues.

***Keywords*:** copper; cobalt; nickel; zinc; *9H*-Pyrido[3,4-*b*]indole; antiproliferative properties;

Introduction

The rational design of innovative metal-containing compounds has been a major goal in cancer research in recent years. While initially most of the studies have been largely focusing on platinum compounds, more recently other noble metals, like gold, osmium and ruthenium have attracted attention [1, 2]. Also silver(I) complexes containing various types of ligands, including carboxylic acids, amino acids, nitrogen-, phosphorus-, or sulfur-donor ligands, have been prepared and studied for their antitumor activity [3, 4]. In fact, some of them exhibit significant *in vitro* antiproliferative properties [3, 4]. Interestingly, for the kinetically more labile first-row transition metals, coordination compounds are known that hold great potential as anticancer agent, and earlier studies supported their drug design [5-8]. Earlier studies by Sigman in the 1990s [9-13], and by Pitié and Meunier [14-16] and several others in recent years [17-23] indicate efficient DNA cleavage and cytostatic properties. Such compounds are also reported to display antiangiogenic activity [24]. Also, studies by some of us with Cu(II) compounds and tridentate pyridine-based ligands have shown very promising results for both DNA cleavage and anticancer activities [25-28].

Within this frame, the results by Ruiz *et al.* hold great promise, and include the group of metal compounds named *casiopeinas*, copper-based coordination complexes with generic structure of $[Cu(N–N)(O–N)]^+$ or $[Cu(N–N)(O–O)]^+$ that have proven cytotoxic to cancer cells sensitive or resistant to cisplatin, and to xenograph tumors in mice [29, 30], and are now in clinical trials. The mode of action for casiopeinas is largely unknown, but it has been suggested that mitochondrial permeability transition may be important [31], as well as DNA damage [32] and others [33].

In general, investigation of first-row transition metal complexes as cytotoxic agents has received increasing attention, because of the development of metallodrugs containing endogenous metal ions (instead of non-physiological ones, with potentially severe side effects) is attractive. Therefore, many studies have been reported during the last 5 years, including those disclosing the 3D structure of the respective compounds [5, 34, 35]. Nevertheless, the mode of action of such metal complexes is still poorly understood; nucleic acids and proteins are likely targets [36-39]. For example, Cu(II), Co(II) and Zn(II) complexes featuring the quinolone antibacterial drug flumequine and N,N'-donor heterocyclic compounds as ligands have also been demonstrated to be able to interact with nucleic acids and serum albumin [40-43]. Instead, cytotoxic Co(III) complexes with substituted phenanthrolines have shown weak binding to nucleic acids [40].

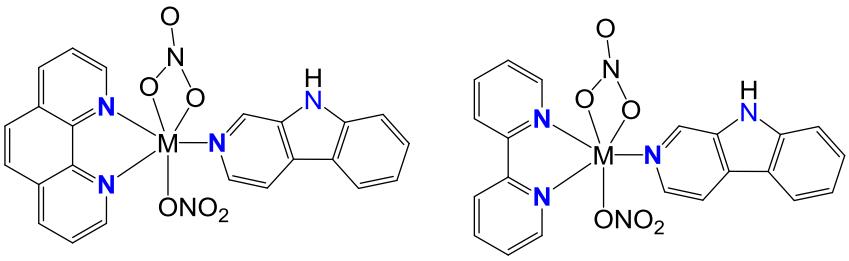
Based on the above-mentioned considerations, in order to design new first-row transition metal compounds with enhanced thermodynamic stability in aqueous solution and with possible antiproliferative activity, we have selected the ligand norharmane (*9H*-Pyrido[3,4-*b*]indole, abbreviated as Hnor and depicted in Scheme 1), consisting of a fused-ring heterocyclic compound that belongs to the alkaloid family β-carbolines (βCs). This molecule has been known for over 50 years [44-49], but it is still poorly studied. It has a remote H-bond donor group, in addition to the metal binding site, and so far it has only been used to coordinate Ru(II) ions [49] and more recently silver(I) ions [50]. In addition to coordination, also other weak forces, like hydrogen bonding and intermolecular non-covalent interactions should be taken into account [51-55]. In this respect Hnor

appears to have a suitable structure to bind metal compounds, where coordination, H-bonding and π-π stacking may act in synergy. Interestingly, so far a wide spectrum of biological, psychopharmacological, and toxicological activities have been reported for Hnor, e.g. anticancer [56], binding to benzodiazepine receptors [57], inhibition of monoamine oxidase [58], and DNA topoisomerase I and II [59].

Despite the fact that several known transition-metal compounds display antiproliferative properties, the combination of certain metals and different (potentially) intercalating ligand remains challenging and worth studying.

Following our recent promising results with Ag(I) complexes of Hnor [50], we have extended here our study to first-row transition metals, and simultaneously added a polypyridine co-ligand, i.e. 2,2'-bipyridyl (bpy), or 1,10-phenanthroline (phen), which are known to be able to interact with DNA and also possess cell-growth inhibition properties [60]. Thus, we report here on the synthesis and characterization of eight new coordination compounds containing the ligands Hnor and 1,10-phenantroline (phen)/2,2'-bipyridyl (bpy) bound to Cu(II), Co(II), Ni(II) and Zn(II) ions (Scheme 1). Notably, the NO_3^- anion was chosen for charge neutrality and as an additional ligand, as this is a potentially weakly coordinating anion, and it is at the same time prone to H-bond acceptance. Thus, the potentially tridentate anionic ligand NO_3^- may act in both monodentate and chelating forms to complete the metal coordination sphere [61]. X-ray diffraction studies allowed elucidating the structure of the copper complex, with phen as co-ligand, **1**.

Some of the obtained compounds were tested for their effects on cell viability against two human cancer cell lines, and evaluation of their apoptotic effects was performed by flow-cytometry. In order to study transport of the compounds *via* copper transporters, the effects of addition of "free" Cu(II) to the cell culture medium were also evaluated; this is a classical competition experiment to assess transport mechanisms [62], but allowed also to relate the observed biological activity to the stability of the metal compounds in solution.



M= Cu, Ni, Co, Zn

$[Cu(Hnor)(phen)(NO_3)_2]$	**1**	$[Cu(Hnor)(bpy)(NO_3)_2]$	**2**
$[Co(Hnor)(phen)(NO_3)_2](MeOH)$	**3**	$[Co(Hnor)(bpy)(NO_3)_2]$	**4**
$[Ni(Hnor)(phen)(NO_3)_2](MeOH)$	**5**	$[Ni(Hnor)(bpy)(NO_3)_2]$	**6**
$[Zn(Hnor)(phen)(NO_3)_2]$	**7**	$[Zn(Hnor)(bpy)(NO_3)_2]$	**8**

Scheme 1 – Schematic description of the metal compounds with Hnor and phen/bpy ligands used in this study.

Experimental Part

Starting Materials and methods

Metal nitrate salts were all used as commercially available; tryptophane was obtained as a racemic mixture and used as obtained. Other standard laboratory chemicals were used as available. Cisplatin was purchased from Sigma. Infrared spectra were recorded as KBr pellets, using a Shimadzu IRAffinity-1 spectrometer with a resolution of 4 cm^{-1}. Ligand field spectra were performed on solid powders (room T, diffuse reflectance mode) in the range 200-1100 nm. Elemental analysis (C,H,N) were performed on a PerkinElmer 2400 Series II CHNS/O system. NMR spectra in DMSO solution were recorded using a JEOL-ECP-400 spectrometer.

Synthesis

The ligand norharmane (Hnor) was synthesized from tryptophane and formaline as described by us before [55] according to the method of Snyder et al. [53]. Yield: 3.2 g (32%), M.P.= 199-200 ºC. ^{1}H NMR (400 MHz, DMSO-d_6, ppm, 293 K):11.65 (1H, s, NH); 8.94 (1H, s, H_1); 8.35(1H, d (J = 5.1 Hz), H_2); 8.22 (1H, d (J = 8.0 Hz), H_4); 8.10 (1H, d (J = 5.1 Hz), H_7); 7.56 (2H, ddd (J = 8.0, 16.8, 7.3 Hz), H_3, H_6); 7.24 (1H, t (J = 14.6 Hz), H_5). Elemental Analysis for $C_{11}H_8N_2$ (%): Calcd. C, 78.55; H, 4.79; N, 16.66. Found C, 78.46; H, 4.71; N, 16.62.

Transition-metal compounds **1-8** (see Scheme 1) with the ligand Hnor were prepared by dissolving the metal salt and Hnor (1:1) in methanol (MeOH) and adding a co-ligand bpy or phen (also in the ratio 1:1). A typical recipe is as follows: 2,2'-Bipyridyl/1,10-phenanthroline (1 mmol) was added to the solution of metal salts (1 mmol) in MeOH, and after 30 min of stirring, Hnor (1 mmol) was added to the mixture and allowed to stir for 12 h and then kept for slow evaporation. The crystalline product obtained was washed with CH_2Cl_2, ether, hexane. The product obtained was dissolved in a MeOH/acetonitrile mixture for re-crystallization.

The new compounds are listed below with their color and elemental analysis and relevant IR and UV data.

Compounds with phen (1,10-phenanthroline) as a co-ligand

1: $C_{23}N_6O_6H_{16}Cu$, Cu(Hnor)(phen)$(NO_3)_2$: green, structurally characterized by XRD (see below); Calcd. % C 51.54; H, 3.01; N, 15.68. Found: C, 51.18; H, 2.97; N, 15.57. Yield: 0.438 g (74%). FTIR (KBr disc, cm^{-1}): 3438, 1635, 1466, 1427, 1385, 1304, 1255, 1034, 849, 723. UV-Vis: λ_{max} (nm) (solid): 257, 301, 371, 663.

3: $C_{23}N_6O_6H_{16}Co$: brown; calcd. % C, 51.17; H, 3.58; N, 14.92 (**3** + MeOH); C, 50.36; H, 3.70; N, 14.68; (**3** + MeOH + 0.5H_2O). Found: C, 50.39; H, 3.41; N, 14.45. Yield: 0.50 g (78%). FTIR (KBr disc, cm^{-1}): 3361, 1631, 1462, 1425, 1385, 1292, 1252, 1038, 845, 725. UV-Vis: λ_{max} (nm) (solid): 272, 374, 527.

5: $C_{23}N_6O_6H_{16}Ni$: yellow-green; calcd. % C, 51.19; H, 3.58; N, 14.92 (**5** + MeOH). Found: C, 51.64; H, 3.55; N, 14.87. Yield: 0.49 g (87%). FTIR (KBr disc, cm^{-1}): 3382, 1632, 1457, 1426, 1384, 1302, 1249, 1041, 847, 726. UV-Vis: λ_{max} (nm) (solid): 253, 291, 357, 617.

7: $C_{23}H_{16}N_6O_6Zn$: yellow; calcd. % C, 51.37; H, 3.00; N, 15.63; and C, 50.52; H, 3.13; N. 15.37; (**7** + 0.5 H_2O); Found: C, 50.29; H, 3.07; N, 15.89. Yield: 0.48 g (78%). FTIR (KBr disc, cm^{-1}): 3396,

1632, 1461, 1427, 1384, 1290, 1250, 1036, 847, 725. ^{1}H NMR (400 MHz, DMSO-d_6, ppm, 293 K): 11.83 (1H, s, NH); 9.17 (1H, d (J = 3.6 Hz)); 8.75 (1H, s); 8.93 (2H, d (J = 8.0 Hz)); 8.32-8.15 (8H, m); 7.59 (2H, ddd (8.0, 14.0, 8.0 Hz)); 7.23 (1H, t (J = 14.8 Hz)). ^{13}C NMR (100 MHz, DMSO-d_6, ppm, 293 K): 149.19, 141.10 140.11, 139.48, 137.65, 133.98, 128.74, 128.27, 127.32, 125.91, 122.10, 120.39, 119.62, 112.12.

Compounds with bpy (2,2'-bipyridine) as a co-ligand:

2: $C_{21}H_{16}N_6O_6Cu$: green; calcd. % C, 49.27; H, 3.15; N, 16.42 and C,48.42; H, 3.29, N, 16.13 (**2 +** 0.5H_2O); Found: C, 48.42; H, 3.02; N, 16.41. Yield: 0.54 g (85%). FTIR (KBr disc, cm^{-1}): 3431, 1633, 1603, 1574, 1474, 1443, 1384, 1286, 1253, 829, 772, 730, 634, 420. UV-Vis: λ_{max} (nm) (solid): 281, 358, 656.

4: $C_{21}H_{16}N_6O_6Co$: brown; calcd. % C, 49.72; H, 3.18; N, 16.57 (and C, 48.85; H, 3.32; N, 16.28 (**4** + 0.5H_2O). Found: C, 48.73; H, 3.34; N,16.56. Yield: 0.495 g (77%). FTIR (KBr disc, cm^{-1}): 3418, 1631, 1606, 1565, 1474, 1451, 1384, 1304, 1248, 826, 767, 733, 631, 422. UV-Vis: λ_{max} (nm) (solid): 246, 258, 358, 504.

6: $C_{21}H_{16}N_6O_6Ni$; Yellow-green; calcd. % C, 49.74; H, 3.18; N, 16.57 and C, 48.43; H, 3.60; N, 16.69 (**6** + 0.5CH_3CN +H_2O). Found: C, 48.82; H, 3.41; N, 16.96. Yield: 0.51 g (83%). FTIR (KBr disc, cm^{-1}): 3394, 1632, 1600, 1567, 1474, 1444, 1384, 1302, 1249, 826, 767, 734, 632, 424. UV-Vis: λ_{max} (nm) (solid): 283, 349, 598.

8: $C_{21}H_{16}N_6O_6Zn$: yellow; calcd. % C, 49.09; H, 3.14; N, 16.36. Found: C, 49.03; H, 3.25; N, 16.54. Yield: 0.464 g (75%). FTIR (KBr disc, cm^{-1}): 3390, 1632, 1608, 1567, 1475, 1442, 1384, 1290, 1249, 827, 767, 733, 633, 423. ^{1}H NMR (400 MHz, DMSO-d_6, ppm, 293 K): 11.80 (1H, s, NH); 8.91 (1H, s); 8.73 (2H, (J = 7.2 Hz)); 8.33-8.16 (7H, m); 7.81 (2H, s), 7.59 (2H, ddd (8.8, 12.4, 15.2 Hz)); 7.24 (1H, t (J = 15.6 Hz)). ^{13}C NMR (100 MHz, DMSO-d_6, ppm, 293 K): 148.44, 141.47, 140.98, 137.78, 133.97, 128.63, 128.11, 127.18, 122.76, 122.05, 120.42, 119.56, 112.15.

X-ray diffraction studies

Suitable single crystals of compound [Cu(Hnor)(phen)$(NO_3)_2$] **1** were obtained by slow evaporation from a mixture of MeOH/acetonitrile. Diffraction and refinement data for compound **1** are summarized in Table 1. Diffraction data were collected using a Rigaku *R*-axis diffractometer equipped with imaging plate area detector utilizing Mo-K_α radiation (λ= 0.71073 Å) with graphite monochromator. The data were collected using ω-scans to a maximum 2θ of 55.0° at 294 K. Preliminary orientation matrices, unit cell determination, data reduction and absorption correction were performed using *CrystalClear* package [63]. The data were empirically corrected for Lorentz and polarization effects. The structure was solved by direct methods and refined by full-matrix least squares on all $|F^2|$ data using *SHELX* package [64]. Aromatic hydrogen atoms were isotropically refined and constrained to ideal geometry, using their appropriate riding model and non-hydrogen atoms were anisotropically refined. The Hnor hydrogen atom on N involved in hydrogen bonding was located from difference Fourier map and refined with distance restraint. One of the nitrate oxygen atoms was found disordered over two positions and refined with a split-atom model of 0.54/0.46 occupancy factor. The descriptive crystallographic figures were created using the *DIAMOND* package [65]. All relevant crystallographic data are summarized in Table 1. Powder X-ray diffraction of the Cu(II) phen compound (**1**) have shown a homogenous phase compared to the pattern simulated from the single-crystal diffraction.

Table 1. *Crystallographic and refinement data for the compound [Cu(Hnor)(phen)(NO_3)$_2$]**1**.*

Compound formula	$C_{23}H_{16}N_6O_6Cu$
Formula weight	535.97
Temperature [K]	294 K
Crystal system	Monoclinic
Space group	*C2/c*
a [Å]	31.3912(8)
b [Å]	8.0795(2)
c [Å]	23.9514(5)
β [°]	130.878(1)
Volume [Å^3]	4593.1(2)
Z	8
Calculated density [mg/mm^3]	1.550
Absorption coefficient [mm^{-1}]	1.004
F(000)	2184
Radiation wavelength	0.71073
Θ range for data collection [°]	3.1, 27.5
Index ranges	-40: 40 ; -10: 10 ; -30: 30
Reflections collected	85470
Independent reflections	4126
Data/restraints/parameters	5263/1/339
Goodness-of-fit on F^2	1.00
Final R indexes [I>=2σ(I)]	5.2
Final R indexes (all data)	14.53
Largest diff. peak/hole [e Å^{-3}]	0.47/-0.60

Cell viability assay

The human lung cancer A549 and human ovarian cancer A2780 cell lines, (obtained from the European Centre of Cell Cultures ECACC, Salisbury, UK) were cultured in DMEM (A549) and RPMI (A2780) complete medium, containing GlutaMax-I and supplemented with 10% FBS and 1%

penicillin/streptomycin (all from Invitrogen), at 37 °C in a humidified atmosphere of 95% of air and 5% CO_2 (Heraeus, Germany). For evaluation of growth inhibition, cells were seeded in 96-well plates (Costar, Integra Biosciences, Cambridge, MA) at a concentration of 10000 cells/well and grown for 24 h in complete medium. Solutions of the compounds were prepared by diluting a freshly prepared stock solution (10^{-2} M in DMSO) of the corresponding compound in aqueous media (RPMI or DMEM depending on the cell lines). Stock solutions of the compounds were stable over several hours. The percentage of DMSO in the culture medium never exceeded 0.2%: at this concentration DMSO has no effect on the cell viability. Afterwards, the intermediate dilutions of the compounds were added to the wells (200 µL) to obtain a final concentration ranging from 0 to 200 µM, and the cells were incubated for 24 h or 72 h. Following drug exposure, 3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells at a final concentration of 0.50 $mg.ml^{-1}$ incubated for 3-4 h, time after which the solution was removed and the violet formazan crystals dissolved in DMSO. The optical density of each well (96-well plates) was quantified in quadruplicate at 550 nm, using a multi-well plate reader, and the percentage of surviving cells was calculated from the ratio of absorbance between treated and untreated cells. The IC_{50} value was calculated as the concentration reducing the proliferation of the cells by 50% and is presented as a mean (± SE) of at least three independent experiments. Statistical comparisons were based on a two-way paired t-test. Before conducting the t-test, the normality of the dataset was checked using a Q-Q plot and a histogram and homogeneity of the variance was assessed using a Barlett's test [66].

Flow Cytometry assays

Confluent A549 cells were seeded at a density of 50000 cells/well in 24-well flat-bottom cell culture plates, under standard conditions for 24 h. Afterwards, the medium was substituted by medium containing the appropriate concentrations of compounds, as described above for MTT detection. After 72 h incubation, cells were detached and collected by centrifugation. An apoptosis Annexin V-APC/PI detection kit (eBiosciences, San Diego, USA) was used to analyze cell apoptosis, according to manufacturer's provided protocol. In short, after centrifugation cells were resuspended in binding buffer and stained with APC-conjugated Annexin V, for 15 min at room temperature, protected from light.

Afterwards, cells were stained with propidium iodide (PI) and immediately analyzed by FACS (BD FACS array™, BD Bioscience, San Jose, USA). Corresponding data were analyzed using the FlowJo software (FlowJo, Ashland, USA). The results shown are representative of at least three independent experiments. Viable cells with intact membranes exclude PI, whereas the membranes of dead or damaged cells are permeable to PI while Annexin V binds to phosphatidylserine, a protein expressed in the extracellular side of the cell membrane of cells undergoing apoptosis. Thus, cells that stain positive for Annexin V and negative for PI are undergoing apoptosis in an early stage, while cells staining positive for both Annexin V and PI are either in a later stage of apoptosis or necrosis. Cells staining positive for only PI are either in a late apoptotic/necrotic or dead and cells negative for both Annexin V and PI are alive and not undergoing measurable apoptosis/necrosis.

UV-vis spectrophotometry in solution

Dilutions of each metal compound were obtained from freshly prepared stock solution (10^{-2} M in DMSO) and added to a 96-well plate. To these solutions, either $CuCl_2$ (from a stock solution of 10^{-1} M in MiliQ water) or MilliQ water were added. The final ratio of compound *versus* $CuCl_2$ was 1:4. The absorption spectra were recorded between 300-900 nm by spectrophotometry (Synergy H1 Hybrid reader, BioTek®) at various time points over 24 h at 37 °C. Obtained values were corrected for two baselines, with and without $CuCl_2$.

Results and discussion

Synthesis and characterization

The new compounds all have the formulae [M(LL)(Hnor)$(NO_3)_2$].The compounds have been prepared by dissolving the metal nitrate, LL and Hnor (ratio 1:1:1) in MeOH according to the procedure reported in the experimental section.

Within a few days single-phase crystalline materials were obtained in yields of ~70-80 %, from which suitable crystals were selected for X-ray diffraction analysis in the case of Cu and phen. The compounds were also characterized by IR and elemental analysis (see Experimental section for details). The IR spectra were all found to be quite similar, and (some representative examples) are depicted in Figures S1-S8. The coordinated nitrate has typical IR bands around 1300 cm^{-1} and agrees with a monodentate and a bidentate nitrate [67]. The N-H peak of the Hnor ligand is generally observed at 3400 +/- 40 cm^{-1}, in some cases split, which is a shift to lower wave numbers compared to the free ligand, as a result of N-H---O hydrogen bonding; such hydrogen bonds do slightly vary from metal to metal. In the case of compound **2**, [Cu(bpy)(Hnor)]$(NO_3)_2$, the NH pattern differs, which may be an indication for a different type of hydrogen bonding. Regretfully, no crystals suitable for X-ray diffraction could be found in this case. Bands due to MeOH could not be assigned.

For further characterization of the compounds for which no single crystals could be grown, ligand field spectra of all Cu, Co and Ni compounds have been recorded in the diffuse reflectance mode. The spectra as such are as expected and are in accordance with octahedrally-based geometries for the metal ions [68]. The Cu compounds have, apart from CT bands above 26000 cm^{-1}, LF maxima at 16000 cm^{-1} (compound **1**) and 16200 cm^{-1} (compound **2**), typical for tetragonal Cu(II). The Co(II) compounds also shows the CT bands at high energy, and d–d bands around 20000 cm^{-1} and just below 9000 cm^{-1}. The Ni(II) compounds show the CT bands above 27000 cm^{-1} and d-d bands at 16500 and 10000 cm^{-1}. However, the degree of distortion from octahedral due to the different ligand cannot be observed for compounds **3**-**6**. In the case of **3** and **5**, the elemental analysis showed the presence of 1 equivalent of MeOH. Ligand field spectra can neither proof nor exclude that MeOH is coordinated to the Co or Ni. The diffuse reflectance spectra are depicted in Figures S9-S14. To learn more on the electronic structure of the 2 Cu compounds, the EPR spectra in the solid state were recorded (spectra are depicted in Figures S15-S16 in the supplementary material). The observed g values are as expected for Cu(II) [69], having g(average) values of 2.089 and 2.087 for compounds **1** and **2**, respectively; g anisotropy and hyperfine splittings are not resolved, most likely due to the relatively close proximity of the Cu ions in the solid state.

Furthermore, the ^{1}H NMR spectra of Zn compounds **7** and **8** were performed in DMSO-d_6 at 400 MHz at 293 K (presented in Figures S17-20 in the supplementary material). The signal appeared at ~11.8 ppm was attributed to NH proton; the characteristic aromatic protons are displayed in the range of ~9-7 ppm (for details, see experimental section above). The signals show marked shifts when compared with the ligand "Hnor" alone. ^{13}C NMR of **7** and **8** complexes was also performed at 100 MHz, which showed all the peaks in the aromatic region of ~140-110 ppm as expected (depicted in Figures S21 and S22, in the supplementary material). Thus, all the peaks were observed in accordance with the proposed structure.

*Description of the molecular structure for compound **1***

The molecular structure of compound **1** was studied using X-ray single crystal diffraction analysis. Descriptive molecular and packing diagrams are presented in Figure 1 and selected geometrical data are summarized in Table 2.

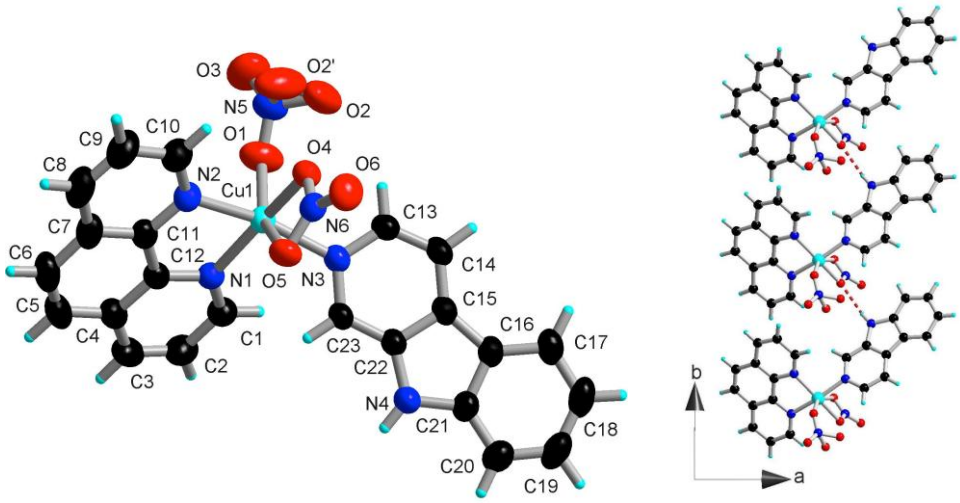


Figure 1: *Molecular and c-axis hydrogen bonding diagrams with the used labeling scheme for compound **1**. Two disordered positions for the O2 atom of the coordinated nitrate are shown. Thermal ellipsoid are at the 50% probability level and hydrogen atoms are represented as spheres of arbitrary radii.*

Table 2. Relevant bond distances (Å) and angles (deg) for compound **1**

	Lengths		Angles
Cu1-O1	2.209(3)	O1-Cu1-O4	100.62(10)
Cu1-O4	2.042(2)	O1-Cu1-O5	153.91(10)
Cu1-O5	2.644(2)	O1-Cu1-N1	92.08(11)
Cu1-N1	2.051(3)	O1-Cu1-N2	96.67(12)
Cu1-N2	2.007(3)	O1-Cu2-N3	98.71(12)
Cu1-N3	1.992(3)	O4-Cu1-O5	53.43(8)

In **1,** the Cu(II) is coordinated in a distorted octahedral way, by a bidentate phen, a monodentate Hnor, a monodentate nitrate and a chelating bidentate nitrate. The Hnor ligand coordinates with the pyridine nitrogen, whereas the N-H group of the ligand, donates a hydrogen bond (intermolecularly) to the coordinated O of the bidentate nitrate of a nearby molecule in C(7) first level chain motif along the [010] base vector, with an N----O contact distance of 3.02(4) Å. The monodentate nitrate is found disordered over 2 rotational positions. The coordination mode of Hnor agrees with our previously found [50] binding to Ag(I) and also with the only transition-metal compound (Ru) known so far for this ligand [49]. Nitrates are well known to be able to coordinate in different modes to metal ions, be it monodentate or bidentate, and two different binding modes in the same compound are also known for quite some time [67]. Given the fact that the ligand has several bands in the 1300-1500 cm^{-1} wavelength range, it was not possible to discriminate between the possible binding modes and mixtures of them from IR. However, given the overall similarities in the IR spectra, we have assumed that the binding modes of the Co, Ni and Zn compounds are similar to that of the Cu(II) compound.

Cytotoxic properties

The antiproliferative properties of the ligand Hnor and the metal complexes **1-5** and **7** (with cisplatin used as a comparison) were assessed by monitoring their ability to inhibit cell growth using the classical MTT assay in human ovarian (A2780) and lung cancer (A549) cell lines. Table 3 summarizes the obtained IC_{50} data, and Fig. 2 shows representative effects on cell viability for compound **3**. Interestingly, the copper compound **1** appears to be the most effective of the series with similar activity as cisplatin in the A2780 cells, and being 10-fold more effective than cisplatin in the A549 lung cancer cell line. This latter result is remarkable since these lung cancer cells are resistant against cisplatin treatment. Moreover, the cytotoxic effects of **1** are already evident after 24 h incubation (IC_{50} = 3.57 ± 0.98 μM), while cisplatin is still poorly active (IC_{50} = 24.2 ± 1.6 μM). Concerning the other complexes with 1,10-phenanotroline ligands, the antiproliferative activities followed the order **7** (Zn) > **3** (Co) >> **5** (Ni). In general, the compounds **2** and **4** (with the bipyridine ligand) were found less effective than their phen analogues. It should be noted that the phen ligand exerts itself important antiproliferative effects, followed by the bpy ligand. Instead, the free Hnor ligand is not toxic in both the tested cell lines (IC_{50} > 200 μM) (Table 3). Notably, the biological activity of **1** and **2** is not only due to the presence of Cu(II) ions, but to the overall compounds' chemico-physical properties (e.g. hydrophilic/lipophilic balance and stability) which may lead to its enhanced accumulation in cancer cells. In fact, treatment of cancer cells with $CuCl_2$ did not show any toxic effect at concentrations higher than 200 μM (Table 3).

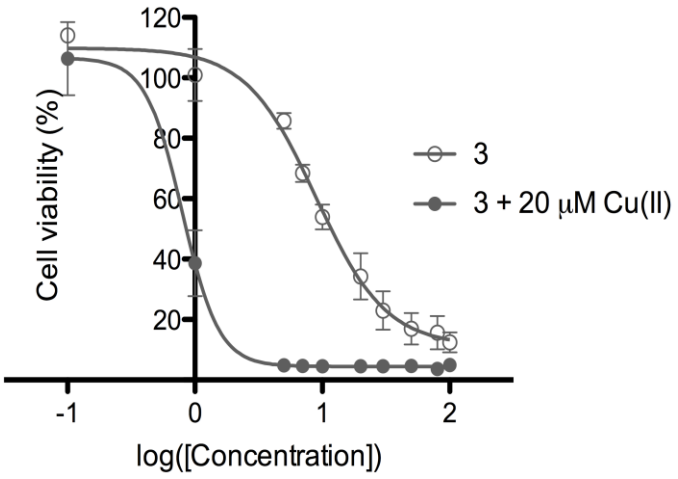


Figure 2 - *Effects of compound **3** on cell growth of A2780 cells. Reported data are from a single representative experiment. The concentration unit is in μM.*

Afterwards, we have used Annexin V/PI flow cytometry detection to determine whether the selected complexes are able to induce apoptosis in A549 cells after 72 h incubation. Compounds were tested at different concentrations around their IC_{50} values (equal, below or higher). Representative results are reported in Fig. 3, showing that the most effective copper compound **1** induces cell death meanly by apoptosis already at 0.5 μM concentration. Instead, the bpy analogue **2** is less effective, causing comparable apoptosis only at 7 μM concentration. In line with the MTT assay results, the cobalt complexes **3** and **4** are much less efficient, inducing apoptosis by only 30% and 27% at a concentration of 7 and 20 μM, respectively.

Mechanism of drug uptake plays a major role in their anticancer properties. In the case of cisplatin and other Pt(II) derivatives exact knowledge of the mechanisms governing their accumulation in cells is still lacking. However, among the proposed pathways, Cu transporters (e.g. CTR1) have been shown to be involved in the cellular import of Pt(II) chemotherapeutic agents in cancer cells, as well as in their resistance mechanisms [70]. In order to study transport of the compounds *via* copper transporters, the effects of addition of "free" Cu(II) ions to the cell culture medium were also evaluated. Thus, we co-administered complexes **1**-**5** and **7** with non-toxic concentrations of $CuCl_2$ (a competitor for transport *via* hCTR1), at the highest non-toxic dose (20 μM), and evaluated the effects on proliferation after 24 or 72 h incubation. If the uptake of the metal compounds goes *via* the same route as for Cu^{2+}, then we would expect a reduced toxic effect due to reduced uptake of metal compounds in cancer cells. However, as it is shown in Table 3, the addition of $CuCl_2$ increases the activity of both copper and cobalt compounds. This effect is especially dramatic for the Co^{2+} compound **3** in both cell lines, where IC_{50} values change from the compound being not active to one of the most potent of the series (see Figure 2 above). Given the fact that Cu(II) is a very strong ligand binder, this would suggest that in these cases the Cu(II) compound is largely responsible for the observed activity.

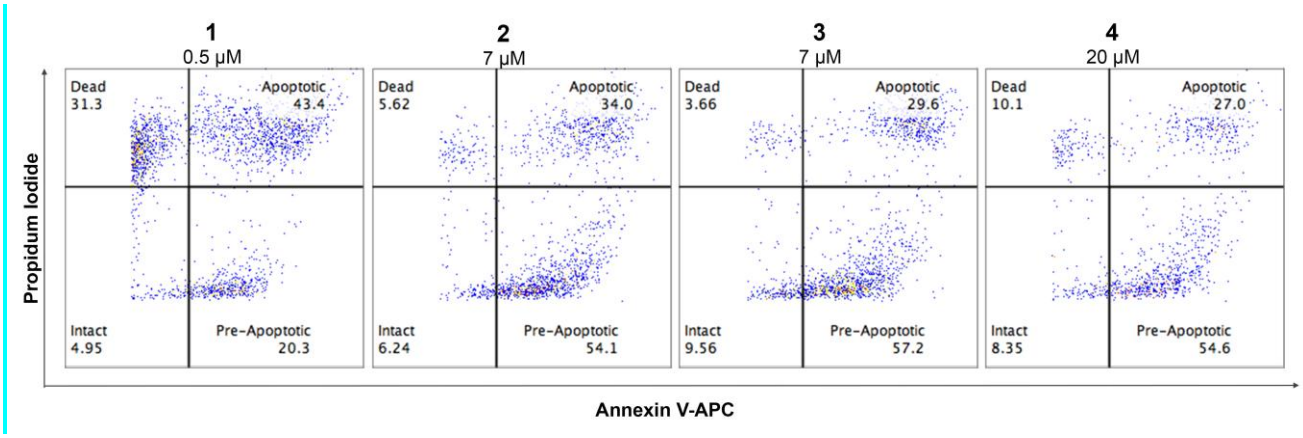


Figure 3. *Representative flow cytometry scheme of apoptotic A549 cells stained with Annexin V-APC/PI. Cells were treated with compounds **1-4** at different concentrations for 72 h. The results are representative of three independent experiments.*

Certainly, according to our results, metal transport *via* copper transporters can be excluded. However, in order to explain the effects of $CuCl_2$ addition on cell proliferation, we hypothesized that upon addition of Cu^{2+} ions either the copper complexes **1-2** are stabilized, or the ligands of the metal compounds **3-5** and **7** prefer binding to copper with respect to their own metal ions. In the latter case, replacement of the metal centre with copper would give rise to the most stable Cu complexes in solution, with enhanced antiproliferative effects. Concerning the Cu complexes **1-2**, the addition of copper renders the compounds more stable to hydrolysis and also may enhance their biological activity. To prove this hypothesis, $CuCl_2$ was added to solutions of each metal complex in a 1:4 ratio and the absorbance of the samples was recorded at different time points over 24 hours by UV-visible spectroscopy. Representative absorption spectra for complex **4** are reported in Fig. 4. As it can be seen from the obtained data, formation of a new band between 700-900 nm could be detected already after 30 min, corresponding to the absorption band of the related copper complex **2**. Further incubation of the solution, did not increase the intensity of the band.

Interestingly, the effects of copper addition on the cytotoxic effects of the various metal compounds follow the expectations according to the Irving-Williams series [71], which refers to the relative stabilities of complexes formed by a metal ion. For high-spin complexes of the divalent ions of first-row transition metals, the stability constant for the formation of a complex follows the order: Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II). Thus, the addition of Cu^{2+} to complexes **1-5** and **7** should induce metal exchange and formation of the most stable copper complex. The propensity of this reaction to occur should follow the order Co(II) > Ni(II) > Cu(II) < Zn(II). This is reflected not only by the UV-vis data but also by the trend in the cytotoxic effects. In fact, the Co complexes are those that have the highest increase in antiproliferative activity after co-incubation with $CuCl_2$ (ca. 10-fold: IC_{50} of **3** = 12.70 ± 0.97 µM and of (**3** +$CuCl_2$) = 0.83 ± 0.29 µM, respectively; see Figure 2). Noteworthy, the value for the (**3** +$CuCl_2$) is comparable to the IC_{50} calculated for **1** (IC_{50} = 1.88 ± 0.21 µM), consistent with the formation of Cu complexes in the cell culture medium. It is worth

mentioning that metal complex instability in aqueous media, favored by the addition of Cu(II) ions, may also affect the concentration of “free” phen ligand which is also toxic.

Table 3. IC_{50} of the metal compounds **1-5** and **7**, and related ligands, determined with and without co-incubation with 20 μM $CuCl_2$ in human ovarian carcinoma A2780 and human lung carcinoma A549 cells. Values for cisplatin and $CuCl_2$ are reported for comparison.

IC_{50} (μM)[a]

	A2780		A549	
Compound	24 h	72 h	24 h	72 h
1	3.57 ± 0.98	1.88 ± 0.21	9.40 ± 0.36	3.03 ± 0.12
1 + $CuCl_2$	1.94 ± 0.51*	0.83 ± 0.10*	3.50 ± 0.44*	2.40 ± 0.36
2	25.1 ± 4.9	9.85 ± 1.38	53.47 ± 3.01	22.05 ± 2.91
2 + $CuCl_2$	13.87 ± 0.72*	6.25 ± 1.58*	43.3 ± 4.5*	17.7 ± 2.1**
3	>100	12.70 ± 0.97	>200	7.70 ± 3.64
3 + $CuCl_2$	1.87 ± 0.97	0.83 ± 0.29*	5.27 ± 1.89	2.50 ± 0.36**
4	>100	41.9 ± 7.8	>200	79.2 ± 14.0
4 + $CuCl_2$	44.9 ± 3.0	9.18 ± 1.83*	>200	38.3 ± 2.0*
5	-	96.1 ± 4.2	-	-
5 + $CuCl_2$	-	35.6 ± 5.8	-	-
7	-	1.26 ± 0.07	-	-
7 + $CuCl_2$	-	1.23 ± 0.01	-	-
Free phen	-	3.70 ± 1.50	-	3.72 ± 1.51
Free bpy	-	10.70 ± 1.80	-	11.50 ± 0.95
Free Hnor	> 200	> 200	> 200	> 200
$CuCl_2$	> 200	> 200	> 200	> 200
cisplatin	24.2 ± 1.6	1.9 ± 0.6	34.7 ± 2.5	8.0 ± 0.5

*Values are the average ±SEM of at least three independent experiments, *p<0.05, **p>0.07.*

Similar effects are observed for the Co-bpy analogue **4**. The effect of $CuCl_2$ on the nickel complex **5** is more moderate (only *ca*. 3-fold increase in potency) in accordance with the higher stability of the compound with respect to metal substitution. Although reactivity of the zinc complex, **7**, with $CuCl_2$ could also be observed *via* absorption spectroscopy, since the zinc complex, **7**, is already *per se* very cytotoxic, marked effects induced by addition of $CuCl_2$ could not be recorded, and we cannot even exclude that **7** may be exerting important effects *per se*. Nevertheless, it is also possible that metal substitutions may also occur *in vivo* in the presence of physiological copper ions

already available in the cell culture medium. Finally, just as expected the biological effects of the copper complexes, **1-2** do not vary substantially in the presence of $CuCl_2$.

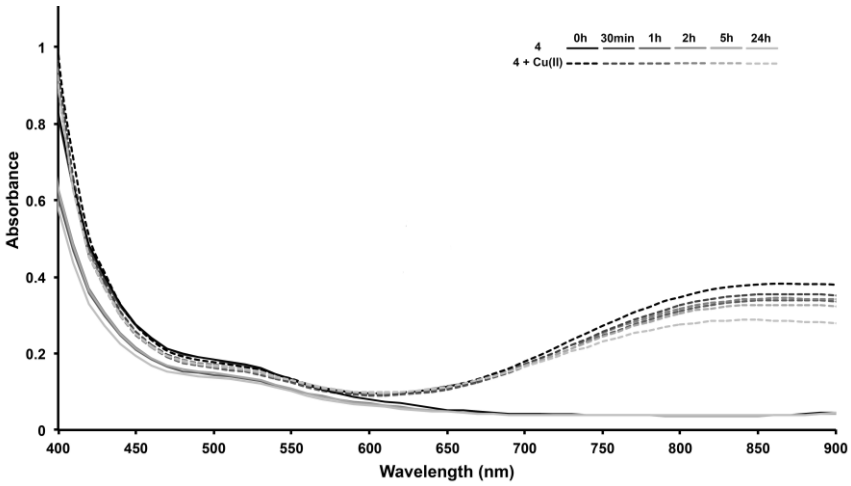


Figure 4. *UV-Absorption spectra of the Co compound **4** after addition of $CuCl_2$ (1:4 stoichiometric ratio) recorded at different times over 24 h.*

Concluding Remarks

Heterocyclic ligands with N-donor groups and nearby N-H groups have shown to be an interesting ligand series to be used in binding studies with metal ions where the coordination to transition metal ions can also be influenced by hydrogen bonding of the N-H group to e.g. anions; as mentioned, such a phenomenon is already known and described [55]. As for pyrazoles, for all the reported compounds, intramolecular H bonds are also visible, as shown by XRD and deduced from IR; while intermolecular hydrogen bonding to the anion must also occur. Concerning the anticancer properties, it appears that the marked antiproliferative activity of the copper complexes **1-2** and of the zinc compound **7** has to be ascribed to at least the presence of significant amounts of the metal in combination with both ligands. Overall, this trend in activity is in accordance with previously reported studies on similar first-row transition metal compounds [61]. While transport of the reported compounds *via* copper transporters may be excluded, our results allowed to identify the most active divalent metal complexes within the first-row transition metals, which can be related to the order of stability within the Irving-Williams series. Further studies are necessary to identify the pathways leading to apoptosis. However, it is worth mentioning that, inside the living cell, metal binding and redox events enter into an exciting, and highly complicated interplay, often resulting in extensive cellular signaling pathways and control networks. Thus, the application of coordination compounds of endogenous metal ions may be even more relevant to induce important intracellular alterations at physiological concentrations. Although the mechanism of antiproliferative activity is not well established for kinetically labile metal ions, the observed biological effects of these complexes, also as a result of the competition with other endogenous metal ions for ligand binding, appears as promising.

Table of less common abbreviations

hCTR1	Human copper transporter 1
Hnor	*9H*-Pyrido[3,4-*b*]indole
DMEM	Dulbecco's Modified Eagle Medium
RPMI	Roswell Park Memorial Institute
FACS	Fluorescence-activated cell sorting
PI	Propidium iodide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
bpy	2,2'-bipyridyl
phen	1,10-phenanthroline

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Appendix A.

Supporting information.

Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC1415868. Copies of available material can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (fax: +44-(0)1223-336033 or Email: deposit@ccdc.cam.ac.uk.

The supplementary information connected with this manuscript contains IR spectra (Figures S1-S8); ligand-field diffuse reflectance spectra for the Cu, Co and Ni compounds (Figure S9-S14), and the EPR spectra for compounds **1** and **2** (Figure S15-S16). ^{1}H NMR spectra of compound **7** and **8** (Figure S17-S20). ^{13}C NMR spectra of compound **7** and **8** (Figure S21-S22). Also the cif file and the checkcif files are available as supplementary information.

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