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Antineoplastic Evaluation of Two Mixed Chelate Copper Complexes (Casiopeínas®) in HCT-15 Xenograft Model

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Abstract. Casiopeínas® is a family of copper complexes with the general formulae $[Cu(N\text{-}N)(N\text{-}O)]NO_3$ and $[Cu(N\text{-}N)(O\text{-}O)]NO_3$; where N-N = substituted aromatic diimine (2,2'-bipyridine (*bipy*) or 1,10-phenanthroline (*phen));* N-O = α-aminoacidate or a peptide; and O-O = acetylacetonate (*acac*) or salicylaldehydate. These compounds have shown antiproliferative activity *in vitro* and antitumor activity in several mouse models with promissory results. Efforts have been done in order to understand the role played by ligands in the biological activity. With the aim of finding out the effect of secondary ligand (N-O or O-O), two of the most active complexes *in vitro* assays were selected to perform *in vivo* study on HCT-15 colon adenocarcinoma xenograft model. Both complexes, [Cu(3,4,7,8-tetramethyl-1,10-phen anthroline)(glycinato)]NO_3 (**1**) and [Cu(3,4,7,8-tetramethyl-1,10-phe nanthroline)(acetylacetonato)]NO_3 (**2**) share the same diimine ligand and the secondary ligand changes from glycinate (*gly*) to *acac*. Results show that **2** is effective to reduce tumor size but **1** does not achieve the values required according to protocols, revealing an important difference between compounds attributable to change of ligand from *gly* to *acac.*
Key words: Copper, mixed chelate, anticancer compounds, xenograft models, SAR relationships.

Resumen. Las Casiopeínas® son una familia de complejos de coordinación de cobre (II) con formula general $[Cu(N\text{-}N)(N\text{-}O)]NO_3$ y $[Cu(N\text{-}N)(O\text{-}O)]NO_3$; dónde N-N = diimina aromática sustituida (2,2'-bipiridina (*bipy*) or 1,10-fenantrolina (*phen));* N-O = α-aminoacidato o un péptido; y O-O = acetilacetonato (*acac*) o salicilaldehidato. Estos compuestos han demostrado actividad antiproliferativa *in vitro* y antitumoral en varios modelos murinos con resultados prometedores. Se han realizado importantes esfuerzos para comprender el papel que juegan los ligantes en la actividad biológica. Con el objetivo de averiguar el efecto del ligante secundario (N-O or O-O), se seleccionaron dos de los complejos más activos en los ensayos *in vitro* para realizar un estudio *in vivo* en un modelo de xenotransplantación empleando adenocarcinoma de colon HCT-15. Ambos complejos, [Cu(3,4,7,8-tetrametil-1,10-fenantrolina)(glicinato)]NO_3 (**1**) y [Cu(3,4,7,8-tetrametil-1,10-fenantrolina)(acetilacetonato)]NO_3 (**2**), comparten el mismo ligante diimina y el ligante secundario cambia de glicinato (*gly*) to *acac*. Los resultados mostraron que el **2** es efectivo para reducir el tamaño del tumor mientras que el **1** no alcanza los valores requeridos de acuerdo a los protocolos, revelando una diferencia importante entre los compuestos que es atribuible al cambio de ligante secundario de *gly* a *acac.*
Palabras clave: Casiopeínas, cobre, quelatos mixtos, compuestos antitumorales, modelos de xenotransplantación, relaciones SAR.

Introduction

Metals can play a wide variety of roles in the development of new therapeutic agents for the treatment of cancer and other diseases. Several strategies are employed in the field to exploit their unique characteristics in order to design metal-based drugs effective to treat a wide range of diseases. The number of metal compounds in current clinical use for the treatment of cancer is extremely limited and concerns platinum compounds exclusively; however, in recent years an increasing number of metal compounds has shown interesting biological and bio-medical properties attributable to mechanisms of action distinctly different from platinum drugs [1, 2]. The rational design of metallodrugs requires a judicious choice of targets, metal and ligands in order to combine high effectiveness and low toxicity. An innovator approach is the use of compounds based on essential metals based on the assumption proposal that endogenous metals may be less toxic[3]. Currently, several groups of copper complexes in both oxidation states (I) and (II) have been studied as potential antitumor agents [4-6].

Copper is an essential trace element for the function of several enzymes involved in energy metabolism, respiration and DNA synthesis in the cell [7, 8]. Copper redox properties at physiologic conditions allow that the major functions of biological-active copper compounds involve redox reactions, paradoxically; the toxicity of copper is also related with this property and its ability to produce reactive oxygen species (ROS) inducing the oxidation of biomolecules as proteins, DNA, RNA and lipids [9-12].

Our research group has selected the copper to design copper complexes with antiproliferative activity and potential use as antineoplastics in clinic. This family of copper (II) coordination compounds, patented [13-15] under the name Casiopeínas®, has the general formulae $[Cu(N\text{-}N)(N\text{-}O)]NO_3$ and $[Cu(N\text{-}N)(O\text{-}O)]NO_3$; where N-N = non substituted and substituted aromatic diimine (2,2'-bipyridine (*bipy*) or 1,10-phenanthroline (*phen));* N-O = α-aminoacidate or a peptide and O-O = acetylacetonate (*acac*) or salicylaldehydate. Two compounds of this family [Cu(4,4'-dimethyl-2,2'- bipyridine)(*acac*)]NO_3 (Casiopeína III-ia) and [Cu(4,7-dimethyl- 1,10-phenanthroline)(*gly*)]NO_3

(Casiopeína II-gly) have been evaluated *in vitro* and *in vivo* showing cytotoxic [16-18], genotoxic [16] and antineoplastic [19, 20] activity. These compounds are able to inhibit cell proliferation and produce dose-dependent cell death by apoptosis through mechanisms dependent and independent of caspase activation [20, 21]. In presence of reducing agents the cell growth inhibition, [16, 20] and degradation of DNA [16, 20, 22, 23] are enhanced, simultaneously with ROS increment, suggesting that DNA oxidation might be triggering the cell death. Nevertheless, apoptosis might be the result of several processes acting alone or in concomitance. The evidence supports three main targets: a) generation of reactive oxygen species (ROS) [16, 23, 24] with DNA oxidation and degradation [16, 25], plus depletion of antioxidant defenses like GSH as consequence [24, 26, 27], b) mitochondrial toxicity [28, 29] or c) DNA damage through direct interaction with complex by an intercalative or non intercalative mechanism [23, 30-32]. The mechanism of action is not fully understood and more work will be performed in this area in order to identify the main signals; however, it is important to highlight that several mechanism are possible and they are not mutually exclusive.

Both, biological activity and toxicity of a metal complex are typically related not just to the metal itself but also to the ligands and to the type of complex [33]; the ligand environment can have a marked effect on the overall reactivity of the complex, affecting the pharmacokinetics and pharmacodynamics of metallodrug as consequence. In a QSAR study, the antiproliferative activity on HeLa, SiHa, HCT-15 and MCF-7 cells for compounds with general formula [Cu(N-N)(*acac*)]NO_3 and [Cu(N-N)(*gly*)]NO_3 was determined, and their relationship with acute toxicity (LD50) on ICR mice was analyzed. According with QSAR studies both, acute toxicity and antiproliferative activity are strongly influenced by diimine ligand without apparent influence of the other ligand [34]; additionally, in every *in vitro* assay performed, the activity for compounds which share diimine ligand in their structure was nearly the same [22, 25, 34, 35]. Given the major changes in biological activity and physicochemical properties are controlled by the substituent on diimine ligand, from now on it will be called primary ligand, and the O-O donor or N-O donor will be called secondary ligand.

After this findings, it was proposed that secondary ligand assist the transport of the copper coordination compound across membranes due to an increment in hydrophobicity, acting as carrier in the uptake of copper (II). The cellular uptake of copper (II) for a set of compounds [Cu(4,7-dimethyl-1,10-phenanthroline)(α-aminoacidato)]$^+$ where the structural variation is only due to aliphatic side chain of α-aminoacidate and its relationship with antiproliferative activity was analyzed. Despite the hydrophobicity of the ligands actually increases the cellular uptake of copper (II); IC50 on HeLa did not correlate with copper intracellular concentration, leading to conclude it is not the main limiting factor in the mechanism of action[35]. Although these findings suggest the lack of influence of secondary ligand on biological activity, this ligand could have a key role *in vivo* where thermodynamic and kinetic stability of ternary complexes in physiological conditions, the exchange rate of ligands with endogenous molecules and hydrophobicity become very important to lead the untransformed molecule across physiologic barriers to the final target, playing a major role in pharmacokinetics. However, the exact contribution of the secondary ligand to the activity remained to be fully understood.

With the aim of finding out if the secondary ligand has influence on antitumor activity *in vivo* and confirm the predictions made by QSAR equations, we selected to perform *in vivo* study on mouse xenograft model, two of the most active complexes *in vitro* assays, [Cu(3,4,7,8-tetramethyl-1,10-phenanthroline)(*gly*)]NO_3 **1**, CasVIII-gly] and [Cu(3,4,7,8-tetramethyl-1,10-phenanthroline)(*acac*)]NO_3 **2**, Cas III-J]. Both complexes share the same diimine ligand and the secondary ligand changes from *gly* (**1**) to *acac* (**2**).

Results and Discussion

Figure 1 shows the structure for the complexes studied, as mentioned above, with the aim of understanding the role played by the secondary ligand on the biological activity, both compounds are mixed chelate copper (II) complexes sharing the same diimine ligand 3,4,7,8-tetamethyl-1,10-phenanthroline in their structure; however, **1** has *gly* as N-O donor while **2** has *acac* as oxygen bidentate donor.

Complexes were characterized by conventional techniques and the purity was assessed by elemental analysis. The complexes synthesized exhibit IR absorption bands typical for coordinated ligands: for **1** absorption bands are present in 1533 and 1434 cm^{-1} (aromatic diimine), 828 and 725 cm^{-1} (δ C-H out of plane), 1620 (ν_{as} COO^-), 3395 and 3300 cm^{-1} (ν NH_2), 645 cm^{-1} (δ NH_2); for **2** absorption bands are present in 1618, 1517 and 1430 cm^{-1} (aromatic diimine), 833 and 727 cm^{-1} (δ C-H out of plane), and 1583 (ν_{as} COO^-). Both complexes show and intense absorption band at ≈ 1384 cm^{-1} assigned to NO_3^- as counter ion.

Both compounds showed the same effective magnetic moment (1.78 BM) that agrees with paramagnetic species with one unpaired electron confirming the +2 oxidation state of copper

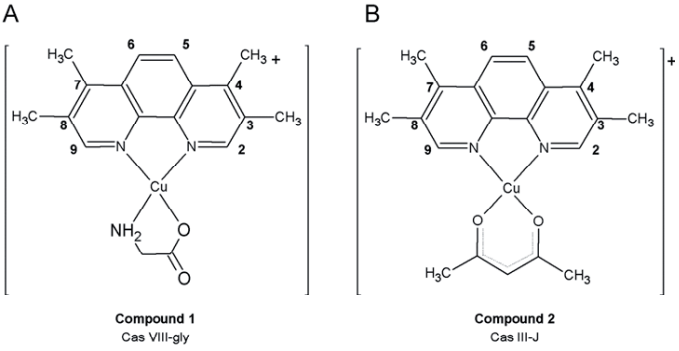
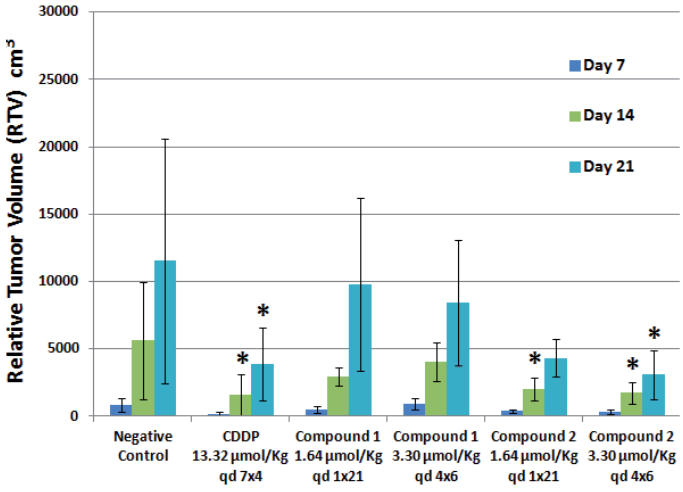


Fig. 1. Structures of the complexes studied. **A**: [Cu(3,4,7,8-tetramethyl-1,10-phenanthroline)(acetylacetonato)]$^+$ and **B**: [Cu(3,4,7,8-tetramethyl-1,10-phenanthroline)(glycinato)]$^+$.

ion. The conductimetric measurements of ternary compounds shown a 1:1 type electrolyte in ethanol which means that NO_3^- is out of coordination sphere as counter-ion.

Acute Toxicity

LD50 for **compound 2** was previously reported as 16.23 ± 2.63 μmol/Kg in male mice ICR. One of the advantages of QSAR studies as tool used to understand and predict activities on biological systems is the reduction of the number of animals used in experiments by making decisions supported on predictions modeled, performing *in vivo* assays only if necessary. QSAR study reported for this type of compounds, proposed that LD50 follows the same trend that cytotoxicity[34]. Acute toxicity has a strong relationship with the nature of diimine ligand without important influence of secondary ligand. If that proposal is accurate, the value of LD50 for **compound 1,** which shares the same diimine ligand with **compound 2**, would be close, and actually it does. The LD50 for **compound 1** is 16.45 ± 2.42 μmol/Kg, confirming QSAR predictions about acute toxicity.



Graph 1. Relative Tumor Volume (RTV) on day 7 (dark blue), 14 (green) and 21 (cyan). Asterisks represent statistically significant difference versus negative control (α = 0.05)

Antitumor activity on mouse xenograft model:

Two administration schedules were employed per drug: A) 1/10 DL50 (1.64 μmol/Kg) daily, 21 doses; and B) 2/10 DL50 (3.30 μmol/Kg) each 4 days, 6 doses. *Cis*-diamino-dichloro platinum (II) (CDDP) 13.32 μmol/Kg each 7 days, 4 doses was employed as positive control according to protocols [36].

Graph 1 shows the relative tumor volume (RTV) calculated on days 7, 14 and 21. On day 7, only the CDDP shows a statistically significant difference with negative control. Nevertheless, on day 14 and 21, both administration schedules of **2** show statistically significant difference compared with negative control. RTV for intermittent treatment with **2**, 3.30 μmol/Kg is smaller than 1.64 μmol/Kg chronic scheme, and there are not differences measured up to CDDP treatment on day 21. Since on day 7 antitumor activity by **2** is not sufficient, but at the end of the experiment (21 day) its antitumor activity is close to reference drug, results lead to think that **2** effect is slower than CDDP. In the other hand, there is not statistically difference between negative control and **1** at neither date nor dose.

Days in doubling size for each tumor were also assessed and reported as mean of 6 individuals per group for every schedule in Table 1; only CDDP shows a considerable delay compared to negative control. Time to increase the tumor size is related to the quantity of tumor cells and their replication rate. Administration of cytotoxic agents will reduce the number of tumor cells able to proliferate and the quantity of daughter cells will be smaller compared with negative control, as consequence the days in doubling size will be greater. Since the time required to doubling tumor size is short because is an early event in the advance of the experiment, and the antitumor effect of a drug could not be immediate, we decided to perform a complete

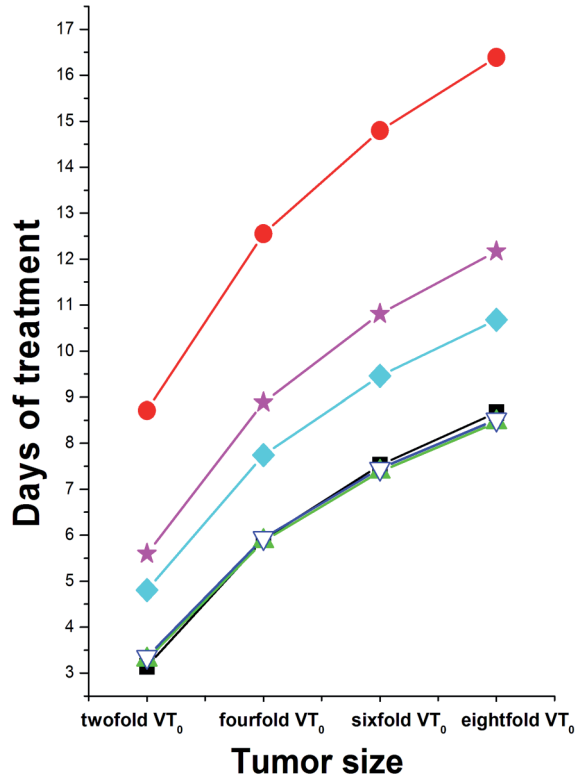
Table 1. Time needed to increase tumor volume (TV). Number of days needed to increase twofold, fourfold, sixfold and eightfold the TV at the beginning of treatment (on day 0). Values are reported as mean of 6 experimental individuals ± SD.

Group	Time to increase twofold TV_0 (days)	Time to increase fourfold TV_0 (days)	Time to increase sixfold TV_0 (days)	Time to increase eightfold TV_0 (days)
Negative Control (distilled water)	3.14 ± 1.17	5.90 ± 1.68	7.52 ± 2.02	8.67 ± 2.27
CDDP (13.32 μmol/Kg qd 7 × 4)	8.71 ± 3.16	12.55 ± 3.86	14.79 ± 4.33	16.39 ± 4.68
Compound 1 (1.64 μmol/Kg qd 1 × 21)	3.29 ± 1.01	5.87 ± 1.20	7.38 ± 1.32	8.45 ± 1.24
Compound 1 (3.30 μmol/Kg qd 4 × 6)	3.35 ± 0.61	5.93 ± 0.90	7.44 ± 1.10	8.52 ± 1.24
Compound 2 (1.64 μmol/Kg qd 1 × 21)	4.80 ± 1.56	7.74 ± 2.10	9.46 ± 2.43	10.68 ± 2.66
Compound 2 (3.30 μmol/Kg qd 4 × 6)	5.59 ± 1.25	8.88 ± 1.95	10.80 ± 2.46	12.17 ± 2.84

analysis concerning the time needed to increase the volume with the aim to be able to establish the moment when the antitumor activity becomes evident. Time needed to increase TV fourfold, sixfold and eightfold was calculated (Table 1) and plotted (Graph 2).

Regarding time needed to increase TV (Graph 2), trend for negative control and both dose schemes for **1** are overlap, indicative of the absence of antitumor activity for **1** in both schemes employed. CDDP (13.32 μmol/Kg qd 7*4) shows a delay in the time needed to increase TV compared with negative control from the beginning of experiment, confirming its antitumor activity. On the other hand, the groups administered with **2** presented a small delay at the beginning but it becomes evident after some time, being the schedule 3.30 μmol/Kg qd 4*6 the most effective. This finding suggests antitumor activity for this compound on HCT-15.

Antitumor function (AF) was also calculated, it determines the capability of compounds to reduce the TV compared with negative control on a certain day of the experiment. Protocols establish AF below 42 to consider a compound as potential antitumor agent. AF on days 7, 14 and 21 are reported on Table 2. Both administration schedules for **1** showed AF values higher than 42 since day 7 till day 21, confirming what the other parameters suggested above, the schedules and doses tested for **1** were not active. In contrast, CDDP and **2** 3.30 μmol/Kg qd 4*6 showed AF values lower than 42 during all long experiment; however, AF for CDDP increases in time while AF for **2** decreases, achieving even better results than CDDP on day 21. Same trend is observed for the 1.64 μmol/Kg schedule, on day 7 the value is small higher than 42 but, after 21 days the value fall below the value required according to protocols. This finding suggests despite the **2** antitumor activity is slower than CDDP; the effect is sustained. A noteworthy fact is that the molar dose of CDDP employed is four times greater than the molar dose employed of **2,** suggestive of a more potent effect.



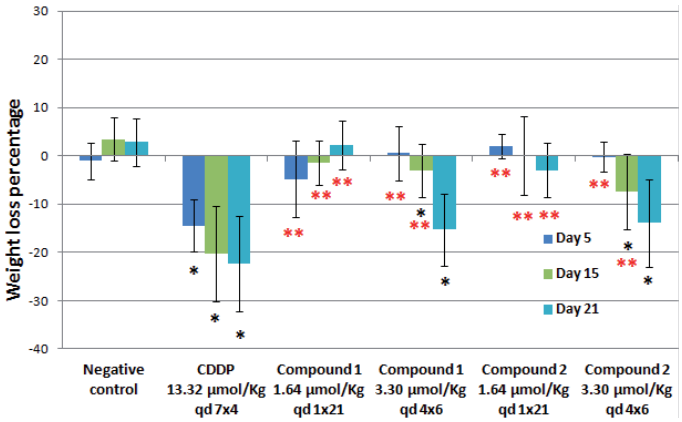
Graph 2. Negative Control (black squares), CDDP 13.3 μmol/Kg (red circles), Compound **1** *1.64 μmol/Kg qd 21* (green solid triangles), Compound **1** *3.2 μmol/Kg qd 4x 6* (open triangles), Compound **2** *1.64μmol/Kg qd 21* (cyan rhombus), Compound **2** *3.2 μmol/Kg qd 4x 6* (pink stars). VT_0: Tumor volume at the beginning of treatment (on day 0).

Table 2. Antitumor Function Rate. AF was calculated on day 7, 14 and 21 of treatment.

COMPOUND	Antitumor function rate (AF)		
	day 7	*day 14*	*day 21*
Negative control	100	100	100
CDDP (13.32 μmol/Kg) qd 7 × 4	20.5	28.3	33.7
Compound 1 (1.64 μmol/Kg) qd 21	57.2	52.3	85.0
Compound 1 (3.30 μmol/Kg) qd 4 × 6	106.0	71.6	72.9
Compound 2 (1.64 μmol/Kg) qd 21	42.9	35.7	37.6
Compound 2 (3.30 μmol/Kg) qd 4 × 6	37.8	30.7	26.8

In order to assess toxicity, percentage of weight loss on days 5, 15 and 21 was calculated (Graph 3). CDDP is the most toxic compound since the beginning of treatment, reaching 22% of weight loss on day 21; the value and early trend to lose weight expose the large toxicity associated with CDDP. Regarding test compounds, **1** present a similar trend to negative control at chronic schedule 1.64 μmol/Kg indicating the low toxicity of test dose; however, it is important to remark that none antitumor activity was observed either. Both (**1**, **2**) increase their toxicity with the augment of dose despite the differences in frequency of administration according to schedules, showing higher percentage of weight loss for intermittent schedules 3.30 μmol/Kg. The magnitude of weight loss employing this scheme is very similar between compounds in agreement with previous findings about acute toxicity[34] discussed above (Table 3). Noteworthy, the toxicity is not directly related with antitumor activity given that AF and VT values for **2** are satisfactory to consider antitumor activity according to protocols but are not sufficient for **1,** both have similar behaviors regarding acute toxicity and weight loss (Table 3).

The aim of the study was to investigate if secondary ligand had influence on antitumor activity *in vivo*. To achieve it, two copper complexes with the same diimine in their structure and *gly* or *acac* as secondary ligand were tested on HCT-15 colon adenocarcinoma xenograft model at the same micromolar doses in order to compare their antitumor activities. The **compound 2** demonstrated that successfully cross barriers and reach site



Graph 3. Weight loss percentage on day 5, 15 and 21. Black asterisks represent statistically significant difference versus negative control ($\alpha = 0.05$) and red asterisks represent statistically significant difference versus CDDP ($\alpha = 0.05$).

Table 3. Comparative summary.

Compound	HCT-15 IC50[a]	DL50	AF[c]	%WL[d]
1	1.4 ± 0.2	16.45 ± 2.42	72.9	–15.28 ± 7.39
2	1.8 ± 0.4	16.23 ± 2.63	26.8	–13.92 ± 8.98
CDDP	21.8 ± 2.4	45.0 ± 5.33[b]	33.7	–22.38 ± 9.84

[a]IC50 (lM) on HCT-15 [34].
b Cisplatin DL50 in mice [47] transformed to µmol/Kg with molecular weight 300 g/mol.
c. Antitumor Function on day 21.
d. Weight loss percentage on day 21.

of tumor, reducing it size compared with negative control; nevertheless, the **1** does not achieve the values required according to protocols, revealing an important difference between compounds that could not be evaluated or noticed *in vitro* before.

Despite xenografts of human tumors grown in immunodeficient mice are not absolutely predictive of drug behavior in the clinic, this model properly used and interpreted can be useful to make choices concerning to pharmacologic and pharmacodynamic attributes of the agent under consideration [37]. The model can serve as useful "filter" for defining the ability of an agent to pass physiologic barriers acting a distance of the site of drug administration affecting the tumors with a positive therapeutic effect [37], and as consequence, these models also work to compare these pharmacologic abilities between two complexes with similar structures. Therefore, if both compounds have comparable IC50 on HCT-15 cell line (Table 3) and other human tumor cell lines[34], the differences found *in vivo* might be attributable to pharmacokinetics. Half life ($t_{1/2}$) for two analogous compounds was previously evaluated, [Cu(4,7-dimethyl-1,10-phenanthroline)(*gly*)]NO_3 and [Cu(4,7-dimethyl-1,10-phenanthroline)(*acac*)]NO_3, they share the same diimine ligand but the secondary ligand changes equal to the compounds analyzed in the current work. *Gly* compound has a half life of 39.37 minutes [38], whereas 27.49 ± 2.34 hours is reported for *acac* compound [39]. Consequently, it is reasonable hypothesize that the slower elimination of latter might be reflected on antitumor activity; however, the pharmacokinetic study for **1** and **2** must be performed in order to unequivocally conclude about this phenomenon. Differences on half life and other pharmacokinetic parameters could be attributable to differences in stability constants, usually higher for *acac* complexes than *gly* complexes [40].

According to evidence obtained from *in vitro* assays [22, 25], QSAR[34] and SAR studies [35], the pharmacophoric group of this family of compounds is the copper-diimine moiety. Despite the mechanism of action is not fully understood, strong evidence supports that both, copper redox reactions to generate ROS [22, 26, 27] and the interaction of the complex with DNA [23], are necessary for the antiproliferative activity and successful results *in vitro*. As reported before the type of diimina (*bpy or phen*) and its substituents control the electronic environment around copper atom, the redox reactivity as consequence, and the interaction *in vitro* with DNA, becoming to this ligand the dominant factor in the mode of action of Casiopeínas® [34]. However, secondary ligand seems to own a more important role on distribution *in vivo*, and final effects as potential antitumor drugs are controlled by the equilibrium between this two pharmacological properties, tumor response to drug and access to tumor site.

Experimental

All chemicals and solvents were purchased from Sigma-Aldrich Chemical Co. and GFS Chemicals Inc., and were used without further purification. Elemental analysis was done in a Fission Instruments Analyzer EA 1108, IR spectra were obtained employing Nicolet Avatar 320 FT-IR, conductimetric and magnetic determinations were done in a Jenway 4330 Conductivity and pH meter and a Mkl magnetic balance from Sherwood Scientific respectively, UV-vis spectra were recorded in a Hewlett Packard 8452 diode array spectrophotometer. The conductimetry were recorded in 1 mM ethanolic solution at 298 K.

Synthesis of copper complexes. All compounds were synthesized following the reported patents [13-15].

Aqua(3,4,7,8-tetramethyl-1,10- phenanthroline)(glycinato) copper(II) nitrate hydrate (Casiopeina VIII-gly) (compound 1). Yield 90%.Elemental analysis data: calculated (%) for $CuC_{18}H_{20}N_4O_5$ (435.9): C, 47.63; N, 12.34; H, 4.88. Found (%): C, 47.27; N, 12.76; H, 4.95. IR (KBr, ν/cm^{-1}): 3395, 3300, 2937, 1620, 1533, 1434, 1385 (NO_3^-), 828, 725, 645. µeff = 1.78 BM. Λ (EtOH) = 35.0 µS.

Aqua(3,4,7,8-tetramethyl-1,10- phenanthroline)(acetylacetonato) copper(II) nitrate hydrate (Casiopeina III-J) (compound 2). Yield 90%.Elemental analysis data: calculated (%) for $CuC_{23}N_3O_6H_{23}$ (478.99): C, 52.66; N, 8.77; H, 5.26. Found (%): C, 52.79; N, 8.95; H, 5.41. IR (KBr, ν/cm^{-1}): 3338, 3081, 2920, 1618, 1583, 1517, 1430, 1384 (NO_3^-), 833, 727, 622. µeff = 1.78 BM. Λ (EtOH) = 37.32 µS.

In vivo experiments

All animal studies were carried out in the animal facility of Facultad de Química-UNAM, UNEXA (Unidad de Experimentación Animal) under controlled environmental conditions: temperature (22 ± 1 °C), 50-60% relative humidity, 12/12 h, light-darkness cycles. Water and food were supplied *ad libitum* and consisted in Sterilizable Harlan Teklad Global Diet 20185 and water purified by reverse osmosis and acidulated with HCl to pH = 2.5 for gastrointestinal parasites control.The acute toxicity and the nude mouse research were performed according to Mexican guides for experimental animal care (NOM-062-ZOO-1999)[41] and international guides to the care and use of experimental animals[42].

Acute Toxicity: Male mice Hsd:ICR (CD-1®) were purchased from Harlan Mexico Inc. with average weight 23 ± 3 g and from 12 to 15 weeks old. For **compound 1**, six different doses were tested in groups of 10 ICR male mice per group. The drug was administered intraperitoneally (ip) with a single dose of complex and mice were kept on observation during 14 days. The surviving mice per group were counted at 24 h and medial lethal dose (LD50) was computed by sigmoidal fit in a dose-quantal response graphic (*Microcal Origin 6.0, Microcal software Inc*.).

Tumor cell lines. HCT- 15 (Colorectal adenocarcinoma) cell line was purchased from the American Type Culture Collection (ATCC) and propagated in Dulbecco's Modified Eagle Medium (D-MEM, Gibco Invitrogen Corporation) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen Corporation) according to standard protocols [43, 44].

Antitumor activity: 46 Male, 6-week-old Hsd:athymic nude mice (nu/nu) (Harlan Inc) were kept under specific pathogen-free conditions with free access to autoclaved food (Harlan Teklad Global Diet (Madison, Wisconsin) 2018S) and water. All mice were injected subcutaneously with 2.5×10^6 HCT-15 cells into the low left flank. When the tumor reached a 0.3 cm diameter, the mice were randomly allocated in one of the following groups: Control (n = 10) distilled water every 24 h for 21 days; CDDP (n = 6) 4 mg/kg (13.32 μmol/kg) once every 7 days,4 doses; **compound 1** (n = 6) 0.74 mg/Kg (1.64 μmol/Kg) daily, 21 doses; **compound 1** (n = 6) 1.48 mg/Kg (3.30 μmol/Kg) once every 4 days, 6 doses; **compound 2** (n = 6) 0.78 mg/Kg (1.64 μmol/Kg) daily, 21 doses; **compound 2** (n = 6) 1.56 mg/Kg (3.30 μmol/Kg) once every 4 days, 6 doses. The drug application was intraperitoneal. The animals were weighed and the tumors measured in length and width using Vernier calibrators every day. Using the established formula [length (cm) × width^2 (cm)^2 × π]/6, tumor sizes were converted in tumor volume (TV). Then, the relative tumor volume (RTV) was calculated on day 7, 14 and 21 as follows: RTV = (tumor volume on day X/tumor volume on Day 0) × 100. Also, the days in doubling size for each tumor were assessed. Antitumor function rate (AF) was calculated on day 7, 14 and 21 as follows: AF = $[(RTV_{\text{experimental group}})/(RTV_{\text{control}})] \times 100$. The percentage of weight loss, as a toxicity indicator, was calculated for each animal as follows: [(weight on day *x*/weight on day 0) - 1] × 100. The experiment finished 24 h after the last drug application in each group and the animals were euthanized in a CO_2 chamber according to AVMA Guidelines on euthanasia[45].

Statistical analysis: Data from *in vivo* experiments was analyzed with the nonparametric Kruskal Wallis test followed by *phi* coefficient[46]; the percentage of weight loss was analyzed with ANOVA followed by Tukey. The accepted statistically significant difference was $p < 0.05$.

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