Synthesis, Characterization, and in Vitro Antitumor Properties of Tris(hydroxymethyl)phosphine Copper(I) Complexes Containing the New Bis(1,2,4-triazol-1-yl)acetate Ligand

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The new sodium bis(1,2,4-triazol-1-yl)acetate ligand, Na[HC(CO$_2$)(tz)$_2$], has been prepared in methanol solution by using 1,2,4-triazole, dibromoacetic acid, and NaOH. Treatment of the [Cu(CH$_3$CN)$_4$][PF$_6$] acceptor with Na[HC(CO$_2$)(tz)$_2$] or Na[HC(CO$_2$)[(pz^{Me2})$_2$], in the presence of the tris(hydroxymethyl)phosphine coligand in methanol/acetonitrile solutions produced unprecedented mononuclear copper(I) complexes of the type [L^n]Cu[P(CH$_2$OH)$_3$]$_2$ (L^1, **2**; L^2, **3**) and [(CH$_3$CN)$_2$Cu(P(CH$_2$OH)$_3$)$_2$]PF$_6$, **4**. These compounds have been characterized by elemental analyses, FTIR, ESI-MS, and multinuclear (^{1}H and ^{31}P) NMR spectral data. The new copper(I) complexes were tested for their cytotoxic properties against a panel of several human tumor cell lines. The results reported here indicate that all the complexes showed in vitro antitumor activity similar or better than that of cisplatin, the most used metal-based antitumor drug. In particular, [HC(CO$_2$)(pz^{Me2})$_2$]Cu[P(CH$_2$OH)$_3$]$_2$, **3** showed IC$_{50}$ values markedly lower than the reference compound against all tumor cell lines. Chemosensitivity tests performed on cisplatin sensitive and resistant cell lines have demonstrated that all these Cu(I) complexes were able to overcome cisplatin resistance, supporting the hypothesis of a different mechanism of action compared to that exhibited by the reference drug. Flow cytometric analysis on 2008 human ovarian carcinoma cells revealed that complex **3**, chosen as the best candidate, induced a marked enlargement of both cell size and granularity, and a significant increase in the fraction of G2/M cells that, differently from cisplatin, was not accompanied by the appearance of a relevant sub-G1 fraction. Besides, no evidence of caspase-3 activation was detected in cells treated with complex **3**. We hypothesize that the cytotoxic activity of the new copper(I) complex may be correlated to its ability to trigger paraptosis, a nonapoptotic mechanism of cell death.

It is known that copper is an essential trace metal for living organisms.[1] This metal plays a crucial role in different enzymes (i.e., cytochrome-c oxidase, superoxide dismutase, ceruloplasmin, etc.) that catalyze oxidation/reduction reactions correlated with the antioxidant system of the organism.[2] It has been reported that certain copper complexes catalyze radical formation, while others seem to have efficacy as antioxidants.[3] The different behaviors depend upon the chemical environment and nature of the chelating agent. In this field, our attention has been focused on copper(I) complexes containing "scorpionate" ligands.[4−6] Poly(pyrazolyl)borates[7] and related scorpionates[8] are potentially tridentate ligands extensively employed as anionic σ-donor chelates in a variety of metal complexes. These ligands have a general structure [RR′B(pz)$_2$]$^-$, where pz is either an unsubstituted or C-substituted pyrazolyl group that can coordinate metals to give complexes of the type [RR′B(μ-pz)$_2$ML$_n$] (L$_n$ = metal coligands). If neither R nor R′ is pz, the ligand is called heteroscorpionate, a description that also includes ligands where R′ is a pyrazolyl group different from the other two bridging pyrazolyl units. Modifications of poly(pyrazolyl)-borates can be made by replacement of the boron bridging atom by other elements such as carbon,[9] silicon,[10] or phosphorus.[11,12] Recent contributions are related to the heteroscorpionate ligands derived from bis(pyrazol-1-yl)methane, with [RR′C(pz)$_2$] as general structure and bearing a coordinating moiety (R′) such as acetate,[13−19] dithioacetate,[19,20] sulfonate,[21] ethoxide,[22,23] phenolate,[24−27] thiolate,[28−30] or other classes of moieties.[31−34]

On this basis, we have focused our attention on the study of the coordinative ability of the monoanionic heteroscorpionate acetate ligand, bis(3,5-dimethyl-pyrazol-1-yl)acetate ([HC(CO$_2$)-(pz^{Me2})$_2$]$^-$), toward copper(I) acceptors. Moreover, we have designed and synthesized the new triazole-based heteroscorpionate ligand, bis(triazol-1-yl)acetate ([HC(CO$_2$)(tz)$_2$]$^-$); triazole-based ligands are electron-withdrawing relative to their pyrazole-based counterparts, and the *exo*-ring-nitrogen atoms may bridge between metal centers or may take part in hydrogen-bond interactions, assisting the formation of two-dimensional water-intercalate or water-layer clathrates,[35,36] thus leading to water soluble species.[37−39]

Besides, because it is of great importance to obtain complexes that are soluble and stable under physiological conditions, an interesting solution is the use of water-soluble phosphine coligands bringing highly polar functional groups such as $-SO_3^+$, $-$COOH, $-NR_3^+$, or $-$OH.[40]

This paper, as part of our investigations into the copper(I) coordination chemistry,[4−6] describes the synthesis and the spectroscopic and analytic characterization of new triazole-based scorpionate copper(I) complexes containing the tris(hydroxymethyl)phosphine coligand.

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Table 1. Relevant IR, ^{31}P NMR, and ESI-MS Data of Copper(I) Complexes **2**−**4**

cmpd	IR[a] (cm^{-1})	δ ^{31}P NMR[b]	ESI MS[c] *m/z* (%)
2	3302 br (OH)	−10.36 (s)	312 (100) $\{Cu[P(CH_2OH)_3]_2\}^+$
	3107 m (CH)		149 (100) $\{[HC(tz)_2]\}^-$
	1660 br s (ν_{asym} CO_2)		193 (50) $\{[HC(CO_2)(tz)_2]\}^-$
3	3370 br m (OH)	−10.28 (s)	435 (100) $\{[HC(CO_2)(pz^{Me2})_2]$-$Cu[P(CH_2OH)_3] + H\}^+$
	3182 m (CH)		559 (80) $\{[HC(CO_2)(pz^{Me2})_2]$-$Cu[P(CH_2OH)_3]_2 + H\}^+$
	1647 br s (ν_{asym} CO_2)		247 (100) $\{[HC(CO_2)(pz^{Me2})_2]\}^-$
4	3360 br (OH)	−11.85 (br s)	312 (100) $\{Cu[P(CH_2OH)_3]_2\}^+$
	3134 m (CH)	−145.14 (septet, $J_{(F-P)}$ = 709.5 Hz, PF_6)	145 (100) $\{PF_6\}^-$
	2253 m (CN)		313 (30) $\{Na[PF_6] + PF_6\}^-$
			477 (20) $\{[Cu(CH_3CN)_3](PF_6)_2\}^-$

[a] In nujol mull. [b] In D_2O solution at 293 K. [c] Major positive and negative ions in H_2O solution.

We have also evaluated the in vitro antitumor activity of these new water soluble Cu(I) complexes in comparison with cisplatin, which is the most widely used metal-based antitumor drug.[41] It is well-known that cisplatin effectiveness is often severely limited by the occurrence of cellular resistance.[42] Thus, one of the main goals in the search for new metal-based anticancer agents is the circumvention of cisplatin resistance. In this study, we have investigated the cytotoxicity profile of several copper(I) complexes containing scorpionate and phosphine ligands against a panel of human tumor cell lines, also including cisplatin-resistant ovarian (C13*) and cervix (A431-Pt) carcinoma cells in which different molecular mechanisms underlie the resistance.[43,44] DNA flow cytometric analysis and biochemical investigations were performed to study the effects on the cell cycle and the induction of apoptosis. These investigations were aimed at gaining insight into the mechanism of action of this new series of copper(I) complexes.

Experimental Section

Materials and General Methods. All syntheses and handling were carried out under an atmosphere of dry oxygen-free dinitrogen, using standard Schlenk techniques or a glove box. All solvents were dried, degassed, and distilled prior to use. Elemental analyses (C,H,N,S) were performed in house with a Fisons Instruments 1108 CHNS-O elemental analyzer. Melting points were taken on an SMP3 Stuart Scientific Instrument. IR spectra were recorded from 4000 to 100 cm^{-1} with a Perkin-Elmer System 2000 FTIR instrument. ^{1}H and ^{31}P NMR spectra were recorded on a Oxford-400 Varian spectrometer (400.4 MHz for ^{1}H and 162.1 MHz for ^{31}P). Electrospray mass spectra (ESIMS) were obtained in positive- or negative-ion mode on a series 1100 MSD detector HP spectrometer.

Syntheses. All reagents were purchased from Aldrich and used without further purification. Sodium salt of the donor $Na[HC(CO_2)(pz^{Me2})_2]$[45] and the tris(hydroxymethyl)phosphine coligand[46,47] were prepared in accordance with the literature methods.

$Na[HC(CO_2)(tz)_2]$ (1). A methanol solution (100 mL) of 1,2,4-triazole (3.000 g, 43.4 mmol) was added to a solution of NaOH (3.600 g, 90.0 mmol) in 40 mL of absolute ethanol. After 1 day of stirring, a solution of dibromoacetic acid (4.730 g, 21.7 mmol) in 40 mL of absolute ethanol was added dropwise to the sodium salt so obtained, and the mixture was stirred for 24 h, allowed to cool to rt, concentrated, and then filtered, eventually affording a pale yellow solid. The crude product was recrystallized from diethyl ether/acetone (1:2), yielding $Na[HC(CO_2)(tz)_2]$ (**1**) as pale yellow microcrystalline needles; yield 72%; mp 219−220 °C dec.

$[HC(CO_2)(tz)_2]Cu[P(CH_2OH)_3]_2$ (2). To a methanol/acetonitrile (1:2) solution (50 mL) of $[Cu(CH_3CN)_4][PF_6]$ (0.373 g, 1.0 mmol) and $P(CH_2OH)_3$ (0.248 g, 2.0 mmol), $Na[HC(CO_2)(tz)_2]$ (0.216 g, 1.0 mmol) was added at room temperature. After the addition, the reaction mixture was stirred for 5 h, and solvent was removed under vacuum. The resulting solid was treated with chloroform (50 mL), and salt was removed by filtration. The solution was concentrated under vacuum, and a colorless solid was filtered off and recrystallized from $CHCl_3$/*n*-hexane (1:5) to give complex **2** in 71% yield; mp 175−176 °C.

$[HC(CO_2)(pz^{Me2})_2]Cu[P(CH_2OH)_3]_2$ (3). Complex **3** was prepared analogously to compound **2** by using $[Cu(CH_3CN)_4][PF_6]$ (0.373 g, 1.0 mmol), $P(CH_2OH)_3$ (0.248 g, 2.0 mmol), and $Na[HC(CO_2)(pz^{Me2})_2]$ (0.270 g, 1.0 mmol). The product was recrystallized from $CHCl_3$/diethyl ether (1:3) in 65% yield; mp 113−114 °C.

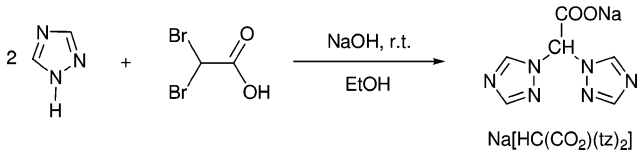
$[(CH_3CN)_2Cu(P(CH_2OH)_3)_2]PF_6$ (4). To an acetonitrile solution (40 mL) of $[Cu(CH_3CN)_4][PF_6]$ (0.372 g, 1.0 mmol), a methanol solution (20 mL) of $P(CH_2OH)_3$ (0.249 g, 2.0 mmol) was added at room temperature. After the addition, the reaction mixture was stirred for 24 h. The resulting white solid was filtered off, washed with acetonitrile, and recrystallized from acetonitrile/methanol to give complex **5** in 89% yield; mp 124 °C dec.

Characterization of complexes **2**−**4**, including elemental analysis, ^{1}H and ^{31}P NMR and IR spectroscopies, and ESI mass spectometry is reported in the Supporting Information and in Table 1.

Experiment with Human Cells. $[HC(CO_2)(tz)_2]Cu[P(CH_2OH)_3]_2$, **2**, $[HC(CO_2)(pz^{Me2})_2]Cu[P(CH_2OH)_3]_2$, **3**, and $[(CH_3CN)_2Cu(P(CH_2OH)_3)_2]PF_6$, **4**, complexes and the corresponding uncoordinated ligands were dissolved in purified water just before the experiment; cisplatin was dissolved in DMSO just before the experiment and a calculated amount of drug solution was added to the growth medium containing cells to a final solvent concentration of 0.5%, which had no discernible effect on cell killing. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and cisplatin were obtained from Sigma Chemical Co., St. Louis, MO.

Cell Cultures. A549, MCF7, and LoVo are human lung, breast, and colon carcinoma cell lines, respectively (ATCC, Rockville, MD), along with melanoma (A375) and human promyelocytic leukemia (HL60) cell lines; 2008 and its cisplatin resistant variant, C13*, are human ovarian cancer cell line, and they were kindly provided by Prof. G. Marverti (Dept. of Biomedical Science of Modena University, Italy); A431 and A431-Pt are sensible and resistant human cervix carcinoma, respectively. They were kindly provided by Prof. Zunino (Division of Experimental Oncology B, Istituto Nazionale dei Tumori, Milan, Italy). Cell lines were maintained in the logarithmic phase at 37 °C in a 5% carbon dioxide atmosphere, using the following culture media: (i) RPMI-1640 medium (Euroclone, Celbio, Milan, Italy) containing 10% fetal calf serum (Biochrom-Seromed GmbH&Co, Berlin, Germany) and supplemented with 25 mM HEPES buffer, L-glutamine, and the antibiotics penicillin (50 units·mL^{-1}) and streptomycin (50 μg·mL^{-1}) for HL60, MCF7, 2008, C13*, A431, and A431-Pt cells; (ii) F-12 HAM'S (Sigma Chemical Co.) containing 10% fetal calf serum, L-glutamine, penicillin (50 units·mL^{-1}), and streptomycin (50 μg·mL^{-1}) for LoVo cells; (iii) D-MEM (Dulbecco's modified eagle's medium; Euroclone) supplemented with 10% fetal calf serum (Euroclone), L-glutamine, penicillin (50 units·mL^{-1}), and streptomycin (50 μg·mL^{-1}) for A549 cells.

Scheme 1. Synthesis of Sodium Bis(1,2,4-triazol-1-yl)acetate, Na[HC(CO$_2$)(tz)$_2$], **1**



Cytotoxicity Assay. The growth inhibitory effect toward tumor cell lines was evaluated by means of MTT (tetrazolium salt reduction) assay.[48] Briefly, 3−8 × 10^3 cells/well, dependent upon the growth characteristics of the cell line, were seeded in 96-well microplates in growth medium (100 μL) and then incubated at 37 °C in a 5% carbon dioxide atmosphere. After 24 h, the medium was removed and replaced with a fresh one containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After 48 h, each well was treated with 10 μL of a 5 mg·mL^{-1} MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) saline solution, and after 5 h of incubation, 100 μL of a sodium dodecylsulfate (SDS) solution in 0.01 M HCl was added. After overnight incubation, the inhibition of cell growth induced by the tested complexes was detected by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 microplate reader. Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted versus drug concentration. IC$_{50}$ values represent the drug concentrations that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells.

Flow Cytometric Analysis. Drug-induced cell cycle effects and DNA fragmentation were analyzed by flow cytometry after DNA staining with propidium iodide (PI) according to Nicoletti et al.[49] Briefly, 2008 cells (5 × 10^5 cells) were exposed for 1−48 h to tested compound concentrations corresponding to IC$_{50}$ values. One mL of a PI solution, containing 50 μg/mL of PI, 0.1% m/v of Triton X-100, and 0.01% m/v of sodium citrate, was added to cells and then incubated for 25 min at 4 °C in the dark. Induced cell death was determined as a percentage of hypodiploid nuclei counted over the total cell population measured by FACSCalibur flow cytometer (Becton−Dickinson, CA) using a 550−600 nm filter. Analysis was performed by Cell Quest software (Becton−Dickinson, CA).

Caspase-3 Activity. Caspase-3 activity was detected by using the ApoAlert Caspase-3 Fluorescent Assay Kit (Clontech) according to the manufacturer-recommended procedures. In the amount of 10^6, 2008 cells were collected following 12 or 24 h of incubation of tested compounds (at concentrations corresponding to IC$_{50}$ values) and lysed on ice in 50 μL of lysis buffer for 10 min and then treated with 50 μL of reaction buffer containing dithiothreitol (DTT) and 5 μL of caspase-3 substrate solution (Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin [DEVD-AFC], Clontech). The fluorescence was determined with a Perkin-Elmer 550 spectrofluorometer (excitation 440 nm, emission 505 nm). The caspase-3 activity was expressed as the increase of the AFC-emitted fluorescence. The Student's t-test was used for data analysis.

Results and Discussion

Syntheses and Characterization of Cu(I) Complexes. The sodium salt of the bis(1,2,4-triazol-1-yl)acetate ligand, Na[HC(CO$_2$)(tz)$_2$], **1**, has been synthesized by treating a methanol solution of 1,2,4-triazole with a solution of NaOH in 40 mL of absolute ethanol, followed by a solution of dibromoacetic acid in absolute ethanol (Scheme 1). Compound **1** is an air- and moisture-stable pale yellow microcrystalline solid; it is soluble in protic solvents as water, methanol, and ethanol. The infrared spectra showed weak absorptions near 3100 cm^{-1} due to the pz ring C−H stretching, and medium to strong absorptions in the range 1520−1560 cm^{-1} related to ring "breathing" vibrations. The presence of the carboxylate moiety is detected by two intense, broad absorptions at 1661 cm^{-1}, due to the asymmetric CO$_2^-$ stretching mode. The ESI-MS positive-ion spectrum of **1** in water is dominated by fragments attributable to the aggregation of two (100%), three (60%), four (70%), and five (20%) sodium ions to the ligand. In the negative-ion spectrum of **1**, the major peak at *m*/*z* 409 (100%) is due to the species {2[HC(CO$_2$)(tz)$_2$] + Na}$^-$; other minor peaks are attributable to the free anionic ligand [HC(CO$_2$)(tz)$_2$]$^-$ (20%) and to the decarboxylated species {[HC(tz)$_2$]}$^-$ (40%).

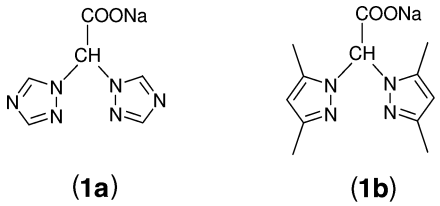


Figure 1. Structure of the ligands employed in this work: (**1a**) sodium bis(1,2,4-triazol-1-yl)acetate, Na[HC(CO$_2$)(tz)$_2$]; (**1b**) sodium bis(3,5-dimethylpyrazol-1-yl)acetate, Na[HC(CO$_2$)(pz^{Me2})$_2$].

Complexes **2** and **3** have been synthesized by reaction of Na[HC(CO$_2$)(tz)$_2$] (**1a**; Figure 1) or Na[HC(CO$_2$)(pz^{Me2})$_2$] (**1b**; Figure 1), with [Cu(CH$_3$CN)$_4$][PF$_6$] and P(CH$_2$OH)$_3$ in methanol/acetonitrile solutions (eq 1).

$$\mathrm{Na[L^{\textit{n}}] + [Cu(CH_3CN)_4][PF_6] + 2\ P(CH_2OH)_3 \rightarrow (L^{\textit{n}})Cu(P(CH_2OH)_3)_2} \quad (1)$$
2,**3**

(**2**) L^1 = [HC(CO$_2$)(tz)$_2$]$^-$. (**3**) L^2 = [HC(CO$_2$)(pz^{Me2})$_2$]$^-$.

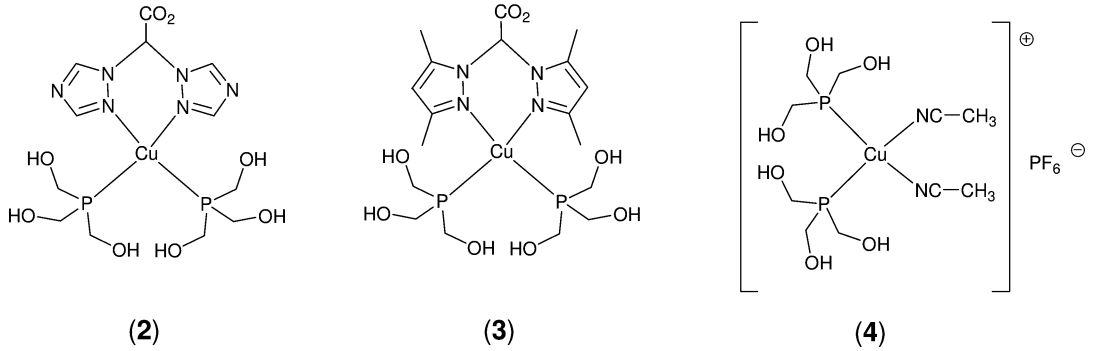
Similar reactions carried out in acetonitrile at room temperature, but without addition of the heteroscorpionate ligand (eq 2), afforded the bis-solvato complex [(CH$_3$CN)$_2$Cu(P(CH$_2$OH)$_3$)$_2$]PF$_6$, **4**. Addition of an excess of P(CH$_2$OH)$_3$ did not modify the stoichiometry of the resulting compound.

$$\mathrm{[Cu(CH_3CN)_4][PF_6] + 2\ P(CH_2OH)_3 \rightarrow [(CH_3CN)_2Cu(P(CH_2OH)_3)_2]PF_6} \quad (2)$$
4

Complexes **2**−**4** are stable in air and soluble in alcohols and water. The identity of these compounds has been established by ESI-MS, IR, and multinuclear (^{1}H, ^{31}P) NMR spectral studies (see Supporting Information and Table 1).

The infrared spectra showed all the expected bands for the scorpionate and the phosphane ligands: (i) weak absorptions in the range 3100−3200 cm^{-1} due to the pz ring C−H stretching, (ii) medium to strong absorptions in the range 1550−1560 cm^{-1} related to ring "breathing" vibrations, (iii) broad peaks in the range 3300−3370 cm^{-1} due to the hydroxylic groups of the phosphanes. In derivatives **2** and **3**, the presence of the COO moiety was detected by the appearance of intense absorptions at 1661 and 1647 cm^{-1}. No significant shifts were observed upon complex formation compared to those exhibited by uncoordinated ligands. In the IR spectrum of complex **4**, a medium peak at 2253 cm^{-1} was assigned to the stretching vibration of the CN groups.

The room-temperature ^{1}H NMR spectra of derivatives **2** and **3**, in D$_2$O solution, exhibited only one set of signals for the triazole rings protons. Upon coordination of the scorpionate ligands with the copper(I) acceptor, both azole rings and C*H*COO protons did not experience large chemical shift variation, suggesting very weak interaction (or lack of direct bond) of these rings and/or of the carboxylate group with the metal. In the ^{31}P NMR spectrum of complexes **2**−**4**, only one broad peak is observed at 293 K in the range of −10.28 to



(**2**) (**3**) (**4**)

Figure 2. Proposed structure of complexes **2**−**4**.

Table 2. Cytotoxic Activity[a]

	IC$_{50}$ (μM) ± S.D.				
cmpd	HL60	A549	MCF−7	A375	LoVo
[HC(CO$_2$)(tz)$_2$]Cu[P(CH$_2$OH)$_3$]$_2$, **2**	34.31 ± 3.9	25.13 ± 1.1	11.29 ± 1.7	26.66 ± 2.0	32.28 ± 3.6
[HC(CO$_2$)(pz^{Me2})$_2$]Cu[P(CH$_2$OH)$_3$]$_2$, **3**	10.60 ± 2.5	2.10 ± 1.3	1.55 ± 0.19	2.55 ± 0.9	7.83 ± 1.3
[(CH$_3$CN)$_2$Cu(P(CH$_2$OH)$_3$]$_2$]PF$_6$, **4**	21.15 ± 2.8	14.81 ± 0.9	16.7 ± 2.7	21.52 ± 1.7	47.17 ± 2.9
Na[HC(CO$_2$)(tz)$_2$], **1a**	ND	ND	ND	ND	ND
P(CH$_2$OH)$_3$	58.67 ± 2.3	72.97 ± 2.4	64.21 ± 4.2	88.71 ± 3.8	65.21 ± 3.2
Na[HC(CO$_2$)(pz^{Me2})$_2$], **1b**	ND	ND	88.81 ± 3.9	ND	ND
cisplatin	19.9 ± 2.5	39.27 ± 1.9	30.18 ± 1.5	20.28 ± 1.3	24.97 ± 1.5

[a] ND = not detectable. S.D. = standard deviation. IC$_{50}$ values were calculated by probit analysis ($P < 0.05$, χ^2 test). Cells (5−8 × 10^4·mL^{-1}) were treated for 48 h with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test.

−11.85 ppm (typical of a CuP$_2$ coordination core),[50] significantly downfield shifted if compared to the signal at δ −24.7 ppm exhibited by uncoordinated P(CH$_2$OH)$_3$. In the spectrum of [(CH$_3$CN)$_2$Cu(P(CH$_2$OH)$_3$]$_2$]PF$_6$, **4**, the additional septet at −145.14 ppm (J_{PF} = 709.5 Hz) was assigned to the PF$_6$ counterion.

Electrospray ionization mass spectroscopy was used to probe the existence of aggregates of the scorpionate ligand with Cu(I) and phosphane coligand in solution. Both positive-ion and negative-ion spectra of complexes **2**−**4**, dissolved in water, were recorded at low voltage (3.5−4.0 kV); under these experimental conditions, the dissociation is minimal and most of the analyte is transported to the mass spectrometer as the intact molecular species. The positive-ion spectrum of compounds **2** and **4** were dominated by the fragment {Cu[P(CH$_2$OH)$_3$]$_2$}$^+$ at *m*/*z* 312 (100%). Instead, in the positive-ion spectrum of compound **3** we observed the peak due to the protonated species {[HC(CO$_2$)(pz^{Me2})$_2$]Cu[P(CH$_2$OH)$_3$]$_2$ + H}$^+$, together with the peak attributable to the fragment ion {[HC(CO$_2$)(pz^{Me2})$_2$]Cu[P(CH$_2$OH)$_3$] + H}$^+$, generated by the loss of only one tris(hydroxymethyl)phosphane coligand. The negative-ion spectra of compounds **2** and **3** were dominated by the fragments due to the free scorpionate ligands, together with the decarboxylated species {[HC(tz)$_2$]}$^-$ in the case of compound **2**.

The spectroscopic and analytical data for the Cu(I) complexes utilized in this study suggest the tetrahedral molecular structures outlined in Figure 2.

Cytotoxicity Studies. [HC(CO$_2$)(tz)$_2$]Cu[P(CH$_2$OH)$_3$]$_2$, **2**, [HC(CO$_2$)(pz^{Me2})$_2$]Cu[P(CH$_2$OH)$_3$]$_2$, **3**, and [(CH$_3$CN)$_2$Cu(P(CH$_2$OH)$_3$)$_2$]PF$_6$, **4**, and the corresponding uncoordinated ligands were examined for their cytotoxic properties against a panel of human tumor cell lines containing examples of lung (A549), colon (LoVo), and breast (MCF-7) cancer, leukemia (HL60), and melanoma (A375). For comparison purposes, the cytotoxicity of cisplatin was evaluated under the same experimental conditions. IC$_{50}$ values, calculated from the dose-survival curves obtained after 48 h of drug treatment from the MTT test, are shown in Table 2.

Uncoordinated ligands proved to be quite ineffective in all tumor cell lines; P(CH$_2$OH)$_3$ gave detectable IC$_{50}$ values that were, anyway, markedly higher than those of the corresponding copper(I) complexes. Compounds **2** and **4** showed IC$_{50}$ values comparable with those exhibited by cisplatin against HL60, A375, and LoVo cell lines, whereas their cytotoxic activity was significantly higher against A549 and MCF-7 cell lines. This general behavior is magnified in the case of complex **3**, whose cytotoxic potency exceeded that of the parent drug by a factor ranging from about 2 to 15.

The encouraging results obtained against the in-house panel of cell lines prompted us to test the cytotoxic activity of our Cu(I) complexes onto two additional cell line pairs, which were selected for their resistance to cisplatin, 2008/C13* ovarian cancer cells, and A431/A431-Pt cervix carcinoma cells. Although cisplatin resistance is multifactorial, the main molecular mechanisms involved in resistance in these sublines have almost been defined. In C13* cells, resistance is correlated to reduced cellular drug uptake, high cellular glutathione levels, and enhanced repair of DNA damage.[43] In human squamous cervix carcinoma A431-Pt cells, resistance is due to defect in drug uptake and to decreased levels of proteins involved in DNA mismatch repair (MSH2), causing an increased tolerance to cisplatin-induced DNA damage.[44] Cytotoxicity of tested compounds in sensitive and resistant cells was assessed after a 48-h drug exposure by the MTT test (Table 3). Cross-resistance profiles were evaluated by means of the resistance factor (RF), which is defined as the ratio between IC$_{50}$ values calculated for the resistant cells and those arising from the sensitive ones. Remarkably, all the tested copper(I) complexes exhibited a different cross-resistance profile than that of cisplatin. Against the ovarian cancer, RF values were about 6 to 18 times lower than those calculated for cisplatin, while against the cervix cell line pair, they were 3 to 5 times lower. The overcoming of cross-resistant phenomena in both cell line pairs strongly supports the hypothesis of a different pathway of action of these copper(I) complexes from that of cisplatin. Also, in this set of experiments, complex **3** appeared to be the most effective

Table 3. Cross-Resistance Profiles[a]

Compound	2008 IC_{50} (μM)	C13* IC_{50} (μM)	R.F.
$[HC(CO_2)(tz)_2]Cu[P(CH_2OH)_3]_2$, **2**	26.36 ± 1.9	12.40 ± 2.2	0.47
$[HC(CO_2)(pz^{Me2})_2]Cu[P(CH_2OH)_3]_2$, **3**	6.6 ± 1.1	8.16 ± 2.5	1.23
$[(CH_3CN)_2Cu(P(CH_2OH)_3]_2]PF_6$, **4**	24.04 ± 1.8	19.12 ± 1.9	0.79
cisplatin	12.69 ± 1.7	89.18 ± 4.5	7.02
Compound	A431 IC_{50} (μM)	A431/Pt IC_{50} (μM)	R.F.
$[HC(CO_2)(tz)_2]Cu[P(CH_2OH)_3]_2$, **2**	49.27 ± 2.3	32.44 ± 2.0	0.65
$[HC(CO_2)(pz^{Me2})_2]Cu[P(CH_2OH)_3]_2$, **3**	7.04 ± 0.9	5.72 ± 2.1	0.81
$[(CH_3CN)_2Cu(P(CH_2OH)_3]_2]PF_6$, **4**	43.63 ± 1.4	23.09 ± 1.9	0.52
cisplatin	22.0 ± 1.0	57.76 ± 1.8	2.62

[a] S.D. = standard deviation. IC_{50} values were calculated by probit analysis ($P < 0.05$, χ^2 test). Cells ($3-5 \times 10^4$·mL^{-1}) were treated for 48 h with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test. Resistant factor (R.F.) is defined as IC_{50} resistant/parent line.

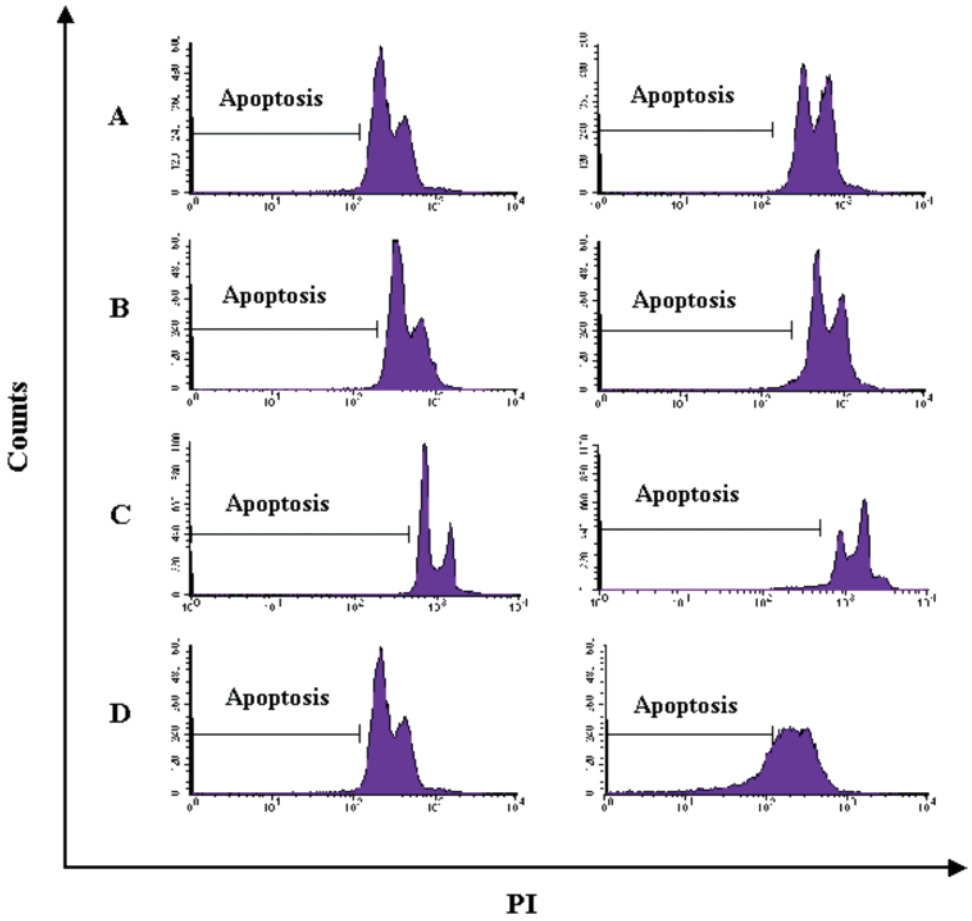


Figure 3. Cell cycle analysis of 2008 human ovarian cancer cells. Panels A−C represent different time points (18, 24, and 48 h, respectively) of control cells (left) and cells treated with IC_{50} concentrations of complex **3** (right). Panel D represents control cells after 18 h in normal medium (left) and cells treated with IC_{50} concentrations of cisplatin (right).

derivative, giving IC_{50} values 2−3 times lower than the reference drug on the parental sensitive cell lines and 10 times lower on the cisplatin-resistant ones. The lower cytotoxicity displayed by complexes **2** and **4** might be related to their moderate stability (see mass spectrometric and IR and NMR spectroscopic data), which likely arises from partial in vitro hydrolysis of these species, thus diminishing their availability for cellular uptake and intracellular trafficking.

Cell Cycle and Apoptosis Studies. We have consequently studied further the cellular effects induced by the representative complex **3** in terms of cell cycle modifications and induction of apoptosis, aiming at the understanding of a possible mechanism of action. Up to now, there are very few mechanistic studies dealing with the anticancer activity of copper(I) compounds. Cu(I) complexes of the type [Cu(dppey)]Cl (where dppey denotes *cis*-$Ph_2PCH{=}CHPPh_2$) have been supposed to act on mitochondria by uncoupling the oxidative phosphorylation by inner mitochondrial membrane depolarization.[51] More recently, the potent cytotoxic activity promoted by $[Cu_2(DPPE)_3(CH_3CN)_2](ClO_4)_2$ (DPPE = $Ph_2PCH_2{-}CH_2PPh_2$) against human lung cancer cells was found to be due to a p53-mediated cell cycle arrest and apoptosis.[52]

To explore complex **3** mode of action on induced cell death, we performed a time-dependent evaluation of the cell-cycle profile of 2008 human ovarian carcinoma cells treated with complex **3** by fluorescence-activated cell sorting (Figure 3). Cisplatin was used under the same experimental conditions as positive control. Treatment of cells with IC_{50} concentrations of complex **3** resulted in a time-dependent increase of G2/M subpopulation within 1−48 h (Figure 3, panels A−C). In

Table 4. Cell Cycle Phases[a]

	18 h			24 h			48 h		
	ctrl	**3**	*P* values	ctrl	**3**	*P* values	ctrl	**3**	*P* values
SUB-G1%	3.34 ± 0.89	3.21 ± 1.20	>0.05	2.31 ± 0.22	10.43 ± 1.90	0.03	2.44 ± 0.17	9.29 ± 0.46	<0.001
G1%	63.88 ± 0.57	50.02 ± 0.99	<0.001	46.28 ± 0.11	28.25 ± 1.33	0.003	59.51 ± 1.39	24.32 ± 1.55	<0.001
G2/M%	33.23 ± 0.95	48.26 ± 0.54	<0.001	52.27 ± 0.14	62.43 ± 0.55	0.002	38.65 ± 1.24	67.09 ± 1.66	<0.001

[a] Percentage of cells in different cell cycle phases as a function of time exposure to IC_{50} concentrations of complex **3** vs control cells (*P* values were determined by the Welch t-test).

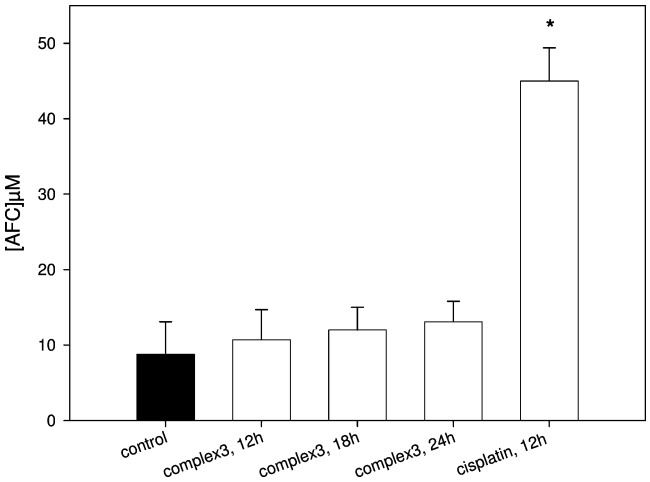


Figure 4. Induction of caspase-3. The 2008 cells were incubated with IC_{50} concentrations of complex **3** or cisplatin and then submitted to the test on caspase-3 induction as described in Experimental Section. Data are the means of at least three independent experiments. Error bars indicate standard deviation. *$P < 0.01$ compared to untreated cells.

particular, after 48 h treatment, the percent of cells in the G2/M phase was significantly higher (about 30%) than that of control cells (Table 4). However, a reduction of G1 cells fraction was observed even at 18 h incubation (Figure 3, panel A). On the contrary, the percentage of 2008 cells with hypodiploid DNA markedly increased by treatment with cisplatin; in particular, the percentage of cells with fragmented DNA was higher (27%) compared to the control samples (Table 4). These results, pointing out a scarce presence of classical signs of apoptosis, suggest that complex **3**-treated cells mainly died through a nonapoptotic cell death mechanism.

Caspase-3 is a well-known executor enzyme in apoptosis,[53] and cisplatin may cause apoptosis associated with increased caspase-3 activity.[54] Complex **3** was tested for its ability to induce caspase-3 activation in lysates of 2008 cells over a period of 24 h (Figure 4). Treatment with IC_{50} concentrations of complex **3** did not result in a significant enhancement of enzyme activity with comparison to untreated cells at any incubation time tested. In contrast, in cisplatin-treated cells, caspase-3 activity became significantly higher than that detected in control cells already at 12 h, being about 6 times the control value.

An additional insight into the cytotoxic potential of the new copper(I) complex has been offered by forward and side light scatter that are used as indices of cell size and cell granularity, respectively. After exposure to IC_{50} concentrations of complex **3**, 2008 cells showed a marked increase of cell size and of cell granularity (Figure 5, upper right quadrant); these alterations became extremely pronounced after a 48 h treatment (Figure 5, panel C). These remarkable morphological changes may be related to the ability of complex **3** to cause an extensive cytosolic vacuolization. Actually, it is well-known that cells with increased light scattering properties may contain structures such as vacuoles or aggregated proteins.[55] Taken together, all our data appear to be in agreement with the hypothesis that the new

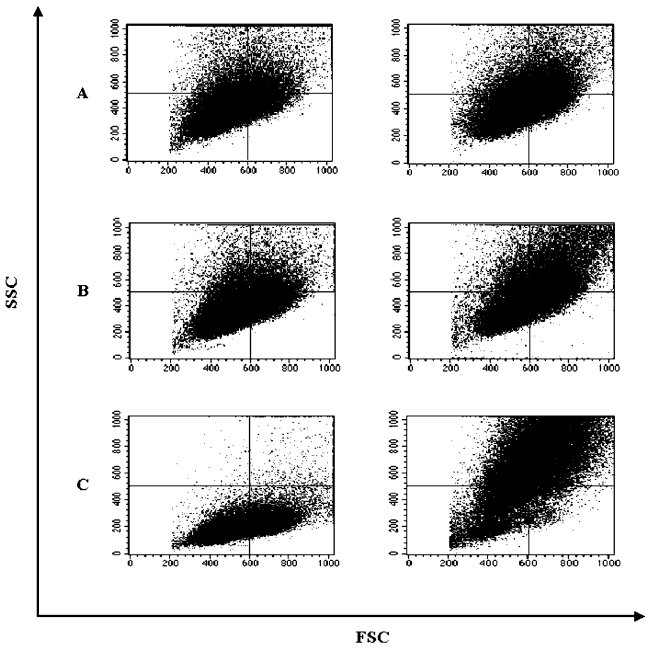


Figure 5. Forward scattering vs side scattering as a function of time in 2008 cells untreated (left) and treated with IC_{50} concentrations of complex **3** (right).

copper(I) complex may activate a cell death pathway distinct from apoptosis, namely, paraptosis. This alternative programmed cell death has been recently characterized by cytoplasmatic vacuolation and, typically, does not involve activation of caspases, the formation of apoptotic bodies, or other characteristics of apoptosis.[56] A similar parapoptotic cell death mechanism has been recently described in human fibrosarcoma cells treated with a Cu(II) thioxotriazole complex, which caused many cytoplasmic vesicles that progressively enlarged and coalesced until the late phases of the cell death process.[57]

The delineation of a cell-death mechanism different from that elicited by cisplatin makes this water-soluble copper(I) complex **3** a very interesting candidate for the development of new metal-based drugs with improved pharmacokinetic properties that is able to overcome the intrinsic or acquired resistance of several tumors to platinum drugs.

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Supporting Information Available: Complete characterization details of compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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