In Vitro Antitumor Activity of the Water Soluble Copper(I) Complexes Bearing the Tris(hydroxymethyl)phosphine Ligand

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Monocationic hydrophilic complexes $[Cu(thp)_4]^+$ **3** and $[Cu(bhpe)_2]^+$ **4** were synthesized by ligand exchange reactions starting from the labile $[Cu(CH_3CN)_4][PF_6]$ precursor in the presence of an excess of the relevant hydrophilic phosphine. Complexes **3** and **4** were tested against a panel of several human tumor cell lines. Complex **3** has been shown to be about 1 order of magnitude more cytotoxic than cisplatin. Chemosensitivity tests performed on cisplatin and multidrug resistance phenotypes suggested that complex **3** acts via a different mechanism of action than the reference drug. Different short-term proliferation assays suggested that lysosomal damage is an early cellular event associated with complex **3** cytotoxicity, probably mediated by an increased production of reactive oxygen species. Cytological stains and flow cytometric analyses indicated that the phosphine copper(I) complex is able to inhibit the growth of tumor cells via G2/M cell cycle arrest and paraptosis accompanied with the loss of mitochondrial transmembrane potential.

Introduction

Phosphine complexes of group 11 metals, especially those including the gold(I) ion, have been proposed as alternative anticancer drugs to the cisplatin reference drug.[1,2] In general, the higher drug tolerance profiles of gold-based agents compared to the severe toxic effects on normal tissues or the occurrence of intrinsic or acquired resistance induced by cisplatin have represented a hopeful prospect for the chemotherapeutic application of gold-based drugs. Despite this promising outlook, clinical trials in humans of gold compounds still remain elusive.

The introduction of the first metal phosphine complex auranofin (a thioglucose derivative of triethylphosphine gold(I)) into clinical use as an antiarthritic drug has represented the basis for a series of investigations focused on the biological chemistry and anticancer properties of phosphines and their related metal complexes.[3] Auranofin itself was shown to possess in vivo antitumor activity against P388 murine leukemia.[4] More recently, in vitro studies indicated that auranofin is able to overcome cisplatin resistance in human ovarian cancer cells,[5] confirming the early assumption that a mechanism of action different from the DNA damage induced by cisplatin could underlie the cytotoxic activity of phosphine Au(I) drugs. It was found that auranofin, acting as a potent inhibitor of thioredoxin reductase, determines an alteration of the redox state of the cell leading to an increased production of hydrogen peroxide and oxidation of the components of the thyoredoxin system, therefore creating the conditions for augmented apoptosis.[5]

Other phosphine rich gold(I) compounds of the type $[Au(dppe)_2][Cl]$ (dppe[a]= 1,2-bis(diphenylphosphino)ethane) have been reported to show cytotoxic activity against several cell lines, including B16 melanoma, P388 leukemia, and M5076 reticulum cell carcinoma.[6]

A rational extension of diphosphine gold(I) chemistry to the first row congener copper indicated that analogous copper(I) complexes of "CuP_4" stoichiometry could be readily prepared and tested in vitro. Among them, $[Cu(dppe)_2][Cl]$ and $[Cu(dppey)_2][Cl]$ (dppey = 1,2-bis(diphenylphosphino)ethylene) produced cytotoxic effects on cell metabolism comparable to those exhibited by Au(I) analogues, which were finally postulated to be the result of the uncoupling of mitochondrial oxidative phosphorylation.[7] However, the presence of several phenyl groups appended to the phosphorus donors in both $[Cu(dppey)_2][Cl]$ and $[Au(dppe)_2][Cl]$ species caused undesired nephrotoxicity[8] and cardiovascular toxicity[9] in animal models, respectively, thus precluding clinical trials in humans.

Partial substitution of the CuP_4 coordination sphere was attempted using heterocyclic thiones[10] or acetonitrile,[11] producing "mixed compounds". Among these derivatives, $[Cu_2(dppe)_3(CH_3CN)_2][ClO_4]_2$ exhibited a potent in vitro cytotoxicity in H460 human lung carcinoma cells, comparable to that displayed by adriamycin.[11]

Following a similar "mixed ligand" approach, we have recently coordinated several N_2-scorpionate ligands to a vacant lipophilic CuP_2 moiety (P_2 = bidentate dppe or two monodentate arylphosphines), trying to enhance the hydrophilic character of the overall phosphine−scorpionate copper species.[12] These less

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[a] Abbreviations: bhpe, bis[bis(hydroxymethyl)phosphino]ethane; DABCO, 1,4-diazabicyclo[2.2.2]octane; dppe, 1,2-bis(diphenylphosphino)ethane; dppey, 1,2-bis(diphenylphosphino)ethylene; DMSO, dimethyl sulfoxide; HE, hematoxylin−eosin; MDR, multidrug resistance; MI, mitotic index; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NR, neutral red; PBS, phosphate buffered saline; PI, propidium iodide; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TB, trypan blue exclusion; thp, tris(hydroxymethyl)phosphine; TMRM, tetramethylrhodamine methyl ester.

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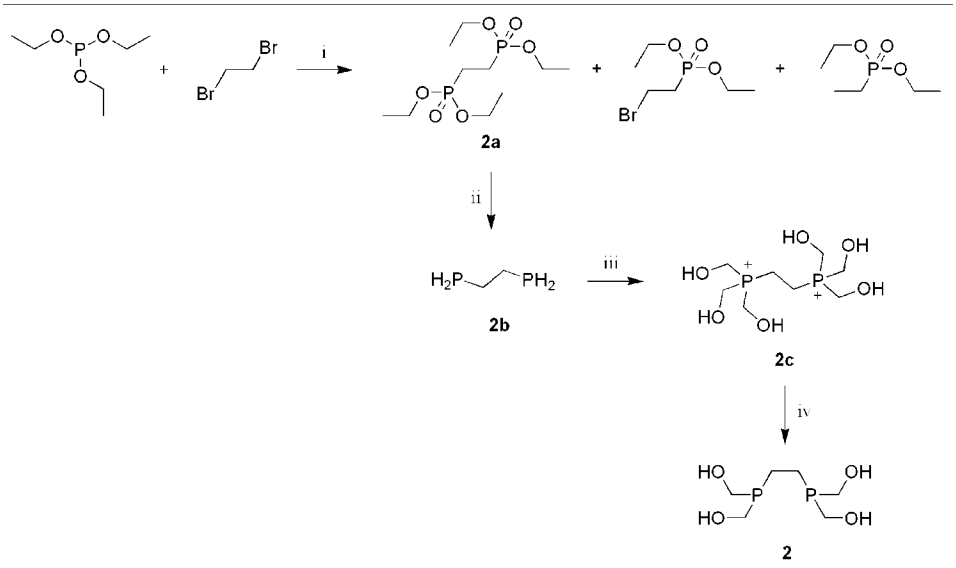
Scheme 1. Preparation of bhpe, **2**[a]



[a] Reaction conditions: (i) 180 °C, reflux; (ii) 0 °C, Et_2O, $LiAlH_4$; (iii) room temp, EtOH, 40% HC(O)H, 3 M HCl; (iv) room temp, 1 M $NaHCO_3$.

Table 1. Selected IR, $^{31}P\{^1H\}$ NMR, and ESI-MS Data of thp **1**, bhpe **2** Ligands, and Copper(I) Complexes **3** and **4**

compd	IR (cm^{-1})[a]	δ $^{31}P\{^1H\}$ NMR[b]	ESI-MS *m/z* (%)[c]
thp **1**		−24.70 (s)	
bhpe **2**	3458 br (OH)	−22.18 (s)	ESI(−): 250 (100) [bhpe + Cl]$^-$
$[Cu(thp)_4][PF_6]$ **3**	3360 mbr (OH)	−5.35 (q, J_{PCu} = 780 Hz)	560 (6) $[Cu(thp)_4]^+$
	3134 m (CH)	−145.14 (septet, J_{PF} = 709 Hz)	436 (65) $[Cu(thp)_3]^+$
	560 s, 477 sbr		312 (100) $[Cu(thp)_2]^+$
	455 w		
$[Cu(bhpe)_2][PF_6]$ **4**	3448 br (OH)	+9.67 (dbr)	492 (100) $[Cu(bhpe)_2]^+$
	3197 w (CH)	−144.05 (septet, J_{PF} = 709 Hz)	
	565 m, 452 sbr		
	376 w		

[a] In Nujol mull. [b] In D_2O solution at 293 K. [c] In water/methanol solutions. Positive ions unless otherwise stated.

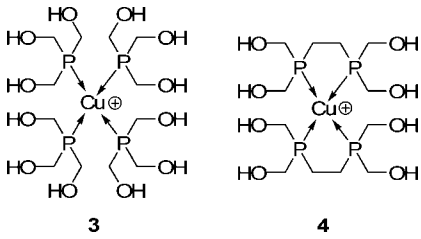


Figure 1. Molecular structure of copper complexes **3** and **4**.

hydrophobic Cu(I) compounds were proved to be easier to handle during the in vitro tests and, more importantly, retained cytotoxic activity against a panel of human tumor cell lines including, for instance, A549 carcinoma cells that show poor sensitivity to cisplatin treatment.[12] In a subsequent work, we established that the substitution of hydrophobic phosphines (e.g., dppe or triphenylphosphine) with hydrophilic tris(hydroxymethyl)phosphines did not affect the stability of the resulting mixed Cu(I) complexes and permitted the preparation of water soluble species with improved cytotoxic properties. In particular, $[HC(CO_2)(pz^{Me2})_2]Cu[P(CH_2OH)_3]_2$ was found to have a significant antiproliferative activity against different human tumor cells, also including examples of cisplatin resistant cancers. Our results have suggested that the cytotoxic activity of this copper(I) complex may be correlated to its ability to induce a nonapoptotic mechanism of cell death.[13]

In the present study, we have prepared two copper complexes of the "CuP_4" type,[14,15] namely, $[Cu(thp)_4][PF_6]$ (**3**) (thp = tris(hydroxymethyl)phosphine) and $[Cu(bhpe)_2][PF_6]$ (**4**) (bhpe = bis[bis(hydroxymethyl)phosphino]ethane). These Cu(I) complexes have been characterized by elemental analysis, FTIR, ESI-MS, and multinuclear (^1H, $^{31}P\{^1H\}$, $^{13}C\{^1H\}$, and ^{63}Cu) NMR spectral data, and they indeed showed high solubility and stability in aqueous solutions. We have assessed the antitumoral properties of these copper complexes in a large panel of human cancer cell lines belonging to a variety of different tumor types and also including cisplatin and multidrug resistance (MDR) phenotypes. Our results showed that complex **3** is endowed with a remarkable in vitro antitumor activity significantly higher than that of cisplatin which was used as reference drug in all our investigations. In particular, chemosensitivity tests performed on the resistant sublines suggested that $[Cu(thp)_4][PF_6]$ may act with a different mechanism compared to cisplatin. The detection of complex **3** mediated cytotoxicity, by means of different assays measuring the damage to various subcellular organelles, revealed that lysosomal damage represents the early cellular event associated with complex **3** induced cell death. Moreover, this event may be mediated by an increased production of reactive oxygen species (ROS) as attested by measuring the tumor cell viability of complex **3** treated cells in the presence of different ROS scavengers. Finally, in order to throughly explore the sequence of cellular events responsible for the antiproliferative action of complex **3**, we have investigated the effects on cell cycle modulation and the cell death mechanism by means of flow cytometric analysis. In previous studies, the antitumor properties of the isostructural gold(I) cation $[Au(thp)_4]^+$ had been ascribed to its ability in selectively elongating (or arresting) the G1 phase of the cell cycle.[16] In the present work, we have

Table 2. Cytotoxic Activity[a]

	$IC_{50} \pm SD$ (μM)								
compd	HL60	A549	MCF-7	Daudi	HepG2	A375	CaCo2	HCT-15	HeLa
$[Cu(thp)_4][PF_6]$ **3**	0.60 ± 0.02	9.11 ± 2.71	11.08 ± 0.52	6.94 ± 0.18	1.26 ± 0.10	4.58 ± 2.41	1.08 ± 0.12	2.00 ± 0.03	8.21 ± 1.50
$[Cu(bhpe)_2][PF_6]$ **4**	47.40 ± 2.92	57.60 ± 2.19	49.71 ± 2.03	65.5 ± 1.22	78.23 ± 1.11	68.21 ± 1.23	52.50 ± 0.81	57.36 ± 1.31	62.41 ± 1.33
thp	68.63 ± 2.44	72.91 ± 2.44	64.23 ± 4.29	>100	98.71 ± 3.63	88.70 ± 3.88	>100	>100	>100
bhpe	83.72 ± 3.23	>100	>100	>100	71.71 ± 1.64	73.21 ± 1.22	84.11 ± 2.22	91.71 ± 4.01	>100
KPF_6	>100	>100	>100	>100	>100	>100	>100	>100	>100
cisplatin	15.91 ± 1.51	29.21 ± 1.92	19.04 ± 1.51	23.97 ± 2.51	21.50 ± 1.41	20.33 ± 1.33	35.42 ± 1.40	25.34 ± 1.31	10.50 ± 1.51

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

Table 3. Cross-Resistance Profiles[a]

Human Ovarian Adenocarcinoma Cells

	$IC_{50} \pm SD$ (μM)		
compd	2008	C13	RF
$[Cu(thp)_4][PF_6]$ **3**	1.48 ± 0.21	2.88 ± 1.07	1.9
cisplatin	12.69 ± 1.72	89.18 ± 4.50	7.02

Human Cervix Squamous Carcinoma Cells

	$IC_{50} \pm SD$ (μM)		
compd	A431	A431/Pt	RF
$[Cu(thp)_4][PF_6]$ **3**	14.37 ± 1.41	13.26 ± 0.80	0.92
cisplatin	22.06 ± 1.62	57.76 ± 1.81	2.61

Human Colon Adenocarcinoma Cells

	$IC_{50} \pm SD$ (μM)		
compd	LoVo	LoVo-MDR	RF
$[Cu(thp)_4][PF_6]$ **3**	1.54 ± 0.03	2.9 ± 0.1	1.88
doxorubicin	1.46 ± 2.30	44.89 ± 0.90	30.74

[a] SD = 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 (RF) is defined as IC_{50} resistant/parent line.

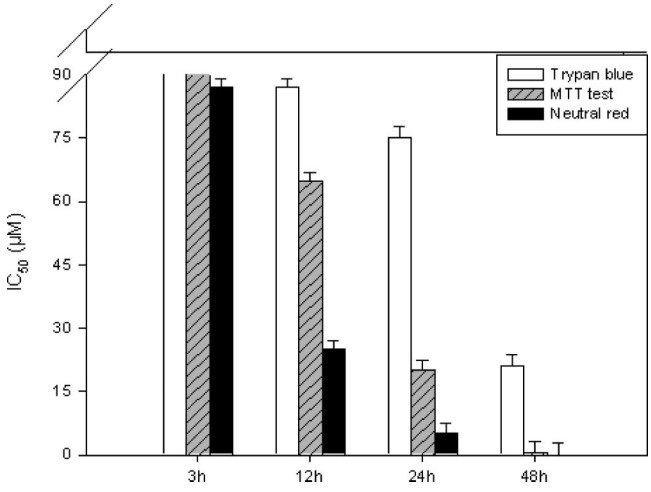


Figure 2. Comparison of IC_{50} values detected by MTT, NR, and TB tests after incubation of 2008 cells with complex **3** for 3, 12, 24, and 48 h. Error bars indicate standard deviation.

demonstrated that the coordination of monophosphine ligands to copper(I) gives rise to a metallodrug able to inhibit the growth of tumor cells via G2/M cell cycle arrest and paraptosis accompanied by the loss of mitochondrial transmembrane potential.

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 glovebox. All solvents were dried, degassed, and distilled prior to use. Description of the instrumentation is reported in the Supporting Information (see also ref 13).

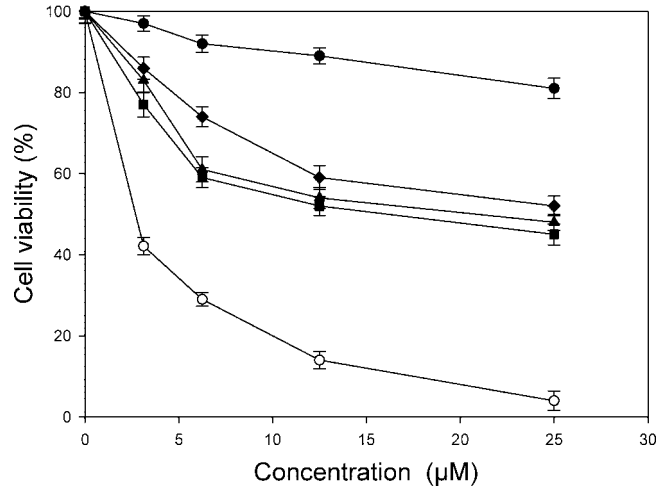


Figure 3. Effects of ROS scavengers on complex **3** cytotoxicity against 2008 cells. Mannitol (1 mM, ▲), DABCO (1.2 mM, ■), tocopherol + ascorbic acid (1 mM, ●), and sodium selenite (0.1 μM, ◆) were added to cell cultures for 48 h in concomitance with complex **3**. Results from complex **3** alone are represented by the open circle (○). MTT test was used to measure cell viability. Results are expressed as percentage viability of control cells.

Synthesis. All reagents were purchased from Aldrich and used without further purification. The tris(hydroxymethyl)phosphine (thp) **1** was prepared in accordance with the literature methods.[17,18] 1,2-Bis[bis(hydroxymethyl)phosphino]ethane (bhpe) **2** was synthesized using a multistep procedure different from that originally reported by Katti and co-workers.[19]

Synthesis of 1,2-Bis[bis(hydroxymethyl)phosphino]ethane (bhpe) (2). Triethyl phosphite (50 mL, 0.3 mol) and 1,2-dibromoethane (53 mL, 0.6 mol) were refluxed at 180 °C overnight. After cooling, the solution was distilled under vacuum. The residual product of distillation was a colorless mobile liquid identified as 1,2-bis(diethylphosphonyl)ethane (**2a**). Freshly distilled anhydrous diethyl ether (25 mL) was added to a two-necked round-bottom flask containing **2a** (1.000 g, 3.309 mmol) under a dinitrogen atmosphere. The flask was cooled to 0 °C, and $LiAlH_4$ (20 mL of a 1.0 M Et_2O solution) was slowly added. The ice bath was removed, and the whitish cloudy mixture was stirred at room temperature for 30 min. The flask was cooled again to 0 °C, and a saturated water solution of Na_2SO_4 was added dropwise until evolution of dihydrogen stopped. Anhydrous Na_2SO_4 was added to the white suspension. The mixture was then filtered off under a dinitrogen atmosphere, and the filtrate was washed with diethyl ether. The ethereal phase was characterized by $^{31}P\{^1H\}$ NMR spectroscopy ($CDCl_3$, 293K: δ –132.79 s) as the primary phosphine $H_2PCH_2CH_2PH_2$ (**2b**). Degassed EtOH (10 mL), formaldehyde (1.2 mL of a 40% m/v H_2O solution), and HCl (2 mL of a 3 M solution) were added to the ethereal solution of **2b** under a dinitrogen atmosphere. The reaction mixture was stirred at room temperature

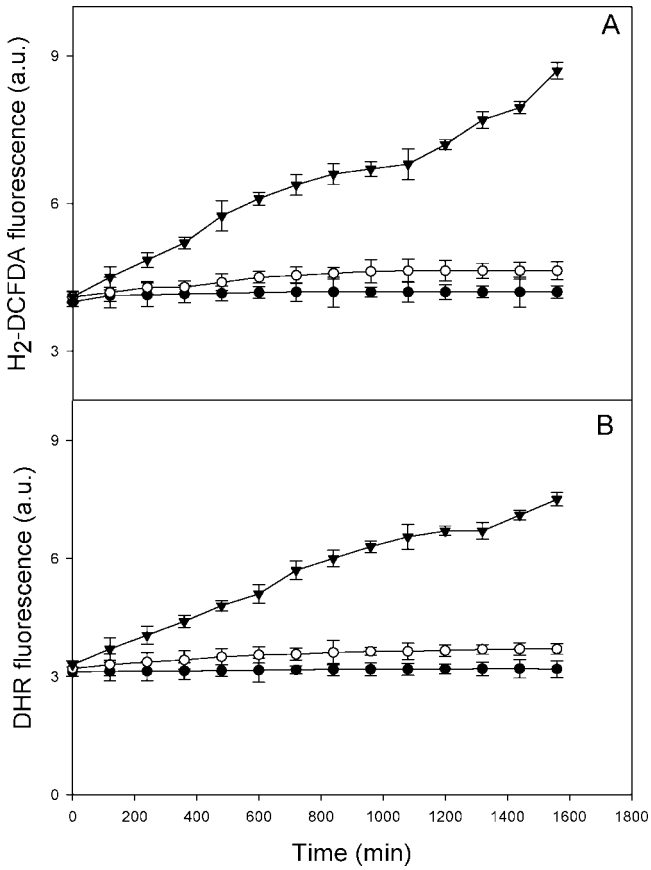


Figure 4. Effect of IC_{50} concentrations of complex **3** on ROS production in 2008 cells. Cells were loaded with the fluorescent probes H_2DCFDA (10 μM) (A) and DHR (50 μM) (B) as described in Experimental Section. Symbols are as follows: ●, control; ○, complex **3**; ▼, antimycin (3 μM).

for 1 h to obtain a white solid, which was filtered off under a dinitrogen atmosphere, and was identified as 1,2-bis[tris(hydroxymethyl)phosphonium]ethane chloride (**2c**). $NaHCO_3$ (1 M solution) was added to a water solution (3 mL) of **2c** to yield a basic solution (pH 9). The solvent was removed in vacuo, and the residue was recrystallized from methanol to afford $[(HOCH_2)_2PCH_2CH_2P(CH_2OH)_2)]\cdot 2NaCl$ **2** as a white solid. Yield 52%. Anal. ($C_6H_{16}Cl_2Na_2O_4P_2$) C, H.

Synthesis of [Cu(thp)$_4$][PF$_6$] (3). To an acetonitrile solution (40 mL) of $[Cu(CH_3CN)_4][PF_6]$ (0.373 g, 1.0 mmol), a methanol solution (20 mL) of thp (0.496 g, 4.0 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/ethanol to give complex **3** in 80% yield. Anal. ($C_{12}H_{36}CuF_6O_{12}P_5$) C, H.

Synthesis of [Cu(bhpe)$_2$][PF$_6$] (4). To an acetonitrile solution (40 mL) of $[Cu(CH_3CN)_4][PF_6]$ (0.373 g, 1.0 mmol), **2** (0.662 g, 2.0 mmol) was added at room temperature. After the addition, the reaction mixture was stirred for 2 h. The resulting white solid was filtered off, washed with methanol, and recrystallized from methanol/acetonitrile to give complex **4** in 78% yield. Anal. ($C_{12}H_{32}CuF_6O_8P_5$) C, H.

Experiments with Human Cells. $[Cu(thp)_4][PF_6]$ (**3**) and $[Cu(bhpe)_2][PF_6]$ (**4**) complexes and the corresponding uncoordinated ligands and counteranions 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. Human lung (A549), breast (MCF-7), liver (HepG2), colon (CaCo-2, HCT-15), and cervix (HeLa) carcinoma cell lines along with melanoma (A375), Burkitt lymphoma (Daudi), and promyelocytic leukemia (HL60) cell lines were obtained from ATCC, Rockville, MD. The 2008 and its cisplatin resistant variant, C13*, are human ovarian cancer cell lines, and they were kindly provided by Prof. G. Marverti (Department of Biomedical Science of Modena University, Italy). A431 and A431-Pt are sensible and resistant human cervix carcinoma cells, respectively; they were kindly provided by Prof. Zunino (Division of Experimental Oncology B, Istituto Nazionale dei Tumori, Milan, Italy). LoVo human colon-carcinoma cell line and its derivative multidrug-resistant subline (LoVo MDR) were kindly provided by Prof. Franca Majone (Department of Biology of Padova University, Italy). Cell lines were maintained in the logarithmic phase at 37 °C in a 5% carbon dioxide atmosphere using the following culture media containing 10% fetal calf serum (Biochrom-Seromed GmbH&Co, Berlin, Germany), antibiotics (50 units$\cdot$mL^{-1} penicillin and 50 μg$\cdot$mL^{-1} streptomycin), and 2 mM L-glutamine: (i) RPMI-1640 medium (Euroclone, Celbio, Milan, Italy) with 25 mM HEPES buffer for HL60, MCF-7, HCT-15, Daudi, 2008, C13*, A431, and A431-Pt cells; (ii) F-12 HAM'S (Sigma Chemical Co.) for HeLa, LoVo, and LoVo MDR cells; (iii) D-MEM medium (Euroclone) for A549, A375, HepG2 cells; (iv) MEM medium (Sigma) for CaCo2 cells. LoVo MDR culture medium also contained 0.1 μg$\cdot$mL^{-1} doxorubicin.

Cytotoxicity Assays. MTT Test. The growth inhibitory effect toward tumor cell lines was evaluated by means of MTT (tetrazolium salt reduction) assay.[20] Briefly, (3–8) $\times$ 10^3 cells/well, dependent on 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 3, 12, 24, or 48 h, each well was treated with 10 μL of a 5 mg$\cdot$mL^{-1} MTT saline solution, and after 5 h of incubation, 100 μL of a sodium dodecyl sulfate (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 vs 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.

In MTT experiments with ROS scavengers, 24 h after the seeding, cells were treated for 48 h with mannitol (1 mM) or DABCO (1.2 mM) or acid ascorbic plus α-tocopherol (1 mM) or sodium selenite (0.1 μM) in concomitance with increasing concentrations of complex **3**. All chemicals were purchased from Sigma.

Neutral Red uptake (NR) Assay. This assay was performed as described by Borenfreund and Peuerner[21] with slight modification. Briefly, 2008 cells (3 $\times$ 10^3 cells/well) were seeded in 96-well microplates in growth medium (90 μL) and then incubated at 37 °C in a 5% carbon dioxide atmosphere. Twenty-four hours later, following incubation with increasing concentrations of tested compounds for 3, 6, 12, 24, or 48 h, the medium was removed and replaced with a fresh one containing 50 μg/mL neutral red dye (Sigma). After 3 h of incubation, the dye-containing medium was discarded, each well was washed twice with PBS and 200 μL of extractant solution (50% ethanol in acetic acid, 1%) were added. After 20 min the absorbance of each well at 540 nm using a Bio-Rad 680 microplate reader was detected. Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted vs drug concentration. IC_{50} values represent the drug concentrations that reduced the mean absorbance at 540 nm to 50% of those in the untreated control wells.

Trypan Blue Exclusion (TB) Assay. This assay was performed as described by Durkin et al.[22] The 2 $\times$ 10^4 2008 cells were incubated for 3, 12, 24, or 48 h in the presence of increasing

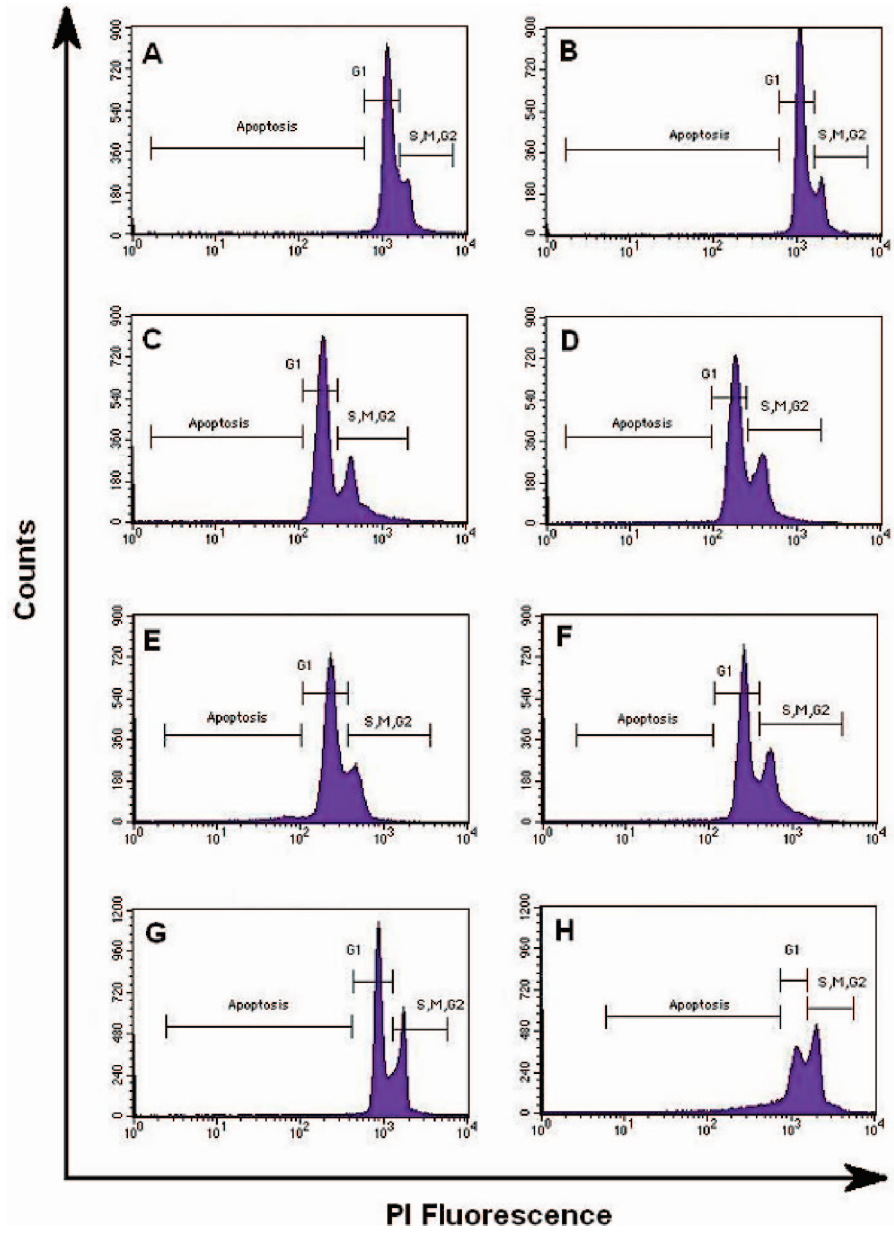


Figure 5. Cell cycle analysis of 2008 cells. Panels A−H represent different time points (3, 12, 24, and 48 h) of control cells (left) and cells treated with IC_{50} concentrations of complex **3** (right).

concentrations of tested compound. Cells were then incubated for 4 min with 0.25% trypan blue (Sigma) and 5% fetal calf serum. Viable cells were identified by their ability to exclude dye, whereas the dye diffuses into nonviable cells. At least 100 cells were counted for each experimental point recorded. The controls were triplicate samples of untreated cells, and their viability ranged from 92% to 98%.

Reactive Oxygen Species (ROS) Production. The production of ROS was measured in 2008 cells (104 per well) grown for 24 h in a 96-well plate in RPMI 1640 without phenol red. Cells were then washed in PBS and loaded with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H2-DCFDA) or 50 μM dihydrorhodamine 123 (DHR) (Molecular Probes-Invitrogen, Eugene, OR) for 25 min in the dark. Afterward, cells were washed with the same medium and incubated with IC_{50} concentration of complex **3**. Fluorescence increase was estimated utilizing the wavelengths of 485 nm (excitation) and 527 nm (emission) in a Fluoroskan Ascent FL (Labsystem, Finland) plate reader. Antimycin (3 μM, Sigma), a potent inhibitor of complex III in the electron transport chain, was used as positive control.

Light Microscopy Analyses. Mitotic Index (MI) Determination. MI was determined as previously described.[23] Briefly, 2008 cells were plated at a density of 1×10^5 cells in 5 cm dishes. Next day, they were treated with with IC_{50} concentration of complex **3**. After 12 and 24 h incubation, cells were treated with colchicine (10^{-4} M) for 4 h, harvested, washed with cold PBS, and then treated with 1% sodium citrate for 12 min at room temperature. Cells were fixed with methanol/acetic acid solution (3:1 v/v), spread on clean slides, air-dried, and stained with a 10% Giemsa solution. At least 150 cells were counted for each concentration, and MI was calculated as the ratio of the number of dividing cells to the total number of cells multiplied by 100.

Hematoxylin−Eosin (HE) Staining. The 2008 cells were seeded into eight-well tissue-culture slides (GIBCO) at 10^4 or 5×10^4 cells/well (0.8 cm^2). After 24 h, cells were washed twice with PBS and treated for an additional 24 h with IC_{50} concentration of complex **3**. Then, after being washed with PBS, cells were fixed in 4% freshly prepared, ice-cold paraformaldehyde, postfixed in

Table 4. Cell Cycle Phases[a]

	3 h			12 h			24 h			48 h		
	control	complex **3**	*p*	control	complex **3**	*p*	control	complex **3**	*p*	control	complex **3**	*p*
apoptosis, %	0.83 ± 0.76	0.82 ± 0.51	>0.05	1.1 ± 0.81	1.04 ± 0.97	0.03	4.24 ± 0.71	2.43 ± 0.66	<0.001	1.21 ± 0.73	15.39 ± 0.96	<0.001
G1, %	77.81 ± 1.22	80.51 ± 2.53	<0.001	68.46 ± 1.77	63.23 ± 2.46	0.003	70.92 ± 1.82	60.9 ± 1.35	<0.001	65.57 ± 1.21	41.03 ± 1.39	<0.001
S, G2/M, %	21.72 ± 0.98	18.99 ± 1.43	<0.001	29.74 ± 1.31	34.15 ± 1.39	0.002	20.29 ± 1.11	33.5 ± 1.28	<0.001	32.62 ± 1.46	37.03 ± 1.12	<0.001

[a] Percentage of cells in different cell cycle phases as a function of time exposure to complex **3** vs control untreated cells. The *p* value was estimated by the Welch test.

ethanol, and air-dried. Slides were then stained with hematoxylin and eosin before being examined by light microscopy (Olympus BX41).

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.[24] Briefly, 2008 cells (5×10^5 cells) were exposed for 1–48 h to tested compound concentrations corresponding to IC_{50} values. One milliliter of a PI solution, containing 50 μg/mL PI, 0.1% m/v Triton X-100, and 0.01% m/v 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).

Mitochondrial membrane potential depletion was determined by measuring the fluorescence of cells stained with the dye tetramethylrhodamine methyl ester (TMRM, Molecular Probes). The 2008 cells (10^6), following 24 h of incubation with complex **3**, were washed once with PBS, harvested, and incubated for 15 min at 37 °C in PBS with TMRM at a final concentration of 10 nM, freshly prepared from stock solution 10 mM in DMSO. The TMRM signal was analyzed in the FL2 channel, which was equipped with a bandpass filter at 560 ± 20 nm.

Results and Discussion

Synthesis and Characterization of Phosphine Ligands and Cu(I) Complexes. The ligand bhpe was synthesized following the procedure outlined in Scheme 1, different from that previously reported.[19] Neat triethyl phosphite and 1,2-dibromoethane were refluxed overnight to obtain a colorless liquid that was distilled under vacuum. The first three fractions were discharged, and the remaining colorless liquid, identified as 1,2-bis(diethylphosphonyl)ethane (**2a**), was treated with an excess of $LiAlH_4$ in anhydrous diethyl ether under a dinitrogen atmosphere at 0 °C. The resulting cloudy mixture was treated with anhydrous Na_2SO_4 and filtered. The filtrate containing the pure primary phosphine (**2b**) was combined in degassed ethanol with a slight excess of aqueous formaldehyde in an acidic medium to obtain a white precipitate of the phosphonium salt (**2c**). The addition of an aqueous solution of $NaHCO_3$ to an aqueous solution of **2c** produced the tertiary phosphine **2**.

Copper complexes $[Cu(thp)_4][PF_6]$ (**3**) and $[Cu(bhpe)_2][PF_6]$ (**4**) have been synthesized according to eqs 1 and 2 by mixing the $[Cu(CH_3CN)_4][PF_6]$ precursor with an excess of the phosphine ligand (4 equiv of thp (**1**) or 2 equiv of bhpe (**2**)) in methanol/acetonitrile solutions.

$$[Cu(CH_3CN)_4][PF_6] + 4\ thp \rightarrow [Cu(thp)_4][PF_6]\ (\mathbf{3}) \quad (1)$$

$$[Cu(CH_3CN)_4][PF_6] + 2\ bhpe \rightarrow [Cu(bhpe)_2][PF_6]\ (\mathbf{4}) \quad (2)$$

The choice of the PF_6 counteranion was determined primarily for chemical reasons. The coordination ability of such an anion is negligible, thus preventing its direct coordination to the metal, which is common for other anions as, for example, iodide. In addition, PF_6 ensures good aqueous solubility of the copper salt and displays a distinct septet in the ^{31}P NMR spectrum, allowing easy characterization of complex **3** in deuterated water (see below).

The identity of complexes **3** and **4** has been determined by ESI-MS, IR, and multinuclear NMR spectral studies (^1H, $^{13}C\{^1H\}$, $^{31}P\{^1H\}$, and ^{63}Cu NMR) (see Table 1). The infrared spectra showed broad peaks in the range 3360–3449 cm^{-1} due to the hydroxylic groups of the phosphines. No significant shifts were observed upon complex formation compared to those exhibited by uncoordinated phosphine ligands. The room-temperature ^1H NMR spectra of **3** and **4** in D_2O solution showed

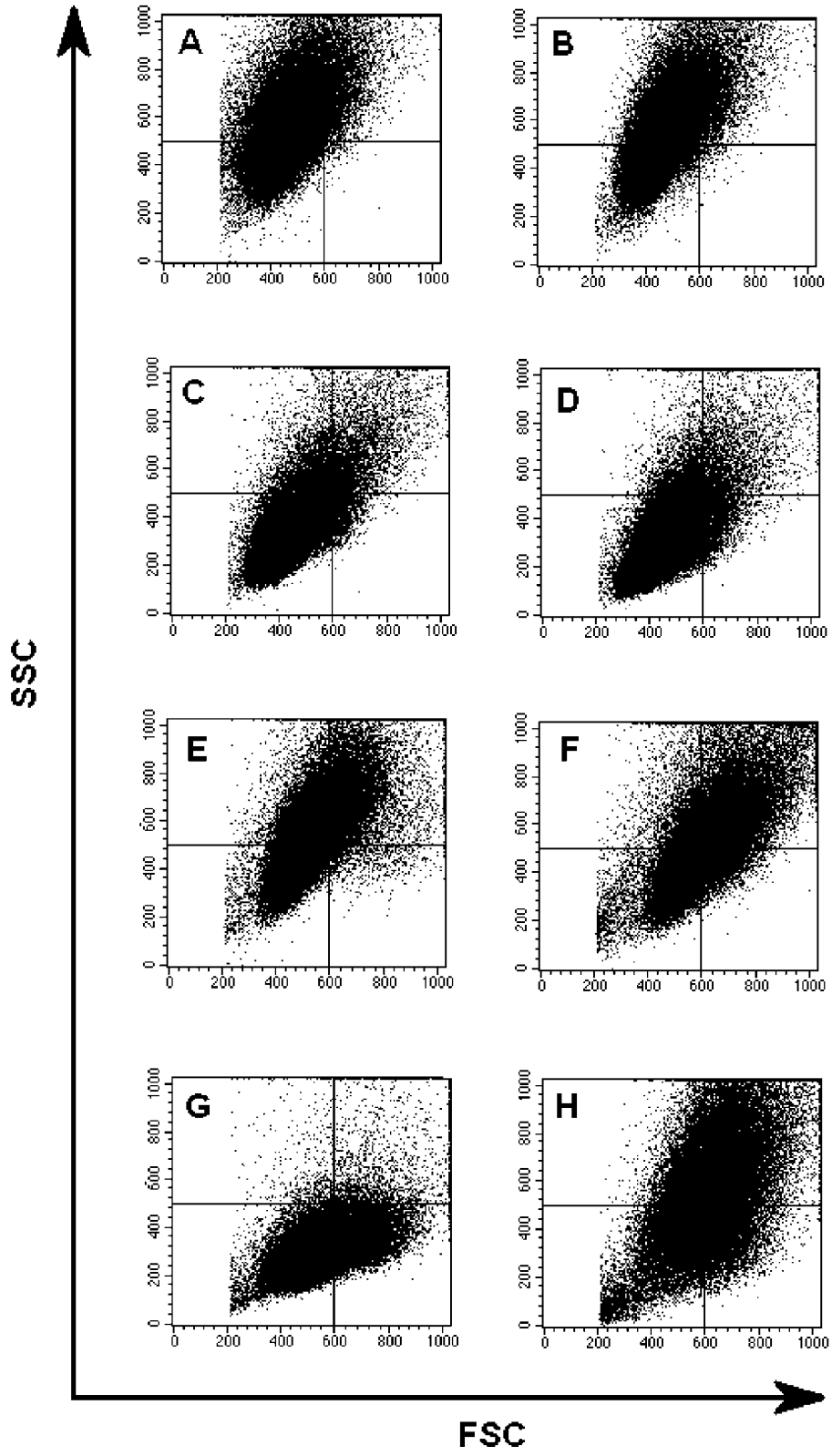


Figure 6. Forward scattering vs side scattering as a function of time in 2008 cells untreated (left) and treated with IC_{50} concentrations of complex **3** (right). Panels A−H represent different time points (3, 12, 24, and 48 h).

rather simple spectra consistent with the formulation (Table 1). The $^{31}P\{^{1}H\}$ NMR spectra gave a four-line signal pattern centered at δ −5.35 and 9.67 ppm for **3** and **4**, respectively, due to the coupling with the $I = {}^{3}/_{2}$ ^{63}Cu and ^{65}Cu nuclei (Supporting Information Figure 1S, bottom), along with a septet centered at about δ −145 ppm due to the PF_6 counteranion. The $^{31}P\{^{1}H\}$ NMR signals of the complexes were significantly downfield shifted compared to the signals at δ −24.70 and −22.18 ppm exhibited by uncoordinated thp and bhpe, respectively. The ^{63}Cu NMR spectrum of **3** displayed a quintet centered at δ 135.2 ppm due to the coupling of copper with four equivalent $I = {}^{1}/_{2}$ phosphorus nuclei (Figure 1S, top). The multiplicities shown in the $^{31}P\{^{1}H\}$ and ^{63}Cu NMR spectra are consistent with the coordination of four phosphines around

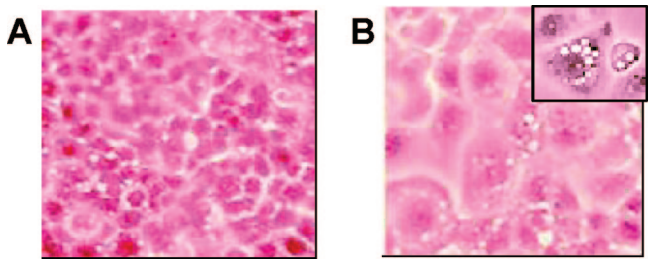


Figure 7. Phase contrast images of 2008 cells stained with hemeatoxylin and eosin: (A) control cells; (B) cells treated with IC_{50} concentration of complex **3** for 24 h (40×), where the inset represents a magnification (100×).

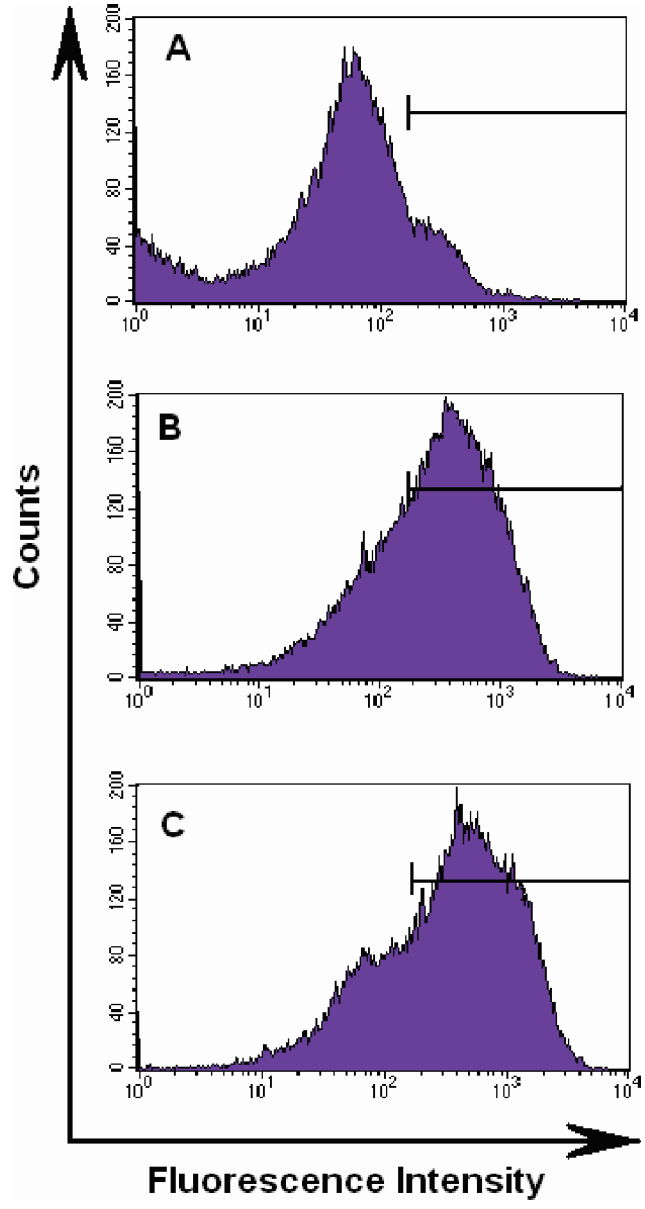


Figure 8. Flow cytometric profiles of 2008 cells untreated (A) and treated with 3.125 (B) or 6.25 (C) μM complex **3** for 24 h and stained with TMRM (10 nM).

copper, the complex adopting a perfect spherical symmetry characteristic of the tetrahedral geometry (Figure 1), which is a common feature in $[CuP_4]^+$ type species.[25] The steric constraints generated by the insertion of the ethylene bridge in the bhpe ligand lower the symmetry in complex **4**. Its $^{31}P\{^1H\}$ NMR signal becomes a broad doublet, and the ^{63}Cu NMR signal is no longer detectable.

The full ESI(+) spectrum of complex **3** showed the molecular ion peak corresponding to the cationic $[CuP_4]^+$ species at 560 *m*/*z* (6%), along with more intense peaks at 436 *m*/*z* (65%) and 312 *m*/*z* (100%) due to the $[CuP_3]^+$ and $[CuP_2]^+$ fragments. Instead, the ESI(+) spectrum of complex **4** showed only the molecular ion peak at 492 *m*/*z* (100%) without detectable fragmentation, indicating that the chelation effect provided by bhpe additionally stabilizes the $[CuP_4]^+$ moiety.

Complexes **3** and **4** are very soluble and stable for days when dissolved in aqueous solutions. Such stability is probably due to water solvation of the complexes, which prevent them from both hydrolysis and disproportionation to Cu(II) and Cu(0). However, although stored under a dinitrogen atmosphere at −20 °C, solid samples of complexes **3** and **4** did not grant reproducible biological results, owing to partial decomposition of the original species in the solid state, as assessed by $^{31}P\{^1H\}$ NMR control experiments. For example, a month old batch of complex **3** exhibited a broad singlet at δ −2.0 ppm overlapped with the original quartet and a significant drop of the PF_6 signal. These data are consistent with the occurrence of partial decomposition of cationic **3** into the corresponding deprotonated neutral $[Cu(thp)_3(thp\text{-}H)]$ complex ($\mathbf{3}_{dec}$) (tph-H = monodeprotonated form of tph),[15] via the intramolecular acid–base reaction reported below.

$$[Cu(thp)_4][PF_6] \rightarrow [Cu(thp)_3(thp - H)]\ (\mathbf{3}_{dec}) + HPF_6 \quad (3)$$

The cytotoxic efficiency of neutral $\mathbf{3}_{dec}$ appears to be drastically reduced. In contrast, lyophilized samples of **3** and **4** gave reproducible biological results.

Cytotoxicity Studies. Complexes **3** and **4** and the corresponding uncoordinated ligands and counteranions were examined for their cytotoxic properties against a panel of human tumor cell lines containing examples of lung (A549), colon (CaCo2, HCT-15), cervix (HeLa), and breast (MCF-7) cancer, leukemia (HL60), lymphoma (Daudi), epatoma (HepG2), 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. Complex **4** showed a relatively low cytotoxic activity against all cancer cell lines, with IC_{50} values roughly 3-fold higher than those of cisplatin. In contrast, complex **3** showed the greatest antitumor efficacy with an average IC_{50} over nine cell types that was over 5 times lower than that of cisplatin. In particular, in HCT-15, HepG2, and HL60 cells IC_{50} values were about 12-, 17-, and 26-fold lower than those calculated for cisplatin, respectively. Remarkably, against CaCo2 cells, cytotoxic potency of complex **3** exceeded by over 30-fold that of the reference drug, thus reflecting a useful selectivity for this human colon adenocarcinoma cell type.

The uncoordinated thp and bhpe ligands and the PF_6 counteranion proved to be hardly effective, even if on certain tumor cell types detectable IC_{50} values were collected; in any case, their values were markedly higher than those displayed by complex **3**. The negligible activity shown by the copper complex **4** could be associated with the high kinetic inertness of this molecule, as assessed by ESI-MS studies. This property determines a low tendency both to dissociate and to play a part in physiologically mediated transport mechanisms which may be relevant for the cellular internalization of Cu(I) species.

The encouraging results obtained against the in-house panel of cell lines prompted us to test the cytotoxic activity of the

most potent derivative, complex **3**, on three additional cell line pairs, two of which (2008/C13* ovarian cancer cells and A431/A431-Pt cervix carcinoma cells) had been selected for their resistance to cisplatin and one (LoVo/LoVoMDR) for its resistance to doxorubicin, thus retaining the MDR phenotype. Cytotoxicity of tested compounds in sensitive and resistant cells was assessed after a 48 h drug exposure by the MTT test. Data are accumulated in Table 3. Cross-resistance profiles were evaluated by means of the resistance factor (RF), which is defined as the ratio of IC_{50} values calculated for the resistant cells to those arising from the sensitive ones. Although cisplatin resistance is multifactorial, the main molecular mechanisms involved in resistance in cisplatin resistant 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.[26] 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.[27] Remarkably, complex **3** showed activity levels very similar on both cisplatin sensitive and resistant cell lines that indicate a different cross-resistance profile than that of cisplatin. Against the ovarian cancer, the RF value was about 4 times lower than that calculated for cisplatin, while against the cervix cell line pair, it was about 3 times lower. The overcoming of cross-resistance phenomena in both cell line pairs strongly supports the hypothesis of a different pathway of action of this copper(I) complex from that of cisplatin.

Acquired multidrug resistance (MDR), whereby cells become refractory to multiple drugs, poses a very important challenge to the success of anticancer chemotherapy. The resistance of LoVo MDR cells to doxorubicin, a drug belonging to the MDR spectrum, is associated with an overexpression of the multi-specific drug transporters, such as the 170 kDa P-glycoprotein (P-gp).[28] Cytotoxicity assays testing complex **3** against this cell line pair allowed the calculation of an RF value about 16-fold lower than that obtained with doxorubicin, thus suggesting that this copper(I) complex is not a potential MDR substrate.

In order to provide information on the antiproliferative effect of complex **3**, we performed a time-dependent evaluation of its cytotoxicity by using three different standard methods that, at relatively short exposure times, assess the damage to different subcellular organelles. The MTT test, detecting the viable mitochondria that are able to reduce the MTT dye to blue formazan, mainly reflects damage to mitochondria. The NR assay, measuring the intracellular accumulation of a weak cationic dye, indicates damage to lysosomes and Golgi apparatus. Finally, the TB test quantifying dead cells by means of their staining after a vital dye treatment reveals damage to the cell membrane. Figure 2 shows the comparison of IC_{50} values detected by MTT, NR, and TB assays in 2008 cells treated with complex **3** for different exposure times. At 3 h, neither of the three methods revealed significant IC_{50} values; otherwise, a time dependent increase in cytotoxicity becomes apparent after longer exposure times. Anyway, the NR test revealed the strongest and earliest effects among those revealed by all three assays; in fact, following the 12 h treatment, IC_{50} values obtained with NR were about one-half and one-third of those calculated from the dose-response profiles by MTT and TB test, respectively. These results suggest that lysosomes/Golgi apparatuses are more sensitive to complex **3** treatment. In contrast, the poor permeability to vital dye indicates that plasma membrane function is still maintained until the late phase of cell death.

It is well-known that copper represents an excellent catalyst of redox cycles, and therefore, it can stimulate cellular ROS formation.[29] To evaluate the contribution of oxidative stress in the execution of tumor cell death, complex **3** mediated cytotoxicity against 2008 cells was investigated in the presence of various ROS scavengers. Mannitol (1 mM) and DABCO (1.2 mM) were respectively used as scavengers of hydroxyl radical and singlet oxygen, the main damaging oxidative species.[30] Ascorbic acid and α-tocopherol (1 mM), working in synergism, act as direct antioxidants toward singlet oxygen and superoxide anion and as indirect antioxidants breaking the chain reaction of lipid peroxidation.[31] Furthermore, selenium, as cofactor of various selenoproteins, has been found to protect, at low concentrations, tumor ovarian cells from oxidative damage induced by hydrogen peroxide.[32] Chemicals were added to 2008 cell growth medium containing different concentrations of complex **3**, and the treatment was extended to 48 h. The results presented in Figure 3 clearly indicate the cytoprotective properties of all ROS scavengers; in particular, the copresence of α-tocopherol and ascorbic acid totally prevent the cytotoxic activity of complex **3**. Taken together, these data suggest the involvement of ROS production in copper(I) complex mechanism.

Cellular production of ROS upon treatment with complex **3** was measured in 2008 cells by the fluorogenic probes H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) and DHR (dihydrorhodamine 123).[33] The 2008 cells, preincubated with H_2DCFDA or DHR, were treated with IC_{50} concentrations of complex **3** for increasing exposure times. Figure 4 shows that no significant increase in ROS level with respect to the basal level of the control cells was detected after either of the exposure times with both H_2DCFDA (Figure 4A) and DHR (Figure 4B) probes. Moreover, also further increasing complex **3** range concentrations, ROS were not detectable (data not shown). Similar negative results had been already obtained by other authors investigating the cellular effects induced by a thioxo-1,2,4-triazolecopper(II) complex.[34] Most probably, as stated in that context, the intracellular ROS production associated with copper complexes needs to be analyzed by other methods. In our case, the potential presence of free phosphines, which are well-known reducing agents in chemical reactions, partially released from the metal center may interfere in ROS determination.

In order to study further the cellular effects induced by complex **3** in terms of cell cycle modifications and induction of apoptosis, a time-dependent evaluation of cell cycle profile was performed by FACS in 2008 human ovarian cancer cells treated with IC_{50} concentrations of complex **3** (Figure 5). Cell cycle analysis revealed a significant time-dependent decrease of the G1 phase. After 48 h of treatment, with respect to control cells, about a 1.8-fold reduction of G1 phase and about a 1.5-fold increase of S, G2, and M phases were observed (Figure 5, panels G and H; Table 4).

PI cytofluorimetric analysis is not able to discriminate the S and M phases from the G2 phase because of the equal DNA cellular content. To better evaluate the influence of complex **3** on the M phase, the mitotic index in 2008 cells treated with IC_{50} concentrations of copper(I) complex for 12 and 24 h was calculated. Mean percentages of mitotic index (42.92 ± 3.11 and 19.33 ± 3.21 at 12 and 24 h, respectively, against an average value of 68.34 ± 3.01 for control cells) attest to a significant time-dependent decrease of the number of treated cells able to complete the mitotic division.

Moreover, by the examination of histograms, no DNA fragmentation was detected following 3, 12, and 24 h exposures (Figure 5, panels A−F). However, cells treated with complex **3** for 48 h showed a slightly appreciable DNA fragmentation signal compared to the control samples (15.4%) (Figure 5, panels G and H, Table 4).

Side scattering and forward scattering based analyses showed a time dependent increase of cell complexity and size in complex **3** treated cells (Figure 6, panels B, D, F, H). Following 48 h of treatment, it can be noted that 36% of cytofluorimetric counts shifted in the high complexity and size fraction of the cytogram (Figure 6, panel H, upper right quadrant). These remarkable morphological changes may be related to the ability of complex **3** to cause an extensive cytosolic vacuolization. Hematoxylin and eosin staining of 2008 cells treated with IC_{50} concentrations of complex **3** for 24 h revealed a striking cytoplasmatic vacuolization (Figure 7), which does not appear if cells were preincubated with the autophagy inhibitor monensin[35] (data not shown). These cellular effects were previously described in tumor cells treated with other copper complexes.[13,34] Taken together, all these results confirm the hypothesis that complex **3** may activate the programmed cell death paraptosis as the main cell death mechanism.[36,37]

Finally, considering that mitochondria play a key role in the pathways to cell death, we investigated the mitochondrial energization of treated tumor cells as the retention of a mitochondrial selective cationic fluorescent probe tetramethyl rhodamine methyl ester (TMRM). Figure 8 shows the flow cytometric profiles of 2008 cells treated with 6.25 and 12.5 μM of complex **3** for 24 h and stained with 100 nM TMRM. The results indicate that copper(I) complex induced a massive increase of the TMRM fluorescence, reflecting a dramatic alteration of mitochondrial membrane potential. The marked increase of $\Delta\Psi_{mt}$ (hyperpolarization) can be attributed to a variety of mechanisms such as increased mitochondrial mass and number per cell[38] or unregulated mitochondrial matrix volume.[39] In particular, mitochondrial membrane hyperpolarization induced by complex **3** might be correlated with the induction of a G2/M phase cell cycle arrest, as previously reported for other anticancer agents.[40]

In conclusion, the study presented herein on the synthesis of hydrophilic phosphine copper(I) compounds coupled with their marked cytotoxic activity against a panel of human cell lines provides the basic chemical background in view of the attractive opportunity to use hydrophilic "hot" phosphine copper (^{64}Cu and ^{67}Cu) complexes as potential therapeutic agents in the radiopharmaceutical field. Several radioactive copper isotopes are available nowadays for biomedical purposes both for radioimaging and for targeted radiotherapy.[41,42] The excellent hydrophilic character of this class of copper(I) complexes may also help to overcome the undesired cardiotoxicity exhibited by previously investigated bis(arylphosphine) copper(I) and gold(I) compounds. Actually, the intravenous administration of $[Au(dppe)_2]^+$ caused myocardial lesions, presumably because of an elevated uptake of the cationic species in the myocardium.[9] It has already been shown that other monocationic lipophilic drugs, such as the tracers ^{99m}Tc-Cardiolite and ^{99m}Tc-Myoview, enter the myocardial cell membrane through a passive diffusion mechanism.[43] The myocardial uptake of hydrophilic copper complexes should be much less marked, thus precluding accumulation in the heart and subsequent heart damage.

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Supporting Information Available: Description of instrumentation and complete characterization details of ligand **2** and copper complexes **3**–**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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