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Dinuclear Cu^{I} complexes of pyridyl-diazadiphosphetidines and aminobis(phosphonite) ligands: synthesis, structural studies and antiproliferative activity towards human cervical, colon carcinoma and breast cancer cells†

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The copper(I) complexes containing phosphorus donor ligands such as diazadiphosphetidine, *cis*-{(*o*-$OCH_2C_5H_4N$)P(μ-N^{t}Bu)}$_2$ (**1**) and aminobis(phosphonite), C_6H_5N{P(OC_6H_3(OMe-*o*)(C_3H_5-*p*))$_2$}$_2$ (**2**, PNP), have been synthesized. Treatment of **1** with copper iodide afforded the 1D coordination polymer [{Cu(μ-I)}$_2${(*o*-$OCH_2C_5H_4N$)P(μ-N^{t}Bu)}$_2$]$_n$ (**3**). Treatment of **3** with 2,2′-bipyridine (bpy) and 1,10-phenanthroline (phen) produced mixed-ligand complexes [(L)$_2$Cu$_2${(*o*-$OCH_2C_5H_4N$)P(μ-N^{t}Bu)}$_2$][I]$_2$ (**4** L = bpy; **5** L = phen) in good yields. The reaction of **2** with copper iodide yielded a rare tetranuclear copper complex [(CuI)$_2$$C_6H_5N(PR_2)_2$]$_2$ (**6**), which on subsequent treatment with various pyridyl ligands produced binuclear complexes [{Cu(μ-I)(py)}$_2$(μ-PNP)] (**7**), [Cu$_2$(μ-I)(bpy)$_2$(μ-PNP)]I (**8**), [Cu$_2$(μ-I)I(bpy)(μ-PNP)] (**9**), [Cu$_2$(phen)(bpy)(μ-PNP)](OTf)$_2$ (**10**), [Cu$_2$(μ-I)I(phen)(μ-PNP)] (**11**) and [Cu$_2$(μ-I)(phen)$_2$(μ-PNP)]I (**12**), in an almost quantitative yield. The new copper(I) complexes (**4**, **5** and **7–12**) were tested for anti-cancer activity against three human tumor cell lines. Compounds **5**, **10** and **12** showed *in vitro* antitumor activity 5–7 fold higher than cisplatin, the most used anticancer drug. These three most potent compounds (**5**, **10** and **12**) were chosen for detailed study to understand their mechanism of action. The copper(I) compounds studied in the present investigation were found to inhibit tumor cell growth by arresting cells at the S-phase of the cell cycle. The characteristic nuclear morphology of treated cells showed signs of DNA damage. The experimental evidence clearly indicated that these compounds initiated apoptosis, which is mediated through the p53 pathway.

Introduction

Ever since the active cancer drug cisplatin was discovered,[1] a variety of transition metal complexes were looked upon as potential anticancer reagents.[2–4] Even today, platinum based drugs such as cisplatin and carboplatin are highly active towards testicular and ovarian cancer, bladder carcinoma and non-small-cell lung cancer.[5–8] However, these compounds present severe toxic and undesirable side effects such as nephrotoxicity and neurotoxicity, and often the treatment failure was also due to the development of resistance to these complexes.[9–12] To make chemotherapy more effective with minimum side-effects, it is necessary to design metallodrugs which are less toxic and highly active in smaller dosages, especially against cell lines that have acquired high resistance towards cisplatin.[13,14] In this context, modification of the ligand structure and employment of other platinum metals were given importance to overcome the toxic side effects.[15] The focus was on group 11 metals for cancer therapy owing to the long known utility of gold complexes in the treatment of arthritis, turberculosis and, in the 1970s, p388 leukemia.[16] Saddler and others have extensively studied antitumor properties of gold(I) as well as ruthenium(II) complexes containing phosphines, N-heterocyclic carbenes and nitrogen based ligands.[8,17–23]

Copper complexes are considered to be important anti-cancer agents because of their bio-friendly nature as copper

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plays a significant role in biological systems. Copper is an essential trace metal for living organisms and plays a major role in several enzymatic activities, in particular redox reactions which have an influence on the antioxidant system of the organism.[24] The medicinal properties of copper complexes in anti-inflammatory, antiarthritic, antiulcer and anticonvulsant diseases including anticancer properties[25–31] are also well documented. Due to the selective permeability of cancer cell membranes to copper complexes and their strict regulation on intracellular concentration encouraged the synthesis of copper-based compounds as probable antiproliferative agents with relatively less severe side effects than the present standard anticancer drugs.[32,33] Several copper(I) complexes containing either phosphorus(III) based compounds or pyridyl-type ligands have been screened for anticancer activity with promising results.[34–43] Recently, we have studied the antitumor activity of gold(I) and copper(I) complexes and also metal-free thio and selenol compounds which inhibit the growth of cancer cells by inducing apoptosis.[44–46] In the present study, several mixed-ligand copper(I) complexes of diazadiphosphetidine or cyclodiphosphazane appended with pyridyl functionalities and aminobis(diphosphonite) ligands along with 2,2′-bipyridine or 1,10-phenanthroline ligands have been synthesized and screened for *in vitro* anticancer activities against human cervical cells (HeLa), breast cancer cells and human colorectal carcinoma cells along with cisplatin, the most widely used antitumor drug[47] for comparison. Among them, the three most potent compounds (**5**, **10** and **12**) were chosen for detailed study to understand their mechanism of action. The copper(I) compounds studied in the present investigation were found to inhibit tumor cell growth by arresting cells at the S phase of the cell cycle.

Results and discussion

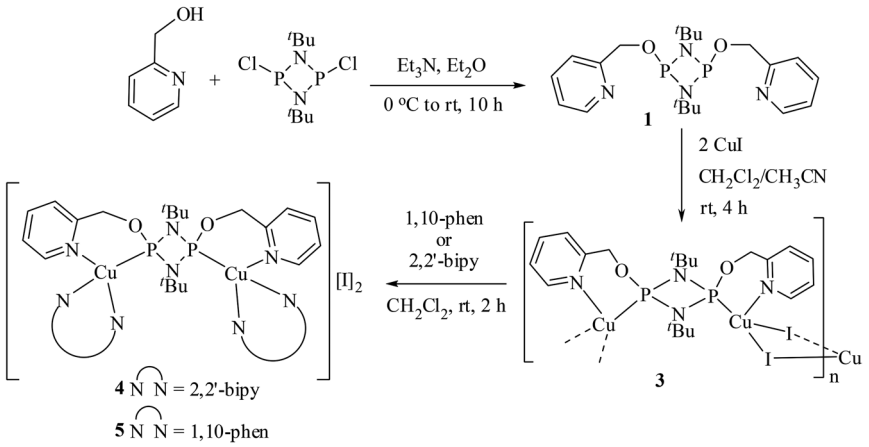
Synthesis of ligands and copper(I) complexes

The reaction of two equivalents of pyridine-2-methanol with *cis*-{ClP(μ-N^{t}Bu)}$_2$ in the presence of triethylamine yielded *cis*-{(*o*-OCH$_2$C$_5$H$_4$N)P(μ-N^{t}Bu)}$_2$ (**1**) in good yield. Compound **1** is a light yellow viscous liquid, sensitive to air and moisture. The ^{31}P{^{1}H} NMR spectrum of **1** displayed a single resonance at 133.5 ppm and the mass spectrum showed the molecular ion peak at *m*/*z* 420.2. The aminobis(phosphonite), C$_6$H$_5$N-{P(OC$_6$H$_3$(OMe-*o*)(C$_3$H$_5$-*p*))$_2$}$_2$ (**2**), was prepared according to the literature procedure.[48]

The treatment of **1** with two equivalents of copper(I) iodide in a 1 : 1 mixture of dichloromethane and acetonitrile resulted in the formation of the copper(I) coordination polymer [{Cu(μ-I)}$_2${(*o*-OCH$_2$C$_5$H$_4$N)P(μ-N^{t}Bu)}$_2$]$_n$ (**3**). Addition of two equivalents of 2,2′-bipyridine or 1,10-phenanthroline to the suspension of **3** in dichloromethane at room temperature yielded cationic dinuclear complexes [(bpy)$_2$Cu$_2${(*o*-OCH$_2$C$_5$H$_4$N)P(μ-N^{t}Bu)}$_2$][I]$_2$ (**4**) and [(phen)$_2$Cu$_2${(*o*-OCH$_2$C$_5$H$_4$N)P(μ-N^{t}Bu)}$_2$][I]$_2$ (**5**) as yellow crystalline solids (Scheme 1). The ^{31}P{^{1}H} NMR spectra of **4** and **5** showed single resonances at 109.8 and 110.7 ppm, respectively.

The reaction of aminobis(phosphonite), C$_6$H$_5$N{P-(OC$_6$H$_3$(OMe-*o*)(C$_3$H$_5$-*p*))$_2$}$_2$ (**2**) (hereafter referred to as 'PNP'), with one or two equivalents of copper(I) iodide in acetonitrile resulted in the formation of a rare tetranuclear copper complex [(CuI)$_2$(μ-PNP)]$_2$ (**6**) irrespective of the stoichiometry of the reactants and the reaction conditions.[49] The reactions of **6** with pyridyl ligands such as pyridine, 2,2′-bipyridine and 1,10-phenanthroline led to the formation of several mixed-ligand complexes. Attempts to prepare mixed-ligand complexes in a one-pot reaction led to the isolation of stable homoleptic amine complexes. In order to prepare mixed-ligand complexes, complex **6** was initially isolated and subsequently reacted with various pyridyl ligands.

The reaction of **6** with an excess of pyridine resulted in the formation of a white crystalline dinuclear complex, [{Cu(μ-I)(py)}$_2$(μ-PNP)] (**7**), whereas the reaction of **6** with 2,2′-bipyridine in 1 : 4 and 1 : 2 molar ratios afforded dinuclear complexes, [Cu$_2$(μ-I)(bpy)$_2$(μ-PNP)]I (**8**) and [Cu$_2$(μ-I)I(bpy)-(μ-PNP)] (**9**), respectively, as bright yellow crystalline solids. In complex **9**, one of the Cu–I bond was cleaved by 2,2′-bipyridine



Scheme 1 Synthesis and copper complexes of *cis*-{(*o*-OCH$_2$C$_5$H$_4$N)P(μ-N^{t}Bu)}$_2$ (**1**).

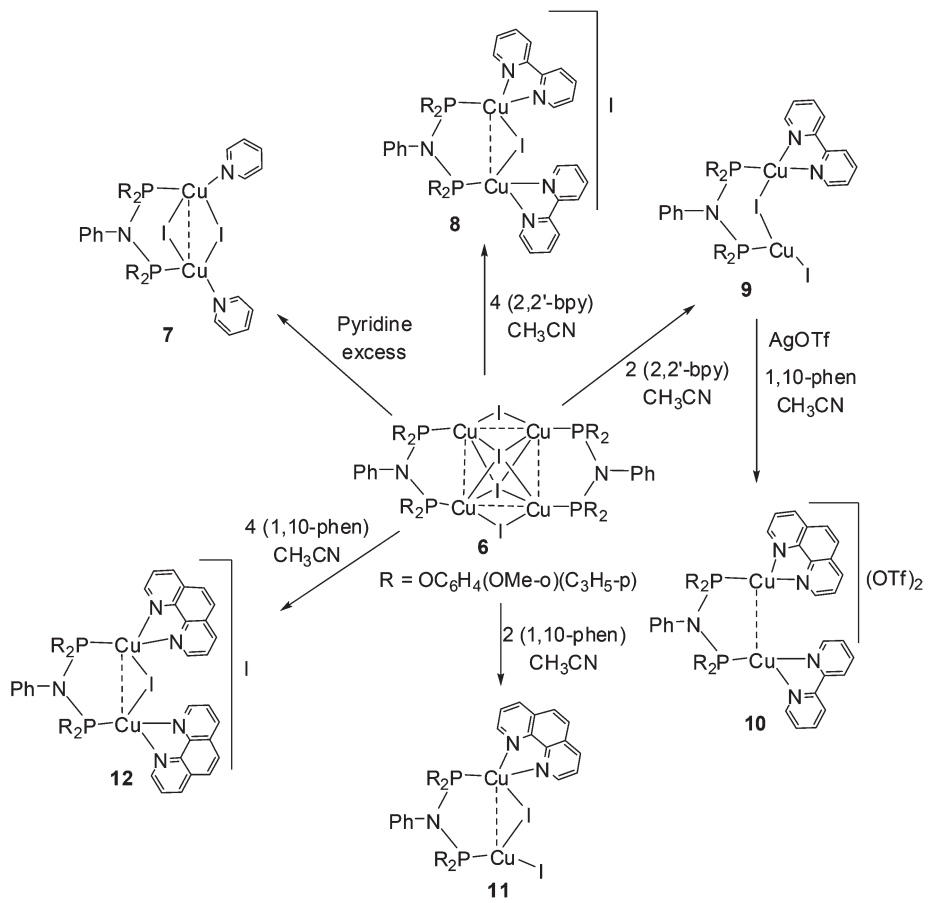
to give both tri- and tetra-coordinated copper centers, whereas in complex **8**, one of the bridging iodides was completely displaced to produce a cationic complex with iodide as the counter anion.[50] Similar reactions of **6** with 1,10-phenanthroline in 1 : 2 and 1 : 4 molar ratios gave $[Cu_2(\mu\text{-}I)I(phen)(\mu\text{-}PNP)]$ (**11**) and $[Cu_2(\mu\text{-}I)(phen)_2(\mu\text{-}PNP)]I$ (**12**), respectively. The reaction of **9** with two equivalents of AgOTf in acetonitrile followed by one equivalent of 1,10-phenanthroline afforded $[Cu_2(phen)(bpy)(\mu\text{-}PNP)](OTf)_2$ (**10**) as a pale yellow crystalline solid (Scheme 2). The $^{31}P\{^1H\}$ NMR spectra of **7**–**9**, **11** and **12** showed broad single resonances around 103 ppm, whereas the $^{31}P\{^1H\}$ NMR spectrum of **10** showed a broad single resonance at 111.7 ppm. The molecular structures of **3**, **9** and **11** were confirmed by single crystal X-ray diffraction studies.

Molecular structures of 3, 9 and 11

The perspective views of molecular structures of **3**, **9** and **11** are shown in Fig. 1–3. The selected bond lengths [Å] and bond angles [°] are given in Tables 1 and 2. Crystallographic data and the details of the structure determinations are given in Table 3.

The yellow crystal of **3** suitable for single crystal X-ray diffraction study was obtained by slow diffusion of copper(I) iodide solution in acetonitrile into a dichloromethane solution of **1** at room temperature. The core structure of **3** consists of cyclic four-membered $\{(o\text{-}OCH_2C_5H_4N)P(\mu\text{-}N^tBu)\}_2$ and $\{Cu(\mu\text{-}I)\}_2$ units arranged alternatively in a zig-zag fashion to form a one dimensional coordination polymer.[45,51,52] The copper centers are in a distorted tetrahedral environment consisting of two bridging iodides, a phosphorus atom from the cyclodiphosphazane ring and a nitrogen atom of the pyridyl group. The Cu···Cu distance in **3** [3.2653(4) Å] is noticeably greater than the sum of the van der Waals radii for the copper(I) ion, thus overruling any metallophilic interactions. The four-membered $[Cu(\mu\text{-}I)]_2$ units are puckered [torsion angle is 13.78(1)°], but the diazadiphosphetidine rings are almost planar. The mean Cu–I distances in **3** [2.6448 Å] is in good agreement with the same observed in analogous complexes $[1,3\text{-}C_6H_4\{OP(\mu\text{-}N^tBu)_2PN(H)^tBu\}_2\{Cu_2(\mu\text{-}I)_2\}_2]_n$,[51] $[\{Cu_2(\mu\text{-}I)_2\}_2\{(OC_6H_4OMe\text{-}o)P(\mu\text{-}N^tBu)\}_2]_n$,[52] $[\{Cu_2(\mu\text{-}I)_2CH_3CN\}_4(\{(NC_4H_8NMe)P(\mu\text{-}N^tBu)\}_2)_8]$[45] and $[Cu(\mu\text{-}I)(dppp)]_2$).[53]

The crystals of **9** and **11** suitable for X-ray diffraction studies were grown from acetonitrile solution. The unit cell of the complex **9** contains two independent molecules with very similar bonding parameters. In both the complexes **9** and **11** the Cu1 and Cu3 have distorted tetrahedral geometry being coordinated to one phosphorus atom, one iodine atom and two pyridyl nitrogen atoms, whereas Cu2 and Cu4 exhibit a trigonal planar environment coordinated by one phosphorus and



Scheme 2 Reaction of **6** with pyridyl ligands.

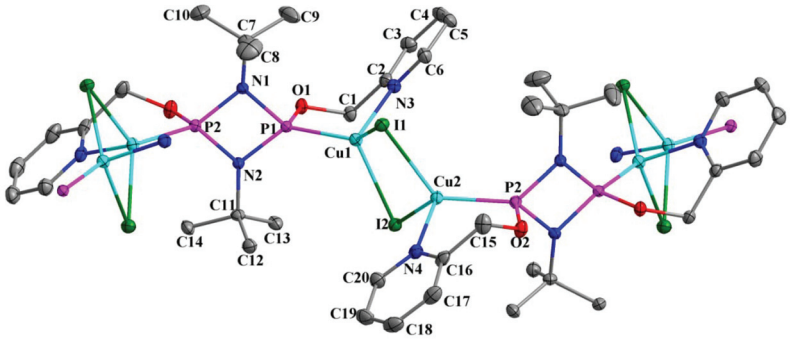


Fig. 1 The molecular structure of $[\{Cu(\mu\text{-}I)\}_2\{(o\text{-}OCH_2C_5H_4N)P(\mu\text{-}N^tBu)\}_2]_n$ (**3**). All hydrogen atoms and lattice solvents are omitted for clarity. Thermal ellipsoids are drawn at the 50% probability level.

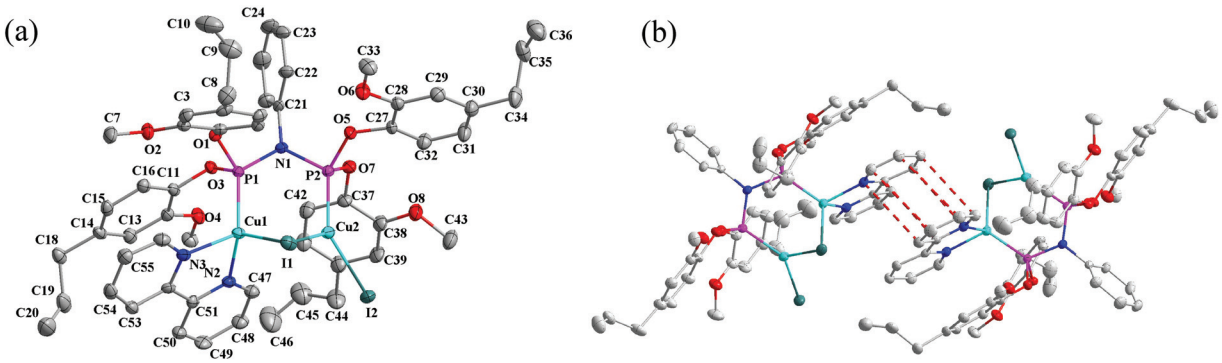


Fig. 2 (a) Molecular structure of $[Cu_2(\mu\text{-}I)I(C_{10}H_8N_2)\{C_6H_5N\{P(OC_6H_3(OMe\text{-}o)(C_3H_5\text{-}p))_2\}_2\}]$ (**9**) showing the atom numbering schemes. (b) Intermolecular π–π stacking in **9**. All hydrogen atoms are omitted for clarity. Thermal ellipsoids are drawn at the 50% probability level.

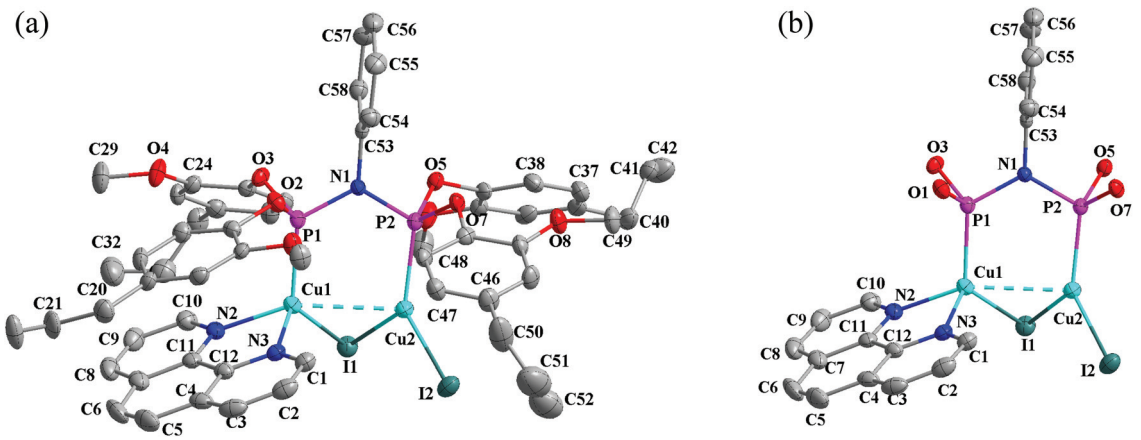


Fig. 3 (a) Molecular structure of $[Cu_2(\mu\text{-}I)I(C_{12}H_8N_2)\{C_6H_5N\{P(OC_6H_3(OMe\text{-}o)(C_3H_5\text{-}p))_2\}_2\}]$ (**11**) showing the atom numbering schemes. (b) Core structure of **11**. All hydrogen atoms and lattice solvents are omitted for clarity. Thermal ellipsoids are drawn at the 50% probability level.

two iodine atoms. The two Cu–P distances differ significantly [Cu1–P1 = 2.1516(1) Å; Cu2–P2 = 2.1921(1) Å (**9**) and Cu1–P1 = 2.1769(1) Å; Cu2–P2 = 2.1913(1) Å (**11**)], and similar is the case for Cu–I distances to the bridging iodide [Cu1–I1 = 2.5760(6) Å and Cu2–I1 = 2.5860(5) Å (**9**) and Cu1–I1 = 2.6433(5) Å and Cu2–I1 = 2.5651(5) Å (**11**)]. The terminal Cu–I distances [Cu2–I2 = 2.5230(6) Å (**9**) and Cu2–I2 = 2.5196(5) Å (**11**)] are shorter than the bridging Cu–I distances. The shorter Cu–P distance in case of four-coordinated copper bonded to 2,2′-bipyridine/phenanthroline is possibly due to the π–π stacking between

Table 1 Selected bond distances [Å] and bond angles [°] for complex **3**

Bond length [Å]		Bond angle [°]	
Cu1–I1	2.6384(4)	I1–Cu1–P1	130.67(2)
Cu1–I2	2.6512(4)	I1–Cu1–N3	102.84(5)
Cu1–P1	2.2166(6)	I2–Cu1–P1	114.90(2)
P1–O1	1.633(2)	Cu1–I1–Cu2	76.30(1)
P2–N1	1.697(2)	I1–Cu1–I2	101.63(1)
P1–N2	1.703(2)	I2–Cu2–P2	122.16(2)
		I2–Cu2–N4	103.11(5)

Table 2 Selected bond distances [Å] and bond angles [°] for **9** and **11**

	9	**11**		**9**	**11**
P1–N1	1.685(4)	1.685(3)	P1–N1–P2	120.1(2)	119.51(18)
P2–N1	1.694(3)	1.689(3)	N1–P1–Cu1	122.75(1)	121.09(11)
Cu1–P1	2.1517(1)	2.1769(1)	N1–P2–Cu2	116.62(1)	115.12(11)
Cu2–P2	2.1921(1)	2.1913(1)	P1–Cu1–I1	114.71(3)	122.54(3)
Cu1–I1	2.5759(5)	2.6433(5)	P2–Cu2–I1	119.12(4)	121.70(3)
Cu2–I1	2.5859(6)	2.5650(5)	P2–Cu2–I2	128.8(4)	127.93(3)
Cu2–I2	2.5230(6)	2.5196(5)	Cu1–I1–Cu2	68.59(2)	64.33(2)
Cu1–N2	2.049(3)	2.062(3)	I1–Cu2–I2	111.83(2)	110.21(2)
Cu1–N3	2.056(4)	2.078(3)	N2–Cu1–N3	80.32(14)	81.25(13)
Cu1–Cu2	2.909(4)	2.7735(6)			

one of the phenoxy group on P1 and the 2,2′-bipyridyl/phenanthroline ligand. The Cu–N bond lengths (Cu1–N2 = 2.048(3) Å and Cu1–N3 = 2.056(3) Å) differ slightly. The distance between two copper centers in the complex **9** is 2.9085(7) Å greater than the sum of the van der Waals radii indicating no metal–metal interaction between the two copper atoms. The complex **11** shows the presence of the cuprophillic interaction between the two copper metals as the Cu–Cu distance is 2.7735(6) Å. The I2–Cu2–I1 and Cu1–I1–Cu2 angles in complexes **9** and **11** are 111.83(2)°, 68.590(1)° and 110.21(2)°, 64.33(2)°, respectively. Complexes **9** and **11** both show intramolecular π–π interactions between one of the phenyl groups and the 2,2′-bipyridine and the 1,10-phenanthroline moiety, respectively. In addition, the complex **9** shows a non-covalent intermolecular π–π interaction between the two 2,2′-bipyridine moieties having parallel displaced π–π stacking alignment with the minimum distance between the two 2,2′-bipyridine rings being 3.59 Å (Fig. 2b).[54] The complex **11** does not show the analogous intermolecular π–π interaction between the two 1,10-phenanthroline groups.

Antiproliferative properties of copper complexes

The copper(I) complexes containing phosphorus based ligands and pyridyl ligands have shown good to moderate antiproliferative activity towards various cancer cell lines.[25,55,56] Recently we reported the antitumor activity of copper(I) complexes containing monocoordinated cyclodiphosphazanes and chelating pyridyl ligands which have shown superior activity towards cervical and breast cancer cell lines compared to cisplatin.[44] In the present investigation we wanted to examine the anticancer properties of two distinct but related aminophosphine systems: (a) cyclodiphosphazane appended with pyridyl functionalities to form the large-bite bis(bidentate) system, and (b) short-bite symmetric bis(phosphino)amine ligands, to assess their anticancer properties. It is anticipated that these chelating ligands would readily form copper(I) ionic complexes and probably show better cytotoxicity. The antiproliferative activity of these two series of copper complexes against the human cervical cancer (HeLa) cell line was checked and different compounds showed varied levels of inhibition as presented in Table 4. Under similar conditions, 10 μM cisplatin inhibited the proliferation of HeLa cells by 44 ± 7%. From these results, the three most potent compounds (**5**, **10** and **12**) were chosen for detailed study to understand their mechanism

Table 3 Crystallographic data for **3**, **9** and **11**

	3	**9**	**11**
Formula	$C_{20}H_{30}Cu_2I_2N_4O_2P_2{\cdot}CH_3CN$	$C_{56}H_{57}Cu_2I_2N_3O_8P_2$	$C_{58}H_{57}Cu_2I_2N_3O_8P_2{\cdot}CH_3CN$
Formula weight	842.35	1342.87	1407.99
Crystal system	Monoclinic	Triclinic	Monoclinic
Space group	*P*21/*n* (no. 14)	$P\bar{1}$ (no. 2)	*P*21/*n* (no. 14)
a [Å]	10.4251(7)	10.9180(9)	12.4841(3)
b [Å]	22.1496(14)	23.2001(18)	17.0323(4)
c [Å]	12.9548(8)	24.1557(19)	27.1967(7)
α [°]	90	65.123(1)	90
β [°]	97.561(1)	81.611(1)	94.464(2)
γ [°]	90	86.293(1)	90
V [Å^3]	2965.4(3)	5491.5(8)	5765.4(2)
Z	4	4	4
ρ_{calc} [g cm^{-3}]	1.887	1.624	1.624
μ (Mo-K$_\alpha$) [mm^{-1}]	3.652	2.013	1.923
F(000)	1640	2688	2828
T (K)	100	100	150
2*θ* range [°]	2.2–28.0	1.9–28.3	3.2–25.0
Total no. of reflns	51 118	95 613	41 807
No. of indep. reflns	7145	19 471	10 122
R_{int}	0.037	0.061	0.059
R	0.0203	0.0433	0.0370
w*R*	0.0467	0.1062	0.0931
S	1.07	1.03	1.03

Table 4 Effects of different copper complexes on the proliferation of HeLa cells after 24 h

Compounds	% Inhibition[a] at 5 μM	% Inhibition at 10 μM
4	38 ± 2	60 ± 2
5	87 ± 2	97 ± 3
7	27 ± 4	36 ± 2
8	25 ± 4	36 ± 3
9	13 ± 3	23 ± 3
10	86 ± 2	93 ± 2
11	33 ± 3	41 ± 3
12	92 ± 2	96 ± 4
Cisplatin	22 ± 3	44 ± 7

[a] % of inhibition of cell proliferation is average of three independently performed experiments. ±SD stands for standard deviation of mean.

Table 5 Half maximal inhibitory concentrations (IC_{50}) of **5**, **10**, **12**

Compounds	IC_{50}[a] (μM) MCF-7	IC_{50} (μM) HeLa	IC_{50} (μM) HCT116
5	1.6 ± 0.3	1.6 ± 0.1	2.3 ± 0.5
10	2.4 ± 0.6	2.5 ± 0.2	3.9 ± 0.5
12	1.7 ± 0.3	2 ± 0.1	1.9 ± 0.1

[a] IC_{50} data are average of three independent experiments for each cell types. ±SD stands for standard deviation of mean.

of action. The antiproliferative activity of **5**, **10** and **12** against the human cervical cancer cell line (HeLa), breast cancer cell line (MCF-7) and human colon carcinoma cell line (HCT116) was determined (Fig. 4). The compounds **5**, **10** and **12**, in culture, inhibited the proliferation of cells in a concentration dependent manner. Half maximal inhibitory concentration (IC_{50}) of **5** was found to be 1.6 ± 0.3, 1.6 ± 0.1 and 2.3 ± 0.5 μM in MCF-7, HeLa and HCT116 cell lines, respectively. Similarly, compounds **10** and **12** inhibited proliferation of MCF-7, HeLa and HCT116 cell lines with IC_{50} values of 2.4 ± 0.6, 2.5 ± 0.2, 3.9 ± 0.5 μM and 1.7 ± 0.3, 2 ± 0.1, 1.9 ± 0.1 μM, respectively (Fig. 4 and Table 5). Under similar conditions, cisplatin was reported to inhibit the proliferation of HeLa, HCT116 and MCF-7 cells with IC_{50} of 8, 16 and 15 μM, respectively.[57] Thus, the antiproliferative efficacies of **5**, **10**, and **12** against cultured tumor cells are much stronger than cisplatin. The structural activity correlation of compounds **4**, **5**, **10** and **12** suggested that the complexes containing 1,10-phenanthroline ligands showed more potency as compared to those containing 2,2′-bipyridine ligands. The better π-stacking ability of 1,10-phenanthroline ligands[58] seems to add more effectiveness to the copper complex than 2,2′-bipyridine ligands (Fig. 4, 5 and Tables 4, 5).

The antiproliferative activity of these compounds is characteristic of metal–ligand complexes as individual ligands and copper iodide fail to show any cytotoxicity. For example, ligand **1** at 6 μM showed 3.5 ± 1% inhibition of cell growth, whereas the ligand **2** and copper iodide did not show any cytotoxicity even at 6 μM concentration. To determine the effect of the copper complexes on cell cycle progression of the MCF-7 cell line, flow-cytometric analysis was performed after 48 h treatment with compounds. The compounds block cell cycle progression at the S phase of the cell cycle (Fig. 6). For example, in the case of the control 17% cells were in the S phase of the cell cycle and the number of cells in the S phase increased to 44%, 48% and 52% in the presence

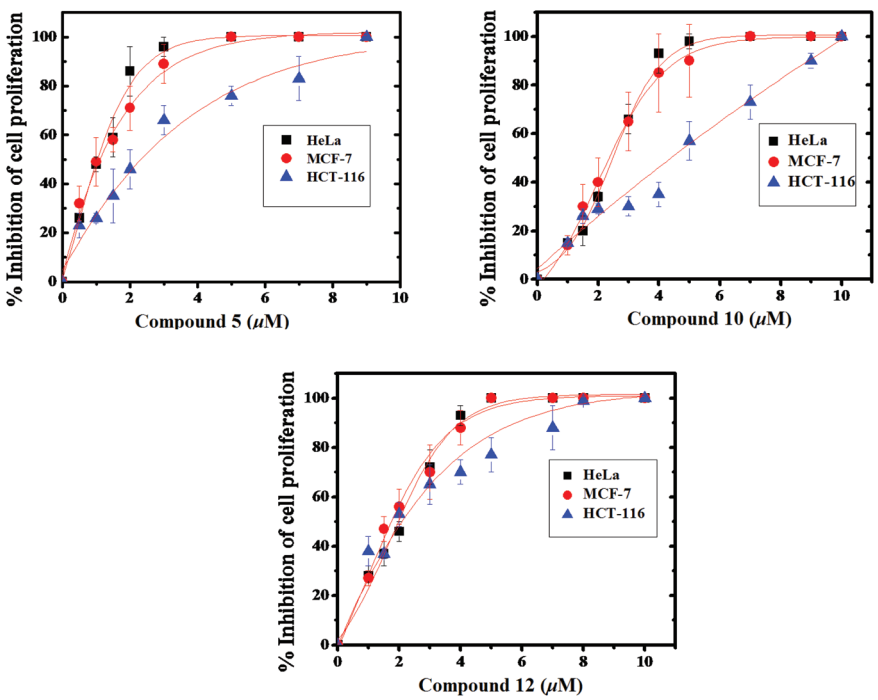


Fig. 4 The effects of **5**, **10** and **12** on human cervical cancer (HeLa), human colon carcinoma (HCT116) and breast cancer (MCF-7) cells after 24, 24 and 48 h.

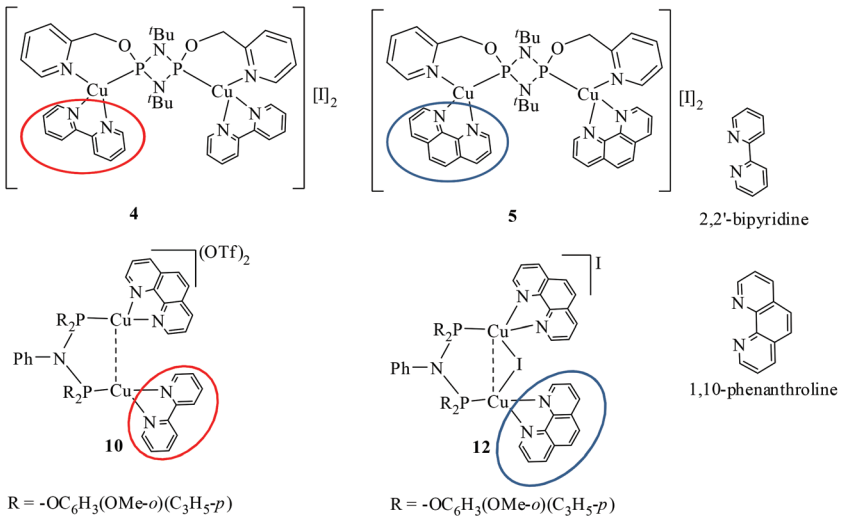


Fig. 5 Structural activity correlation of complexes **4**, **5**, **10** and **12**.

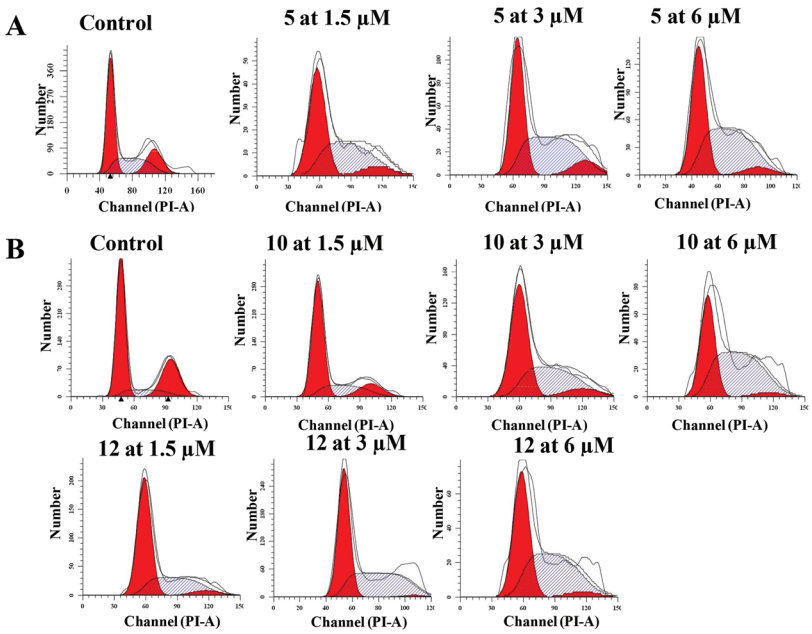


Fig. 6 Effects of compounds **5** (6A) and **10** (upper panels 6B) and **12** (lower panels 6B) on cell cycle of MCF-7 cells.

of **5** at 1.5, 3 and 6 μM, respectively (Fig. 6 and Table 6). FACS analysis for Annexin V and propidium iodide staining was used to confirm apoptosis induction by these complexes. The compounds **5**, **10** and **12** increased the number of cells in early and late stages of apoptosis (Fig. 7 and Table 7).

The apoptosis-causing ability of the compounds was further confirmed by performing DNA fragmentation assays. When a cell undergoes apoptosis, endonucleases cleave the internucleosomal linker DNA that results in fragmentation of the genome.[59] The fragmented genome gives a laddering pattern on running the gel which is a characteristic signature

Table 6 Effects of **5**, **10** and **12** on MCF-7 cell cycle progression

	Compound **5**			Compound **10**			Compound **12**		
	% of cells in different phases of cell cycle								
Conc. (μM)	G1	S	G2	G1	S	G2	G1	S	G2
0	55	17	28	59	11	30	59	11	30
1.5	48	44	8	63	23	14	61	34	5
3	43	48	9	52	41	7	53	46	1
6	43	52	5	39	58	3	46	50	4

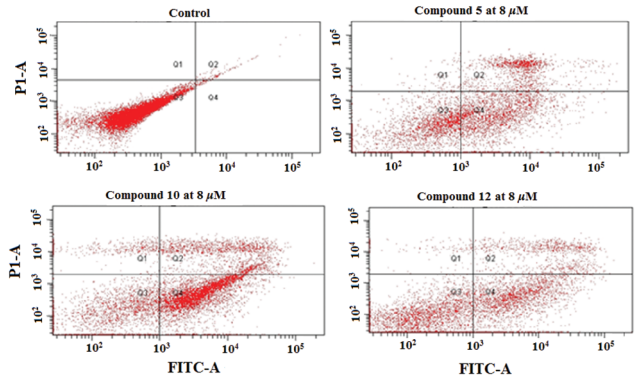


Fig. 7 Apoptosis determination by FACS analysis of MCF-7 cells.

Table 7 The distribution of cells in different states of apoptosis in the presence of copper complexes, **5**, **10** and **12**

Sample	Q1 (% dead Cells)	Q2 (% late apoptosis)	Q3 (% living cells)	Q4 (% earlier apoptosis)
Control	0.4	0.9	98.6	0.5
5 (8 μM)	0.6	23.5	19.1	56.8
10 (8 μM)	2.8	30.1	18.1	49.1
12 (8 μM)	0.9	16.9	33.6	48.4

of apoptosis.[60] In comparison with the control, compounds **5**, **10** and **12** caused DNA fragmentation comparable to etoposide, a known DNA damaging agent (Fig. 8).[61]

Since the role of tumor suppressor p53 is well known in apoptosis,[62,63] we were interested in knowing whether apoptosis by these compounds is mediated through the p53 pathway or not. Although the structures of **10** and **12** are quite similar, compound **12** was considered for this study as it was found to be a more potent cytotoxic agent. The vehicle-treated MCF 7 cells showed compact and circular DNA morphology. In contrast, treatment with **5** and **12** induced disorganization of the nucleus with a granular and hollow appearance clearly indicating the DNA damage as shown in Fig. 9 and S1.† The damage was more pronounced at higher concentrations.

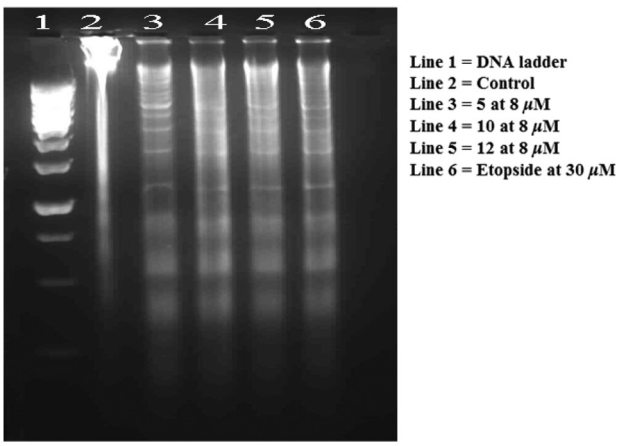


Fig. 8 DNA laddering experiment suggesting fragmentation of genomic DNA.

In comparison with the control, treatment with compounds **5** and **12** at 1.5, 3 and 6 μM concentrations increased nuclear localization of p53 from 1.8% in the control to 25, 33 and 42% and 32, 40, and 47%, respectively (Fig. 9, S1† and Table 8). Since p21 is the downstream protein of p53 and is indispensible for p53 mediated G1/S arrest,[64,65] p21 localization to the nucleus will give an insight into the effect of these compounds on the progression of the cell cycle. In the control, 2% of the cells showed nuclear localization of p21, while treatment with **5** and **12** at 1.5, 3, and 6 μM concentrations increased its nuclear localization to 29, 35, and 45% and 27, 42, and 48%, respectively (Fig. 9 and S1† and Table 8).

In summary, we have identified a few copper complexes as potent anticancer agents. The complexes caused strong S-phase arrest which can be due to the DNA damage. The pathway of apoptosis and S-phase arrest seems to follow the p53 and p21 route. The results are in agreement with the general mechanism followed by G1/S blocking agents of the cell cycle. The stability of complexes under *in vitro* conditions and in aqueous medium for more than 16 h indicates the absence of any covalent interactions with DNA through substitution or hydrolysis. Therefore, non-covalent interactions such as hydrogen-bonding or π–π interactions may be responsible for antiproliferative activities. Recent computational studies[58] have shown that the planar heteroaromatic diimines interact with DNA base pairs by aromatic π–π stacking. The superior activity of 1,10-phenanthroline containing compounds is probably due to better π–π stacking interaction compared to 2,2′-bipyridine containing complexes. Further efforts to definitively unravel the uncertainties about the actual interactions would be warranted.

Conclusions

Di- and tetranuclear copper(I) complexes containing aminobis(phosphonites) and cyclodiphosphazane ligands were prepared and reacted with various pyridyl ligands to form binuclear mixed-ligand complexes. The mixed-ligand copper(I) complexes have shown highly promising antiproliferative activities towards various human cancer cell lines. Copper(I) compounds were found to suppress tumor cell growth by arresting cells at the S-phase of the cell cycle. The nuclear morphology of treated cells clearly showed DNA damage. The experimental evidence clearly indicated that these compounds initiated apoptosis, which is mediated through the p53 pathway. Copper being an essential cofactor in several enzymes and physiological processes, and also not being designated as a carcinogenic element, may be less toxic than other transition metals such as platinum, ruthenium and palladium. Further, the antiproliferative activities and apoptosis induction of the copper complexes presented in this paper are several-fold higher than the cisplatin, in remarkably low concentrations.

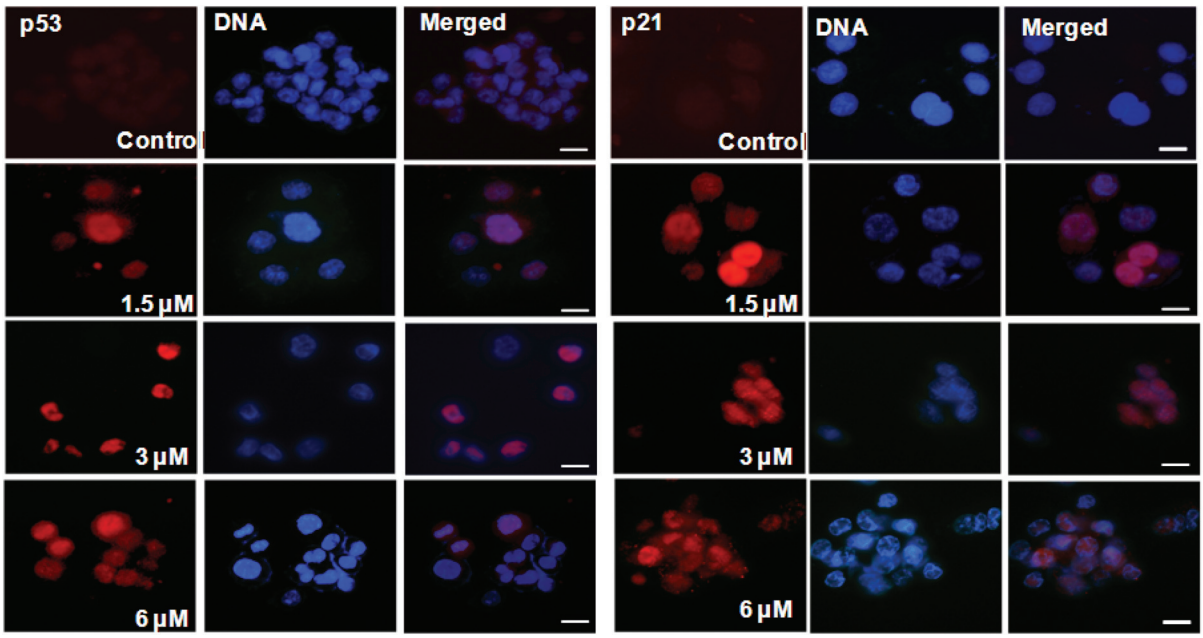


Fig. 9 Compound **5** mediated apoptosis through p53 and p21 pathway. There is prominent nuclear translocation of p53 and its downstream protein p21.

Table 8 Nuclear translocation of p53 and p21 in the presence of copper complexes **5** and **12**

Sample	% cell p53 nuclear localization	% cell p21 nuclear localization
Control	1.75	2
5 (1.5 μM)	25	29
5 (3 μM)	33	35
5 (6 μM)	42	45
12 (1.5 μM)	32	27
12 (3 μM)	40	42
12 (6 μM)	47	48

Experimental section

General procedures

All manipulations were performed using standard vacuum-line and Schlenk techniques under a nitrogen atmosphere unless otherwise stated. All the solvents were purified by conventional methods and distilled prior to use. The compounds *cis*-{ClP(μ-N^{t}Bu)}$_2$ [66] and $C_6H_5N\{P(OC_6H_3(OMe\text{-}o)(C_3H_5\text{-}p))_2\}_2$ (**2**)[48] were prepared according to the published procedures. CuI, AgOTf, 2,2′-bipyridine and 1,10-phenanthroline were purchased from Aldrich Chemicals and used without further purification. Other chemicals were obtained from commercial sources and purified prior to use.

Instrumentation

The NMR spectra were recorded at the following frequencies: 400 MHz (^{1}H), 100 MHz (^{13}C), 162 MHz (^{31}P) using either Varian VXR 400 or Bruker AV 400 spectrometers. ^{13}C and ^{31}P NMR spectra were acquired using broad band decoupling. The spectra were recorded in $CDCl_3$ (or DMSO-d_6) solutions with $CDCl_3$ (or DMSO-d_6) as an internal lock; chemical shifts of ^{1}H and $^{13}C\{^1H\}$ NMR spectra are reported in ppm downfield from TMS, used as an internal standard. The chemical shifts of $^{31}P\{^1H\}$ NMR spectra are referred to 85% H_3PO_4 as an external standard. The microanalyses were performed using a Carlo Erba Model 1112 elemental analyzer. Mass spectra were recorded using Waters Q-Tof micro. The melting points were observed in capillary tubes and are uncorrected.

Synthesis of *cis*-{(*o*-OCH$_2$C$_5$H$_4$N)P(μ-N^{t}Bu)}$_2$ (1)

A mixture of pyridine-2-methanol (1.0 g, 9.16 mmol) and triethylamine (1.3 mL, 0.943 g, 9.32 mmol) in diethyl ether (30 mL) was added to *cis*-{ClP(μ-N^{t}Bu)}$_2$ (1.26 g, 4.58 mmol) also in diethyl ether (30 mL) at 0 °C. The reaction mixture was allowed to stir for 6 h at room temperature. The triethylamine hydrochloride salt thus formed was filtered off and the solvent was removed under reduced pressure to obtain *cis*-{(*o*-OCH$_2$C$_5$H$_4$N)P(μ-N^{t}Bu)}$_2$ (**1**) as a pale yellow viscous liquid. Yield: 91% (1.75 g). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.54 (m, 2H, py), 7.68 (t, $^3J_{HH}$ = 8 Hz, 2H, py), 7.55 (d, $^3J_{HH}$ = 8 Hz, 2H, py), 7.16 (t, $^3J_{HH}$ = 6 Hz, 2H, py), 5.15 (s, 4H, CH_2), 1.24 (s, 18H, ^{t}Bu). $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$): 159.3, 148.9, 136.6, 122.1, 121.2, 64.7, 51.4 (t, $^2J_{PC}$ = 12.5 Hz), 31.1 (t, $^3J_{PC}$ = 9.6 Hz). $^{31}P\{^1H\}$ NMR (162 MHz, $CDCl_3$): δ 133.5 (s). HRMS: Calcd for $C_{20}H_{31}N_4O_2P_2$ [M + H]: 421.1922; Found: 421.1907.

Synthesis of [{Cu(μ-I)}$_2${(*o*-OCH$_2$C$_5$H$_4$N)P(μ-N^{t}Bu)}$_2$]$_n$ (3)

An acetonitrile (10 mL) solution of CuI (0.028 g, 0.147 mmol) was added dropwise to a well-stirred solution of *cis*-{(*o*-OCH$_2$C$_5$H$_4$N)P(μ-N^{t}Bu)}$_2$ (**1**) (0.031 g, 0.074 mmol) in dichloromethane (10 mL) at room temperature and stirring was continued further for 4 h. The solvent was removed under

vacuum and the residue obtained was washed with 2 × 5 mL of petroleum ether to give an analytically pure sample of **3** as a yellow solid. Yield: 80% (0.047 g). Mp: >270 °C. ^{1}H NMR (400 MHz, DMSO-d^6): δ 8.78 (m, py, 2H), 7.66–7.57 (m, py, 6H), 5.20 (d, CH_2, $^3J_{PH}$ = 16 Hz, 4H), 1.26 (s, ^{t}Bu, 18H). ^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$): δ 113.5 (br s). Anal. Calcd for $C_{20}H_{30}N_4I_2O_2P_2Cu_2{\cdot}CH_3CN$: C, 31.37; H, 3.95; N, 8.31%. Found: C, 31.49; H, 3.82; N, 8.42%.

Synthesis of [(bpy)$_2$Cu$_2${(*o*-OCH$_2$C$_5$H$_4$N)P(μ-N^{t}Bu)}$_2$][I]$_2$ (4)

To a suspension of **3** (0.048 g, 0.06 mmol) in dichloromethane (5 mL) was added dropwise a solution of 2,2′-bipyridine (0.019 g, 0.12 mmol) in the same solvent (5 mL) at room temperature. The reaction mixture was stirred for further 2 h, concentrated to 2 mL and layered with petroleum ether (3 mL). The turbid yellow solution was stored at room temperature for 24 h to obtain **4** as a yellow crystalline solid. Yield: 82% (0.054 g) Mp: 210–212 °C (dec). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.89 (s br, 4H), 8.53 (d, $^3J_{HH}$ = 4 Hz, 4H), 7.90 (s br, 4H), 7.67–7.16 (m, 12H), 5.35 (d, $^3J_{PH}$ = 8.2 Hz, 4H), 1.29 (s, ^{t}Bu, 18H). ^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$): δ 109.8 (br s). Anal. Calcd for $C_{40}H_{46}N_8P_2O_2Cu_2I_2$: C, 43.14; H, 4.16; N, 10.06%. Found: C. 43.62; H, 4.05; N, 10.01%.

Synthesis of [(phen)$_2$Cu$_2${(*o*-OCH$_2$C$_5$H$_4$N)P(μ-N^{t}Bu)}$_2$][I]$_2$ (5)

This was synthesized by a procedure similar to that of **4**, using **3** (0.1 g, 0.125 mmol) and 1,10-phenanthroline (0.05 g, 0.25 mmol). Yield: 79% (0.118 g). Mp: 232–234 °C (dec). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.32 (s, br, 4H), 8.48–7.14 (m, 20H), 5.41 (d, $^3J_{PH}$ = 8 Hz, 4H), 1.36 (s, ^{t}Bu, 18H). ^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$): δ 110.7 (br s). Anal. Calcd for $C_{44}H_{46}N_8I_2O_2P_2Cu_2$: C, 45.49; H, 3.99; N, 9.65%. Found: C. 45.07; H, 4.60; N, 9.52%.

Synthesis of [(CuI)$_4${PhN{P(OC$_6$H$_4$(OMe-*o*)(C$_3$H$_5$-*p*))$_2$}$_2$}$_2$] (6)

A solution of cuprous iodide (0.014 g, 0.074 mmol) in acetonitrile (5 mL) was added to a solution of **2** (0.03 g, 0.037 mmol) in acetonitrile (5 mL). After stirring for 4 h, the solvent was evaporated under vacuum to obtain an oily residue which on washing several times with petroleum ether and drying under vacuum gave a white crystalline solid. Yield: 89% (0.0391 g). Mp: 123–125 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74–7.30 (m, C_6H_5, 5H), 6.73 (d, C_6H_3, 4H, $^3J_{HH}$ = 8.0 Hz), 6.58 (s, C_6H_3, 4H), 6.52 (d, C_6H_3, 4H, $^3J_{HH}$ = 6.4 Hz), 5.97–5.90 (m, CH, 4H), 5.10–5.04 (m, CH_2, 8H), 3.63 (s, OCH_3, 12H), 3.30 (d, CH_2, 8H, $^3J_{HH}$ = 8 Hz). ^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$): δ 86.3 (br s). Anal. Calcd for $C_{92}H_{98}N_2I_4O_{16}P_4Cu_4$: C, 46.56; H, 4.16; N, 1.18%. Found: C, 46.23; H, 4.01; N, 1.19%.

Synthesis of [{Cu(μ-I)(py)}$_2${C$_6$H$_5$N{P(OC$_6$H$_3$(OMe-*o*)-(C$_3$H$_5$-*p*))$_2$}$_2$}] (7)

Pyridine (5 mL) was added to compound **6** (0.03 g, 0.0126 mmol) dropwise and allowed to stir at room temperature for 4 h. The resulting yellow solution was evaporated to dryness under vacuum; the residue was dissolved in dichloromethane (2 mL), layered with petroleum ether (3 mL) and stored at −20 °C for a day to give a white crystalline solid. Yield: 82% (0.0278 g). Mp: >230 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.14 (br s, Pyr-4-H, 2H), 7.88 (d, $^3J_{HH}$ = 8 Hz, Pyr-2-6-H, 4H), 7.52 (t, $^3J_{HH}$ = 8 Hz, Pyr-3-5-H, 4H), 6.36–7.39 (m, ArH, 17H), 6.01–5.91 (m. CH, 4H), 5.12–5.06 (m, CH_2, 8H), 3.63 (s, OCH_3, 12H), 3.34 (d, CH_2, 8H, $^3J_{HH}$ = 8 Hz). ^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$): δ 104.7 (br s). Anal. Calcd for $C_{56}H_{59}N_3P_2O_8I_2Cu_2{\cdot}CH_2Cl_2$: C, 47.88; H, 4.30; N, 2.94%. Found: C, 47.58; H, 4.54; N, 3.12%.

Synthesis of [Cu$_2$(μ-I)I(bpy){C$_6$H$_5$N{P(OC$_6$H$_3$(OMe-*o*)-(C$_3$H$_5$-*p*))$_2$}$_2$}] (8)

To a solution of **6** (0.03 g, 0.0126 mmol) in acetonitrile (5 mL) was added dropwise a solution of 2,2′-bipyridine (0.008 g, 0.0504 mmol) in the same solvent (5 mL) and the reaction mixture was stirred for 4 h. The resulting yellow solution was concentrated and stored at −10 °C for 24 h to give an analytically pure product as bright yellow crystals. Yield: 89% (0.0337 g). Mp: 119–121 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.11 (br s, Pyr-3-3′-H, 2H), 7.75 (br s, Pyr-6-6′-H, 2H), 7.52–6.47 (m, ArH, 21H), 5.95–5.87 (m, CH, 4H), 5.13–5.08 (m, CH_2, 8H), 3.64 (br s, OCH_3, 12H), 3.28 (br s, CH_2, 8H). ^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$): δ 103.7 (br s). Anal. Calcd for $C_{66}H_{69}N_5P_2O_8I_2Cu_2$: C, 52.74; H, 4.63; N, 4.66%. Found: C, 53.10; H, 4.67; N, 4.38%.

Synthesis of [Cu$_2$(μ-I)I(bpy){C$_6$H$_5$N{P(OC$_6$H$_3$(OMe-*o*)-(C$_3$H$_5$-*p*))$_2$}$_2$}] (9)

This was synthesized by a procedure similar to that for **8** using 2,2′-bipyridine (0.004 g, 0.0256 mmol) and **6** (0.03 g, 0.0126 mmol). Yield: 85% (0.0288 g). Mp: 166–169 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.01 (br d, Pyr-3-3′-H, 2H), 7.72 (br d, Pyr-6-6′-H, 2H), 7.56 (br d, Pyr-4-4′-H, 2H), 7.46 (br t, Pyr-5-5′-H, 2H), 7.32–6.50 (m, ArH, 17H), 5.97–5.87 (m, CH, 4H), 5.29–5.07 (m, CH_2, 8H), 3.64 (br s, OCH_3, 12H), 3.28 (br s, CH_2, 8H). ^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$): δ 103.5 (br s). Anal. Calcd for $C_{56}H_{57}N_3P_2O_8I_2Cu_2$: C, 50.08; H, 4.28; N, 3.13%. Found: C, 49.78; H, 4.14; N, 3.57%.

Synthesis of [Cu$_2$(phen)(bpy){C$_6$H$_5$N{P(OC$_6$H$_3$(OMe-*o*)-(C$_3$H$_5$-*p*))$_2$}$_2$}](OTf)$_2$ (10)

To a solution of **9** (0.027 g, 0.02 mmol) in acetonitrile (5 mL) was added AgOTf (0.0052 g, 0.02 mmol) and the reaction mixture was stirred for 1 h. The AgI formed was filtered through a frit with celite and a solution of 1,10-phenanthroline (0.004 g, 0.02 mmol) in acetonitrile was added dropwise. The resulting yellow solution was stirred for another 2 h, the solvent removed under *vacuo*, and the residue obtained dissolved in 1 mL of dichloromethane and diluted with 3 mL of petroleum ether to give a yellow precipitate. The precipitate was separated, and dried under *vacuo* to obtain an analytically pure product. Yield: 91% (0.0281 g). Mp: 176–178 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.70–7.10 (m, ArH, 25H), 5.97–5.90 (m, CH, 4H), 5.11–5.06 (m, CH_2, 8H), 3.48 (br s, OCH_3, 12H), 3.22 (br s, CH_2, 8H). ^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$): δ 112.3 (br s).

Anal. Calcd for $C_{69}H_{65}NF_3I_5O_{11}P_2SCu_2$: C, 53.63; H, 4.24; N, 4.53, S, 2.08%. Found: C, 53.39; H, 3.96; N, 4.29, S, 1.91%.

Synthesis of $[Cu_2(\mu\text{-}I)I(phen)\{C_6H_5N\{P(OC_6H_3(OMe\text{-}o)\text{-}(C_3H_5\text{-}p))_2\}_2\}]$ (11)

This was prepared by a procedure similar to that for **8** using 1,10-phenanthroline (0.005 g, 0.0256 mmol) and **6** (0.03 g, 0.0126 mmol). Yield: 87% (0.03 g). Mp: 130–132 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.48 (br s, Pyr-3-3′-H, 2H), 7.93 (br d, Pyr-4-7-H, 2H), 7.73 (br d, Pyr-2-9-H, 2H), 7.41 (s, Pyr-5-6-H, 2H), 7.35–7.18 (m, ArH, 17H), 5.97–5.90 (m, CH, 4H), 5.11–5.06 (m, CH_2, 8H), 3.62 (br s, OCH_3, 12H), 3.22 (br s, CH_2, 8H). $^{31}P\{^1H\}$ NMR (162 MHz, $CDCl_3$): δ 103.6 (br s). Anal. Calcd for $C_{58}H_{57}N_3P_2O_8I_2Cu_2$: C, 50.96; H, 4.20; N, 3.07%. Found: C, 51.18; H, 4.29; N, 3.15%.

Synthesis of $[Cu_2(\mu\text{-}I)(phen)_2\{C_6H_5N\{P(OC_6H_3(OMe\text{-}o)\text{-}(C_3H_5\text{-}p))_2\}_2\}]I$ (12)

This was prepared by a procedure analogous to **8** using 1,10-phenanthroline (0.01 g, 0.0504 mmol) and **6** (0.03 g, 0.0126 mmol). Yield: 79% (0.0308 g). Mp: >160 °C (dec). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.46 (br s, Pyr-3-3′-H, 2H), 7.91 (br d, Pyr-4-7-H, 2H), 7.72 (br d, Pyr-2-9-H, 2H), 7.40 (s, Pyr-5-6-H, 2H), 7.34–7.16 (m, ArH, 17H), 5.99–5.89 (m, CH, 4H), 5.13–5.07 (m, CH_2, 8H), 3.62 (br s, OCH_3, 12H), 3.22 (br s, CH_2, 8H). $^{31}P\{^1H\}$ NMR (162 MHz, $CDCl_3$): δ 103.6 (br s). Anal. Calcd for $C_{70}H_{65}N_5I_2O_8P_2Cu_2$: C, 54.34; H, 4.23; N, 4.53%. Found: C, 54.37; H, 4.03; N, 4.93%.

Antiproliferative studies

Materials. Sulforhodamine B, Hoechst 33258, mouse anti-p53 IgG, mouse anti-p21 IgG antibodies and apoptosis detection kit (Annexin V-propidium iodide) were purchased from Santa Cruz Biotechnology, CA, USA. All other reagents were of analytical grade and obtained from Sigma, MO, USA and Himedia, Mumbai, India.

Cell culture. MCF-7, HeLa and HCT116 cells were grown in MEM (Eagle's minimum essential medium) (Himedia, Mumbai, India). MEM contains 10% fetal bovine serum, 2.2 g L^{-1} sodium bicarbonate and 1% antibiotic–antimycotic solution composed of streptomycin, amphoterecin B and penicillin. Cells were grown at 37 °C in a humidified atmosphere of 5% carbon dioxide and 95% air. Cell lines were maintained in a 25 mL cell culture flask (Nunc) at 37 °C.

Cell proliferation assay. Cytotoxicity of different copper complexes on HeLa cell line proliferation at 5 μM and 10 μM concentrations was determined by sulforhodamine B assay[67,68] Briefly, cells were seeded in 96 well cell culture plates (Himedia, Mumbai, India) for 24 h. Then, the medium was removed and fresh media containing 0.1% DMSO or different concentrations of each compound were added into the wells. The antiproliferative activity of **5**, **10** and **12** against human cervical cancer (HeLa), human colon carcinoma (HCT116) and breast cancer (MCF-7) cells was determined after 24, 24 and 48 h, respectively. The extent of inhibition of cell proliferation was determined by the SRB assay.[67,68] Data are the average of three independent experiments performed in a 96 well plate on different days for each cell line.

p53 and p21 staining for determining p53 mediated apoptotic pathway

MCF-7 cells were incubated with either vehicle or different concentrations of **5** and **12** for 24 h. Cells were collected by centrifugation, fixed with 3.7% formaldehyde and then immunostained using antibody against p53 and p21 (1° 1 : 300, 2° 1 : 300). Secondary antibody was alexa linked anti-mouse antibody generated in rabbit. The DNA was stained with Hoechst 33258. The images were captured at 40× using a Nikon microscope and the processing was done by an image pro plus Software (Media Cybernetics, Silver Spring, MD, USA). The cells following apoptosis through a p53 dependent manner showed red nuclear staining due to the translocation of p53 and p21 into the nucleus.[67]

Annexin V/propidium iodide (PI) staining for apoptosis

Samples for Annexin V/propidium iodide (PI) staining were prepared as described recently.[67,69,70] MCF-7 cells were incubated with either vehicle or 8 μM concentrations of **5**, **10** and **12** for 24 h. The cells were dislodged by trypsin-EDTA treatment, collected by cyto-spinning at 2400 rpm for 10 min at 25 °C, washed twice with PBS and resuspended in 1× binding buffer. Then the cells were incubated with 5 μL of FITC-AnnexinV and 5 μL of propidium iodide for 15 minutes at room temperature in the dark. Annexin and PI content of the live cells was quantified by flow cytometry (FACS Aria Beckton Dickenson) and analyzed using the Modfit LT program (Verity Softwares, USA).

Cell cycle analysis using FACS. MCF-7 cells (~1×10^6 cells mL^{-1}) were incubated without or with different (1.5 μM, 3 μM and 6 μM) concentrations of **5**, **10** and **12**. DNA were stained with 50 μg mL^{-1} propidium iodide and quantified by flow cytometry.[44,67] The data were analyzed by Modfit LT program (Verity Softwares, USA).

DNA fragmentation assay for determination of apoptosis

MCF-7 cells were grown in 25 mL culture flasks, once the cell density reached 1×10^6 cells mL^{-1}, the cells were incubated with **5**, **10** and **12** at 8 μM and etoposide at 30 μM for 48 h. After compound treatment, the cells were dislodged and collected by centrifugation.[71] The pellets were washed with PBS, dissolved in lysis buffer (50 mM Tris, pH 8.0/10 mM ethylenediaminetetraacetic acid–0.5% SL-sarcosine–0.5 mg mL^{-1} of proteinase K), and incubated at 50 °C for 1 h in a heating block. After heat treatment, 5 μL of RNase (1 mg mL^{-1}) was added to the mixture and it was incubated at 50 °C for 1 h. Finally, the mixture was incubated at 65 °C for 2 min, the temperature was lowered to 25 °C and the sample was run on a 1.8% agarose gel. The DNA bands were analyzed using UV gel doc.

X-Ray crystallography

Crystals of each of the compounds **3** and **9** suitable for X-ray crystal analysis were mounted on a Cryoloop with a drop of Paratone oil and placed in the cold nitrogen stream of the Kryoflex attachment of the Bruker APEX CCD diffractometer, whereas crystals of **11** were mounted on a CCD Oxford Diffraction XCALIBUR-S diffractometer equipped with an Oxford Instruments low-temperature attachment. Data were collected at 100(2) K for **3** and **9** and 150(2) K for **11** using graphite-monochromated Mo Kα radiation ($\lambda\alpha$ = 0.71073 Å). The data were collected by the standard ω-2θ scan techniques (for **11**) or APEX2 programme suite (for **3** and **9**)[72] and were scaled and reduced using the SAINT software.[73] Multiple measurements of equivalent reflections provided the basis for an empirical absorption correction as well as a correction for any crystal deterioration during the data collection (SADABS[74]). The structures **3** and **9** were solved by the Patterson method, whereas **11** was solved by direct methods and refined by full-matrix least-squares procedures using the SHELXTL program package.[75] Hydrogen atoms attached to carbon were placed in calculated positions and included as riding contributions with isotropic displacement parameters tied to those of the attached non-hydrogen atoms. The isotropic thermal parameters of the hydrogen atoms were fixed at 1.2 times that of the corresponding carbon for phenyl hydrogen and 1.5 times for $C(CH_3)_3$. In the final refinement, the hydrogen atoms were riding with the carbon atom to which they were bonded. Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 977645 (compound **3**), 977646 (compound **9**), 977647 (compound **11**).

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