Available online at www.sciencedirect.com
ScienceDirect
European Journal of Medicinal Chemistry 41 (2006) 1494–1503
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Laboratory note

Synthesis, characterization and in vitro biological activity studies of Cu–M (M = Cu^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}) bimetallic complexes

K.R. Rupesh [a,1], S. Deepalatha [b,1], M. Krishnaveni [a], R. Venkatesan [b], S. Jayachandran [a,*]

[a] *Department of Biotechnology, Pondicherry University, School of Life Sciences, Kalapet, Pondicherry 605014, India*
[b] *Department of Chemistry, Pondicherry University, Kalapet, Pondicherry 605014, India*

Received 30 June 2005; received in revised form 25 October 2005; accepted 14 November 2005
Available online 29 September 2006

Abstract

Six new bimetallic complexes of the type CuCu, CuCo, CuNi, CuZn and CuMn were prepared. The structures of these complexes and the ligand have been proposed on the basis of FAB mass, elemental analysis, UV–vis, IR, EPR and CV studies. All the complexes completely cleave pBS (SK−) DNA at a concentration of 10 μM; however, even at lower concentrations of 2 μM and 0.1 μM, the complexes (**I** and **Ia**) showed partial cleavage. The results of the fluorescence binding studies of the metal complexes with CT-DNA showed that the presence of aliphatic ligands added additional binding effects including electrostatic, hydrogen binding and vander Waals interactions. Complexes (**I**, **Ia**) showed 50% inhibition of COX-1 and COX-2 activities at as low a concentration as 12.5 μM, 13.5 μM, 14 μM and 14.5 μM. Inhibition assay of top I and top II by different complexes in mutant yeast strains (JN394, JN394 t_{-1} and JN394 t_{2-5}) with all the complexes showed significant inhibition of topoisomerase at 5 μM concentration. Complexes **I** and **Ia** exhibited good anti-microbial activities against all human pathogens tested except *Klebsiella pneumoniae*. The following studies showed that among the synthesized bimetallic complexes, complexes **I** and **Ia** seem to be promising candidates possessing DNA cleavage activities besides anti-microbial and anti-inflammatory properties to serve as chemical nucleases and chemotherapeutic agents.

Keywords: Cu(II) complexes; COX; DNA cleavage; Bimetallic complexes; Anti-microbial

1. Introduction

The interaction of transition metal polypyridyl complexes with DNA has received considerable attention for they serve as potential models of biological systems [1]. The design and development of small- or medium-sized potential therapeutic agents, particularly those designed to target nucleic acids site-specifically or to mimic the function of enzymes that participate in nucleic acid strand cleavage can lead to safer and more rational approaches to novel therapeutic agents for cancer, viral diseases and tools for molecular biology [2,3]. Small molecules interact with double-stranded DNA in a number of ways [4–6] and in most cases it is non-covalent, which involves intercalation of planar aromatic molecules between the base pairs of DNA helix.

In recent years, increasing emphasis has been placed on the screening and tailoring of non-platinum/ruthenium complexes [7–9]. In comparison to the 4d- or 5d-metal analogues, complexes of first row transition elements showing photocleavage activity could find better application at the cellular level. The first copper complex reported to show efficient DNA cleavage activity is bis(1,10-phenanthroline)copper(I) [10]. Subsequently, the chemistry of metal complexes of 1,10-phenanthroline (phen) or a modified phen ligand is particularly attractive for developing new diagnostic and therapeutic agents that can

* Corresponding author. Tel.: +91 413 2655991x428/91 413 2655715 (direct); fax: +91 413 2655265.
E-mail address: sr_jayachandran@yahoo.com (S. Jayachandran).
[1] Authors equally contributed to this work.

0223-5234/$ - see front matter © 2006 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2005.11.016

recognize and cleave DNA [11]. Cationic copper complex [CuI(phen)2]+ in the presence of molecular oxygen and a reducing agent, is known to act as an efficient nuclease with a high preference for double-stranded DNA [12]. It has been suggested that copper complex may partially be intercalated into the DNA duplexes containing mismatched strands of oligonucleotides, since cleavage occurs near the bulges where intercalation of copper complexes is thought to be favored. Also Barton and Raphael have reported that chiral phenanthroline–cobalt(III) complexes recognize different local structures of DNA [13]. Aromatic ring stacking between nucleobases is considered to be a major driving force that leads to binding. The size, electron density of the interacting aromatic rings and the combined effect of hydrophobic and hydrophilic interactions determine the extent of binding [14–16]. Thus by using mixed ligand complexes, it is possible to systematically vary parameters of interest hence changing the properties of the intercalating groups without altering the chemical nature of the intercalating moiety.

Herein we report the synthesis of biologically active homo–hetero bimetallic complexes and the DNA interaction kinetics of these multi-metallic complexes in which the different metal complexes did not lose their individual chemical identity except providing a platform for multiple coordinating sites with DNA. Infrared analysis, absorption spectra and FAB mass analysis, EPR and CV are employed to analyze the prepared complexes. Our results indicate that the synthesized 2,2-bipyridyl based bimetallic complexes completely cleaved pBS (SK−) DNA at 10 μM concentration and the complexes **I** ($CuC_{24}H_{22}N_2O_6$) and **Ia** ($Cu_2C_{32}H_{43}N_8O_3$) are effective in inhibiting the growth of *Candida albicans* and a wide variety of Gram-positive and Gram-negative medically important bacteria except *Klebsiella pneumoniae*. Complexes **I** and **Ia** also inhibited cyclooxygenases (COX-1 and COX-2) and topoisomerases (top I and top II), which are predominantly used as markers in anti-tumor studies.

2. Materials and methods

2.1. Synthesis of 2,2′-bipyridyl based bimetallic complexes

2.1.1. Preparation of 1,10-phenanthroline-5,6-dione (*L1*)

1,10-Phenanthroline-5,6-dione (**L1**) was prepared by oxidation of 1,10-phenanthroline. A round bottom long necked flask containing 1.00 g (5.04 mmol) of 1,10-phenanthroline and 5.95 g (50 mmol) of potassium bromide was placed in an ice bath. To this, cold concentrated sulphuric acid (20 mL) was added followed by the addition of 10 mL of concentrated nitric acid, dropwise along the sides of the flask. The resulting solution was cooled to room temperature and heated over a boiling water bath until the bromine vapours were eliminated. The reaction mixture was again cooled to room temperature and poured into a beaker containing 400 mL of ice cold water. The solution was made neutral by the addition of sodium bicarbonate and extracted with dichloromethane to give 1,10-phenanthroline-5,6-dione as yellow needles [9,17]. Yield: 86% (0.91 g). Melting point: 270 °C. IR (KBr, cm^{-1}): 1681 (C=O stretching), 1559 (C=C stretching), 1200, 1111, 1005 (C–C stretching). ^1H NMR ($CDCl_3$): 9.13 (d, H), 8.55 (d, H), 7.60 (t, 2H).

2.1.2. Preparation of $[Cu(bpy)(H_2O)](NO_3)_2$ (*L2*)

To 10 mmol (2.416 g) of copper(II) nitrate taken in 25 mL of distilled water, 10 mmol (1. 562 g) of 2,2′-bypyridyl in 50 mL ethanol was added and stirred for about 2 h. The resulting blue solution was reduced to 20 mL to precipitate; the desired complex was filtered, recrystallized from acetonitrile/ethanol and dried under vacuum. IR (KBr, cm^{-1}): 1273, 1160 C–C (stretching), 1383 (NO stretching) 772 (NO out of plane bending), 480 (Cu–N stretching).

2.1.3. Preparation of $[Cu(bpy)(L1)(H_2O)(C_2H_5OH)](NO_3)_2$ (*I*)

To 10 mmol (4.16 g) of **L2** in 50 mL water, 10 mmol (2.10 g) of **L1** in 100 mL ethanol was added dropwise and stirred for 4 h. The resulting green coloured solution was reduced to 20 mL. The precipitate was filtered and dried in the desiccator. Analytical data (%) for $CuC_{24}H_{22}N_6O_{10}$, found (calculated): C, 46.12 (46.64); H, 3.25 (3.59); N, 13.56 (13.6); Cu, 9.98 (10.28). IR (KBr, cm^{-1}): 1702 (C=O stretching), 482 (Cu–N stretching). FAB mass: molecular ion peak at 493 (M + 1) for the molecular formula, $CuC_{24}H_{22}N_2O_6$, calculated mass: 492.09.

2.1.4. Preparation of bimetallic complexes (*Ia*, *Ib*, *Ic*, *Id*, and *Ie*)

Compound **I** of 10 mmol (6.08 g) was taken in 250 mL of dry ethanol and was refluxed for an hour to ensure complete solubility and to this 20 mmol (1.76 g) of *N*,*N*-dimethylethylenediamine was added followed by the addition of 10 mmol of the appropriate metal salt and allowed to reflux for 24 h. The resulting solution was cooled to ambient temperature. To the reaction mixture 4.2 equivalents of potassium hexaflurophosphate (KPF_6) was added to precipitate the complex. The precipitate was filtered, washed with excess ethanol, recrystallized from acetonitrile/ethanol and dried under vacuum to give a dark green powder. Addition of appropriate metal ions is followed in each of the preparations, 10 mmol (2.416 g) of copper(II) nitrate for **Ia**; IR (KBr, cm^{-1}): 1611 (C=N Schiffs base stretching), 482 (Cu–N stretching). FAB mass: molecular ion peak at 711 (M − 2) for the molecular formula, $Cu_2C_{32}H_{43}N_8O_3$, calculated mass: 713.21; 10 mmol (2.910 g) of cobalt(II) nitrate for **Ib**, IR (KBr, cm^{-1}): 1625 (C=N Schiffs base stretching), 482 (Cu–N stretching). FAB mass: molecular ion peak at 672 (M − 1) for the molecular formula, $CuCoC_{32}H_{39}N_8O$, calculated mass: 673.19; 10 mmol (2.908 g) of nickel(II) nitrate for **Ic**, IR (KBr, cm^{-1}): 3428 (O–H stretching), 1646 (C=N Schiffs base stretching), 476 (Cu–N stretching). FAB mass: molecular ion peak at 672 (M − 1) for the molecular formula, $CuNiC_{32}H_{39}N_8O$, calculated mass: 672.19; 10 mmol (2.195 g) of zinc acetate for **Id**, IR (KBr, cm^{-1}): 1646 (C=N Schiffs base stretching), 483 (Cu–N stretching). FAB mass: molecular

ion peak at 807 (M + 1) for the molecular formula, $CuZnC_{36}H_{55}N_8O_5$, calculated mass: 806.29; 10 mmol (1.979 g) of manganese(II) chloride for **Ie**, IR (KBr, cm^{-1}): 1625 (C═N Schiffs base stretching), 482 (Cu–N stretching). FAB mass: molecular ion peak at 752 (M − 2) for the molecular formula, $CuMnC_{34}H_{48}N_8O_4$, calculated mass: 750.25.

2.2. Structural analysis

Infrared spectra of the ligands and complexes were recorded on a ABB Bomem MB-104 FTIR spectrometer and Hitachi infrared spectrophotometer using KBr disks. Electronic absorption spectra were recorded on an Ocean Optics miniature Fiber Optics spectrometer. The SD1000 accepts light energy transmitted through the optical fiber and disperses it via a fixed grating across the detector, which is responsive from 200 to 1100 nm. All the studies were done in a 1 cm quartz cuvette. The FAB mass spectra were recorded on a JEOL SX 102/DA-6000 mass spectrometer/data system using argon/xenon (6 kV, 10 mA) as the FAB gas. The accelerating voltage was 10 kV and the spectra were recorded at room temperature. *M*-Nitrobenzyl alcohol (NBA) was used as the matrix.

2.3. DNA–metal binding studies by fluorescence

Fluorescence measurements were performed to study the metal interaction with DNA using a Hitachi F4500 spectrofluorimeter employing right angle detection mode. Ethidium bromide (EB) is one of the most sensitive fluorescence probes that can bind with DNA [18]. Calf thymus-DNA (used in the present studies) does not emit by itself. The fluorescence of ethidium bromide increases after intercalating into DNA. If the metal intercalates into DNA, it leads to a decrease in the binding sites of DNA available for EB and a decrease in the fluorescence intensity of the EB–DNA system [19].

All the experiments were carried out in 0.02 mol L^{-1} phosphate buffer containing 60 mmol sodium chloride at pH 7.0. Calf thymus-DNA (CT-DNA) as sodium salt was purchased from Hi Media and the purity was checked by the absorbance ratio between A_{260} and A_{280} of DNA, which was found to be 1.88 in phosphate buffer at pH 7.0. Ethidium bromide, which binds to DNA by interacting and enhancing the fluorescence intensity, was taken in the concentration of 3.3×10^{-6} M. With the concentration of the DNA and ethidium bromide constant, the fluorescence studies were done with the increasing concentrations of the metal complex, ranging from 1×10^{-6} M to 20×10^{-6} M. The metal complexes did not fluoresce by themselves. An incubation period of 10 min was fixed by trial and error method. All the emission spectra were recorded by exciting the solution at 500 nm. Double distilled water was used throughout.

The emission spectrum for ethidium bromide in the absence and in the presence of DNA was recorded for a concentration range of $1.65–3.3 \times 10^{-6}$ M. A plot of fluorescence intensity vs. ethidium bromide concentration resulted in a straight line with a slope of 19.3. The concentration of metal complexes bound to DNA was calculated using the formula:

$$C_b = I_t - I_m/(V-1)K$$

where C_b is the concentration of the metal complex bound to DNA, I_t is the fluorescence intensity of DNA and EB in the absence of metal complex, I_m is the fluorescence intensity of DNA, EB and metal complex at a particular concentration of the complex ($1–20 \times 10^{-6}$ M), V is the ratio of fluorescence intensity of ethidium bromide bound to DNA and free ethidium bromide, and K is the slope of plot of fluorescence intensity vs. ethidium bromide concentration. The unbound metal concentration was written as:

$$C_{ub} = C_o - C_b$$

where C_o is the total concentration of the metal complexes and C_b is the concentration of metal complexes bound to DNA. The specific binding of the metal complex to DNA was calculated as:

$$\upsilon = C_b/(C_b + [\mathrm{DNA}]).$$

The saturation binding constant was calculated by plotting unbound metal concentration against the specific binding. The number of binding sites and the binding constant were calculated for all the complexes.

The emission spectra were recorded for increasing concentrations of the complexes, by exciting the substance at 500 nm. Upon the addition of the metal complexes, the fluorescence spectra of complexes showed a bathochromic shift of the excitation and emission peaks showed a decrease in the peak intensity.

2.4. Plasmid DNA isolation by alkali lysis method [20]

The pBS (SK–) vector was obtained from the culture collection of the Department of Biotechnology, School of Life Sciences, Pondicherry University, Pondicherry, India. Plasmid Bluescript (pBS) DNA (SK–) was extracted as per the procedure of Sambrook et al. [20]. The purity of pBS (SK–) was confirmed via both agarose gel electrophoresis and UV spectroscopy by determining the ratio of absorbance at 260 nm to the absorbance at 280 nm. The concentration of DNA was determined from the absorbance at 260 nm ($A_{260} = 1.0$ OD for 50 μg/mL).

2.5. Determination of DNA cleavage activity [21]

DNA cleavage assay was performed according to the method described by Sambrook et al. [20] and Yang et al. [21]. The experiment was performed in a 20 μl NaH_2PO_4:-Na_2HPO_4 buffer (pH 6.7) containing 1 μg supercoiled plasmid DNA (pBluescript SK–). The reaction mixture was incubated at 37 °C. After incubating for 25 min, 0.1 M EDTA to a final concentration of 5 mM was added to terminate the reaction. The mixture was subjected to electrophoresis on 1.0% agarose gel at 70 V for 30 min. The DNA cleavage activity was viewed under UV light (254 nm) and measured by digitizing the photographic image and quantified using Bio-Rad Quantity one

version 2000. The degree of DNA cleavage activity was expressed in terms of the percentage of conversion of the scDNA to ocDNA according to the following equation:

$$\%\ \text{DNA cleavage activity} = \frac{[\%\ \text{of scDNA}]\text{control} - [\%\ \text{of scDNA}]\text{sample}}{[\%\text{of scDNA}]\text{control}} \times 100$$

2.6. Topoisomerase inhibitory assay

Saccharomyces cerevisiae mutant cell cultures, JN271-R52, JN271-R52 t_{-1}, JN271-R52 t_{2-5}, JN394 (top I and II positive), JN394 t_{-1} (top I positive) and JN394 t_{2-5} (top II positive) were supplied by Dr. John Nitiss of St. Jude Children's Research Hospital, Tennessee, USA for carrying out anticancer assay. The organisms were cultured in Petri dishes containing YPDA medium (20 mL). The cells from a fully grown plate of each organism were suspended in saline solution (10 mL) and diluted to obtain 1×10^8 CFU/mL. Fifty microliters of this suspension was used to inoculate Petri dishes containing the corresponding media (20 mL). Test compounds were dissolved in DMSO and added to the inoculated dishes (20 μL) at 5 μM concentration. The plates containing cell cultures and compounds were incubated at 27 °C for 48 h. At the end of the incubation period, the zones of inhibition were recorded for each cell suspension used. Solvent control, DMSO (20 μL), was used where necessary.

2.7. Cyclooxygenase inhibitory assay

The procedure was carried out as per the manufacturer's guidelines laid out in the COX inhibitory assay kit provided by Caymen Chemicals, USA.

2.8. Antibacterial and antifungal assays [22]

The agar diffusion method was followed for antibacterial and antifungal susceptibility tests. Petri plates were prepared by pouring 10 mL of Mueller Hinton Agar for bacteria and Sabouraud Dextrose Agar for fungus and allowed to solidify. Plates were dried and 0.1 mL of standardized inoculum suspension was poured and uniformly spread. The excess inoculum was drained and was allowed to dry for 5 min. The discs were then applied and the plates were incubated in BOD incubators at 37 °C for 24 h (bacteria) and at 28 °C for 48 h (fungus). The inhibition zone was measured from the edge of the disc to the inner margin of the surrounding pathogens. Each assay in this experiment was repeated thrice.

3. Results and discussion

3.1. Preparation and characterization of complexes

Synthesis of homo–hetero bimetallic Cu(II) complexes was achieved in two steps. The first step involved the synthesis of monometallic 2,2-bipyridyl, 1,10 phenanthroline-5,6-dione Cu(II) complex and the second step involved template synthesis of bimetallic complexes with *N*,*N*-dimethylethylenediamine and appropriate metal salt followed by the precipitation with PF6 anion (Fig. 1). The six synthesized complexes have been characterized spectroscopically using elemental analysis, FAB mass, IR spectroscopy, EPR, variable temperature magnetic studies and electrochemical studies. Data of the elemental analysis, FAB mass spectra, EPR spectra, IR spectra [23] and electronic absorption of all the six complexes have been reported in detail by Deepalatha et al. [24]. The FAB mass spectra and simulation of mass isotope peaks of the complexes confirm the fragmentation pattern expected from the proposed structure of the complexes. The analytical data of the complexes correlated to the molecular formula $CuC_{24}H_{22}N_6O_{10}$ (**I**, MW − 492.09), $Cu_2C_{32}H_{43}N_8O_3P_4F_{24}$ (**Ia**, MW − 713.21), $CuCoC_{32}H_{39}N_8OP_4F_{24}$ (**Ib**, MW − 673.19), $CuNiC_{32}H_{39}N_8OP_4F_{24}$ (**Ic**, MW − 672.19), $CuZnC_{36}H_{55}N_8O_5P_4F_{24}$ (**Id**, MW − 806.29) and $CuMnC_{34}H_{48}N_8O_4P_4F_{24}$ (**Ie**, MW − 750.25) (Table 1).

3.2. DNA interaction studies by fluorescence

The emission spectra of complex **I** in the presence of CT-DNA excited at 500 nm are shown in Fig. 2. Ethidium bromide (EB) is one of the most sensitive fluorescence probes that can bind with DNA. The fluorescence of free EB is very low and tends to increase after intercalating with DNA. If the metal

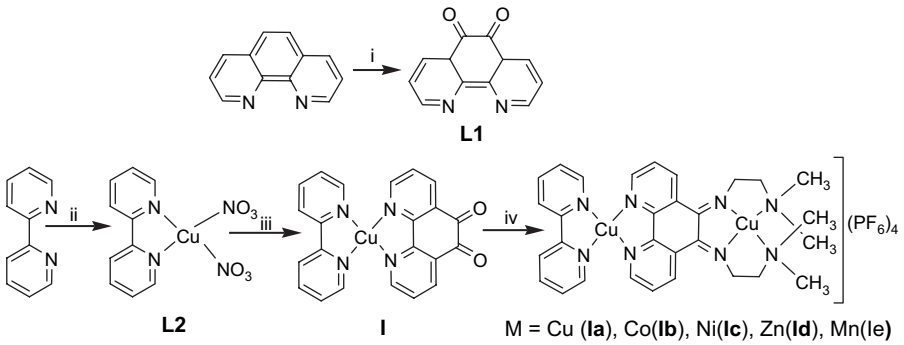

Fig. 1. Synthetic strategy of 2,2-bipyridyl based bimetallic complexes: (i) conc. H_2SO_4/HNO_3, KBr, at 0 °C; (ii) 1 equivalent of $Cu(NO_3)_2$, ethanol/water, stir for 2 h; (iii) **L1**/ethanol, water, stir for 4 h; (iv) 2 equivalents of *N*,*N*-dmen/metal ion/dry ethanol, reflux 24 h under dry conditions; 4.2 equivalents of KPF_6 is added.

Table 1
Elemental data of the complexes

Complexes	Found (calculated) %			
	C	H	N	Cu
I ($CuC_{24}H_{22}N_6O_{10}$)	46.50 (46.64)	3.50 (3.59)	13.56 (13.6)	10.18 (10.28)
Ia ($Cu_2C_{32}H_{43}N_8O_3P_4F_{24}$)	29.60 (29.69)	3.29 (3.35)	8.63 (8.65)	9.89 (9.82)
Ib ($CuCoC_{32}H_{39}N_8OP_4F_{24}$)	30.62 (30.65)	3.19 (3.13)	8.85 (8.94)	5.03 (5.07)
Ic ($CuNiC_{32}H_{39}N_8OP_4F_{24}$)	30.59 (30.65)	3.17 (3.14)	8.83 (8.94)	5.04 (5.07)
Id ($CuZnC_{36}H_{55}N_8O_5P_4F_{24}$)	31.12 (31.14)	3.89 (3.99)	8.01 (8.07)	4.52 (4.58)
Ie ($CuMnC_{34}H_{48}N_8O_4P_4F_{24}$)	30.60 (30.68)	3.59 (3.63)	8.34 (8.42)	4.72 (4.77)

Values given in the brackets are calculated for the given molecular formula.

complex competes and intercalates with DNA, it then leads to replacement of EB from DNA resulting in a decrease in the fluorescence intensity [25]. However, such observation was not seen in fluorescence emission spectrum of denatured DNA samples [26,27]. The slope of the plot of fluorescence intensity of free EB vs. concentration of EB in the presence of DNA was found to be 19.254×10^{-6} M, by fitting a least square fit with $R^2 = 0.98$.

The fluorescence intensity pattern of DNA–EB ($4 \times 10^{-6}\ M^{-1}$ and $3.3 \times 10^{-6}\ M^{-1}$) as a function of concentration of **I** was found to be $1–20 \times 10^{-6}$ M in 0.02 mol L^{-1} of phosphate buffer containing 60 mmol sodium chloride at pH 7.0 at 300 K (data not shown). The saturation binding constant was constructed by fitting nonlinear regression curve for unbound metal concentration against the specific binding. Among the various modes considered for binding of metal with DNA, the fluorescence intensity pattern was found to fit with the one site non-cooperative mode of binding. Uma Maheswari and Palaniandavar [27] have reported that $[Ru(NH_3)_4(diimine)]Cl_2$ showed a decrease in luminescence intensity in the presence of DNA, indicating that the excited state of these complexes acted as strong oxidizing agents that could oxidize guanine base.

Many workers [27,28] were of the opinion that the two major binding modes of interaction of substrate with DNA were (a) the intercalation: wherein the metal complexes squeeze in between the double helix through hydrogen bonding and (b) the covalent binding: where two major sites are available for the metal ion to interact with the DNA, one being the electron-donor groups of the bases, more preferably at the guanine N-7 and the other the phosphate moieties of the ribose–phosphate backbone. Saturation binding plot and Scatchard plot have enabled us to evaluate the required kinetic parameters. The binding constants and the number of binding sites for each of the Cu(II) complexes are shown in Table 2. Bi-copper system **Ia** showed lower binding constant than the corresponding mono-copper system. The lowering of binding constant is the direct consequence of the increased geometrical distortion around the two Cu(II) centers as compared to their corresponding parent mono-copper system [29,30].

In the case of Cu–Co (**Ib**) and Cu–Ni (**Ic**) complexes, the binding constants and binding sites were found to be 0.5669, $0.7336 \times 10^6\ M^{-1}$, and 0.1764, 0.1580, respectively. Even though the extent of geometrical distortion was found to be similar to that of bi-copper system, these complexes showed higher binding constants. This would be explained in terms of the tendency of the Co(II) and Ni(II) ions to have the axial coordination [31]. A possible mode of binding could be the covalent binding of Co(II) with the phosphate groups and the nitrogen donors of the purines, which might facilitate the uncoiling of double strand thus decreasing the DNA–EB fluorescence intensity. Muller et al. [32] and Chen et al. [33] have shown that positively charged mononuclear Ni(II) complexes could promote efficient cleavage of DNA with a pronounced preference for accessible guanines. However, as far as the Ni(II) ion binding details were concerned, it should be pointed out that numerous studies on mononucleotides [34,35] have revealed that Ni(II) ion preferentially coordinated to N-7 nitrogen of purines. Since, N-7 sites of both adenine and guanine remain exposed it is possible that besides the intercalation of aromatic groups attached with Cu(II), additionally the Ni(II) ions might also bind with guanine N-7 resulting in higher binding constant. The binding constants for the complexes, **Id** (Cu–Zn) and **Ie** (Cu–Mn), were found (Table 2) to be in the order of $10^5\ M^{-1}$, an order lower than that observed for other mononuclear complexes, possibly due to coordination kinetics or inherent electronic behavior of d^{10} and d^5 systems [34].

3.3. DNA cleavage activity

It is well documented [36,37] that there are specific chemical compounds that can specifically recognize and cut DNA. The potential scope of utility of these compounds is enormous and range from the creation of synthetic restriction enzymes for use by molecular biologists to the development of chemotherapeutic agents that might be effective against a variety of diseases including cancer.

Derivatives of diazonium salts [36], NCS aglycon [38], (aqua)bis-(dipyridoquinoxaline) and copper(II) [37] have been shown to cleave supercoiled DNA from different bacteria. More appropriately both the naturally occurring DNA cleavage agents and man-made compounds that can specifically recognize and cut DNA have been termed as chemical nucleases [39]. Since DNA cleavage is one of the important mechanism to arrest the growth of bacteria and viruses, in the control of diseases particularly cancer, there is considerable current interest in the development of reagents suitable for cleaving DNA. Further, most often the natural products prove to be toxic to the cells and hence great efforts are

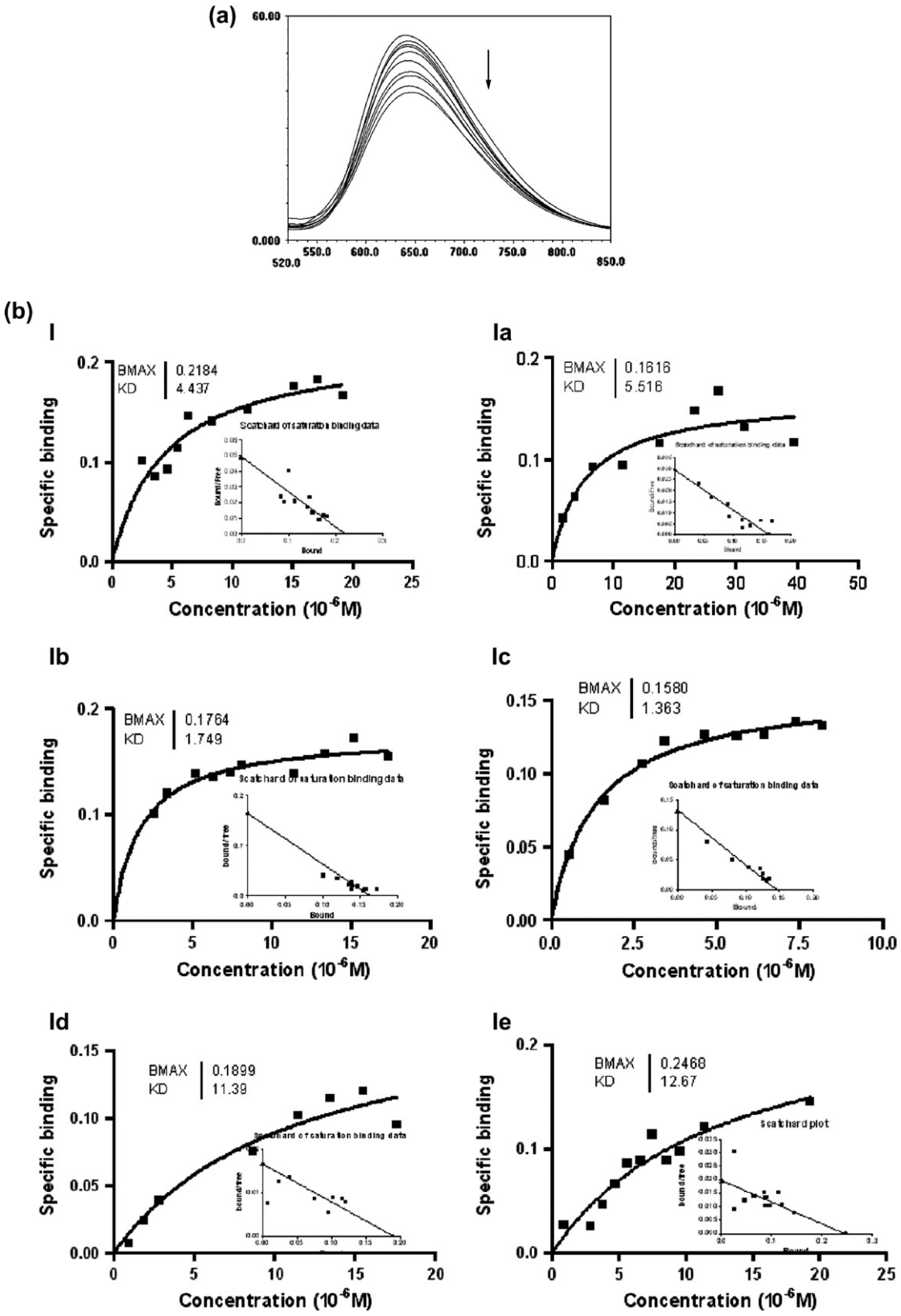

Fig. 2. (a) Fluorescence emission spectra of ethidium bromide in the presence of DNA and complex **I**, excited at 500 nm. $[EB] = 3.3 \times 10^{-6}$ M, $[DNA] = 4 \times 10^{-6}$ M, $[I] = 1{-}20 \times 10^{-6}$ M in 0.02 mol L^{-1} of phosphate buffer containing 60 mmol sodium chloride at pH 7.0 at 300 K. (b) Saturation binding graph and Scatchard plot for the complexes, B_{max} represents the number of binding sites, KD represents the inverse of complex to DNA binding constant, $\lambda_{ex} = 500$ nm, $\lambda_{em} = 640$ nm, $[EB] = 3.3 \times 10^{-6}$ M, $[DNA] = 4 \times 10^{-6}$ M, $[complex] = 1{-}20 \times 10^{-6}$ M in 0.02 mol L^{-1} of phosphate buffer containing 60 mmol sodium chloride at pH 7.0 at 300 K.

Table 2
Kinetic data of binding of metal complexes with calf thymus-DNA from fluorescence emission data at $\lambda_{ex} = 500$ nm, $\lambda_{em} = 640$ nm, [EB] = 3.3×10^{-6} M, [DNA] = 4×10^{-6} M, [complex] = $1-20 \times 10^{-6}$ M in 0.02 mol L^{-1} of phosphate buffer containing 60 mmol sodium chloride at pH 7.0 at 300 K

Complexes	No. of binding sites (B_{max})	K_a (10^6 M^{-1})
I	0.2184	0.2254
Ia	0.1616	0.1813
Ib	0.1764	0.5669
Ic	0.1580	0.7336
Id	0.1899	0.0878
Ie	0.2468	0.0789

directed towards the design of synthetic analogs capable of cleaving DNA in a similar manner without exhibiting the associated toxicity. As a sequel to find out whether any of these synthesized compounds would exhibit DNA cleavage activities in vitro, their effect at concentrations of 10 μM, 2 μM, and 0.1 μM was studied using pBS (SK−) DNA and the results are shown in Figs. 3 and 4. While at 10 μM concentration, the percent DNA cleavage activity occurred as evidenced by the total disappearance of DNA (lanes − 2, 5, 8, 11, 14, 17), at lower concentrations of 2 μM and 0.1 μM, no DNA cleavage activity was observed (lanes − 3, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18, 19). However, as to the optimization of conditions like substrate concentration, concentration of the compound, temperature, period of incubation, pH, etc., more detailed experiments are required to be carried out. Though many workers have reported several mechanisms for action of DNA cleavage such as (a) photolytic formation of phenyl cations [40], (b) alkylation of the purines [36], and (c) hydrolytic cleavage of double-stranded DNA involving a phospho−diester bond [37] and abstraction of a C-5′ hydrogen from thymidylate and adenylate residues [38], the mechanism of action of Cu(II) complexes on DNA cleavage is underway.

The ability of complex **Ia** to cleave pBS (SK−) at 10 μM, 2 μM, and 0.1 μM concentrations is shown in Figs. 3 and 4. As has been observed in the monomer **I**, at the highest concentration of 10 μM employed in present study, complete disappearance of pBS (SK−) DNA was observed (lane 5). A decrease in intensity of pBS (SK−) DNA (lane 6) is a reflection of the mild DNA cleavage activity of the compound. It is not known whether the period of incubation is a rate-limiting factor. In respect to compounds **Ib**, **Ic**, **Id** and **Ie**, similar patterns of DNA cleavage were seen at different concentrations tested (lanes 8−19), showing cent percent cleavage at the highest

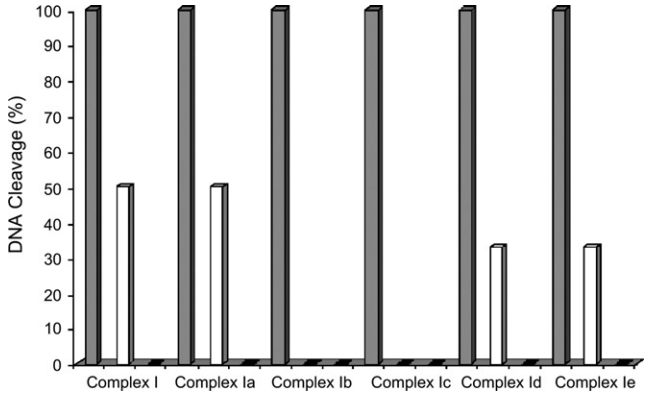

Fig. 4. Percent cleavage of pBS (SK−) DNA in the presence of different Cu(II) complexes at 10 μM (■), 5 μM (▨) and 0.1 μM (□) concentrations.

concentration of 10 μM. However, at lower concentration of 2 μM, **I** and **Ia** alone showed partial DNA cleavage activity.

The decrease in luminescence intensity in the presence of DNA by complex **I** employed in the present study could be due to DNA cleavage activity besides oxidation of guanine base. Thus the mono- and bi-copper complexes (**I**, **Ia**) are promising candidates as chemical nucleases to cleave DNA with far reaching consequences.

3.4. Cyclooxygenase enzymes (COX-I and COX-II) inhibitory assay

Cyclooxygenase (COX) or prostaglandin endoperoxide H synthase (PGHS) enzymes are used widely to measure the anti-inflammatory effects of natural products and are the pharmacological target site for non-steroidal anti-inflammatory drug (NSAID) discovery [41]. The formation of prostaglandins from arachidonic acid by prostaglandin synthase is a well-studied process and the effects of the prostaglandins can result in the stimulation of inflammation and associated pain [41,42]. Two isozymes involved in the conversion of arachidonic acid to prostaglandins are COX-I and COX-II [41,42].

IC_{50}s for inhibition of the COX-1 and COX-2 by different complexes are shown in Fig. 5. While complexes **Ib** and **Ic** were more inhibitory to COX-1, complexes **I**, **Ia**, **Id**, and **Ie** were more inhibitory to COX-2. The rankings shown in Table 3 are reflections of relative selectivity for inhibition of COX-1 or COX-2 by the 2,2-bipyridyl based bimetallic complexes. A

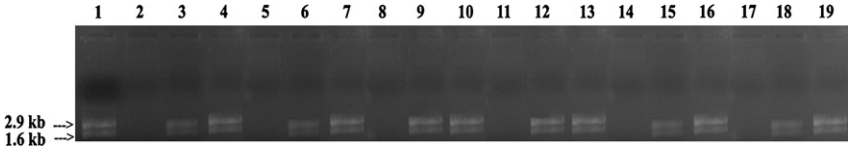

Fig. 3. Agarose gel electrophoresis showing the cleavage of pBS (SK−) DNA (1 μg) by ligand complexes and 0.1 M phosphate buffer and NaCl. Lane 1: control DNA, lane 2: DNA + 10 μM complex **I**, lane 3: DNA + 2 μM complex **I**, lane 4: DNA + 0.1 μM complex **I**, lane 5: DNA + 10 μM complex **Ia**, lane 6: DNA + 2 μM complex **Ia**, lane 7: DNA + 0.1 μM complex **Ia**, lane 8: DNA + 10 μM complex **Ib**, lane 9: DNA + 2 μM complex **Ib**, lane 10: DNA + 0.1 μM complex **Ib**, lane 11: DNA + 10 μM complex **Ic**, lane 12: DNA + 2 μM complex **Ic**, lane 13: DNA + 0.1 μM complex **Ic**, lane 14: DNA + 10 μM complex **Id**, lane 15: +2 μM complex **Id**, lane 16: DNA + 0.1 μM complex **Id**, lane 17: DNA + 10 μM complex **Ie**, lane 18: DNA + 2 μM complex **Ie**, and lane 19: DNA + 0.1 μM complex **Ie**.

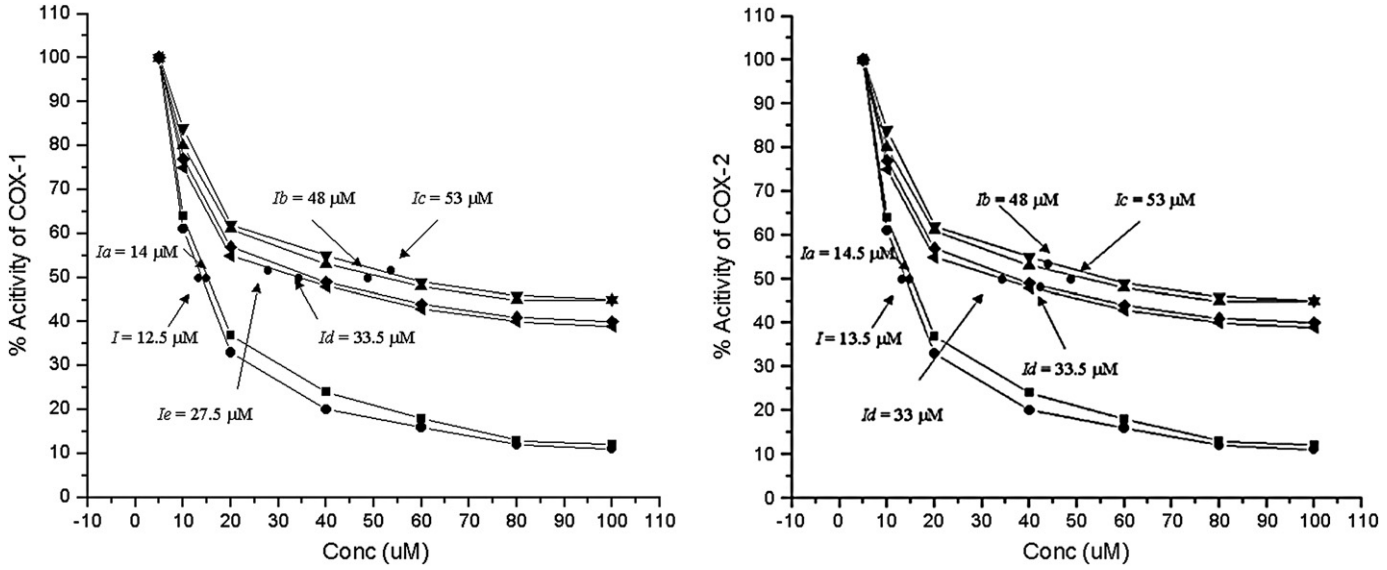

Fig. 5. COX-I and COX-II inhibitory assay by 2,2-bipyridyl based bimetallic complexes. The activities of COX-1 and COX-2 enzymes in the presence of different Cu(II) complexes at various concentrations were estimated. -■- **Ia**, -●- **I**, -▲- **Ib**, -▼- **Ic**, -◆- **Id**, -◄- **Ie**. The IC_{50}s are shown numerically.

ratio of concentration of IC_{50} of COX-1 to IC_{50} of COX-2 close to 1 indicated nearly equal selectivity [41,42]. Employing indomethacin and their analogs, [42], reported varying inhibitions of COX-1 and COX-2 activities suggesting that removing chlorine from the benzene ring or changing its position from *para*, or replacing it with another halogen, affected the ability of the compound to inhibit COX-2.

Likewise in our studies all the analogs that have been developed did not exhibit any marked variation in their inhibition to COX-1 and COX-2. To the best of our knowledge this is the first report wherein the inhibition of COX-1 and COX-2 activities by 2,2-bipyridyl based bimetallic complexes is shown. Apparently the complex **Ia** containing the bi-copper and complex **I** containing mono-copper without the association of other metals prove to be more toxic to cyclooxygenases. In other words the question that remains to be answered is: did the addition of other metals either singly or in combination with **L1** lead to reduction in inhibition of cyclooxygenases? Though contradictory reports on the usefulness of COX-2 inhibition have been published [41,42], the fact that the 2,2-bipyridyl based bimetallic complexes **I**, **Ia**, **Id** and **Ie** selectively inhibited COX-2 activity is encouraging and hold much promise for their use as effective anti-inflammatory agents.

Table 3
Ratio of concentration of complex (IC_{50}) that inhibited 50% of cyclooxygenase-1 (COX-1) activity to the IC_{50} for COX-2 activity

Rank	Complexes	Ratio[a]
1	**Ib** ($CuCoC_{32}H_{39}N_8OP_4F_{24}$)	1.171
2	**Ic** ($CuNiC_{32}H_{39}N_8OP_4F_{24}$)	1.152
3	**Ia** ($Cu_2C_{32}H_{43}N_8O_3P_4F_{24}$)	0.966
4	**I** ($CuC_{24}H_{22}N_6O_{10}$)	0.926
5	**Id** ($CuZnC_{36}H_{55}N_8O_5P_4F_{24}$)	0.838
6	**Ie** ($CuMnC_{34}H_{48}N_8O_4P_4F_{24}$)	0.833

[a] Ratio > 1 indicates that a drug is more COX-1 selective; ratio < 1 indicates that a drug is more COX-2 selective. Ratio represents values obtained for the concentration of complexes (IC_{50}) that inhibited 50% of COX-1 and COX-2 activities.

3.5. Topoisomerase inhibitory assay

DNA topoisomerases are important targets of anticancer chemotherapeutic agents. These enzymes are involved in and are essential for the completion of DNA metabolic activities in cells including replication, transcription and recombination. The enzymes are divided into two classes, namely, type I (topoisomerase I) and type II (topoisomerase II) enzymes. These enzymes catalyze the breakage and rejoining of DNA strands in order to interconvert different topological forms of DNA [43,44].

The topoisomerase inhibitory assay was carried out as per the guidelines stipulated by the manufacturer. The basis for the assay lies in the ability of anticancer compounds to inhibit or poison the activities of topoisomerase I (top I) and topoisomerase II (top II). Cancer cells have been shown to possess elevated levels of top I and top II enzymes [43,44]. Therefore, inhibition of these enzymes would be expected to lead to a decrease in cell proliferation. Extensive reviews on the role of DNA topoisomerases in human as well as in yeast have been published [43,44]. The catalytic activity of DNA topoisomerases by their interference with different cellular processes has also been reviewed [45,46]. It is well known that wild type laboratory yeast strains are not sensitive to most DNA topoisomerase targeted drugs [44,47]. Since many of these drugs convert DNA topoisomerases into cellular poisons, assessing the cytotoxic activity of these agents is dependent upon achieving sufficient intracellular concentrations to interfere with enzyme

activity. However, this initial impediment by using yeast as a model system to establish the mechanism of drug action was overcome by developing drug-sensitive yeast strains, either by manipulating drug permeability or by increasing the level of enzyme activity [44] and yeast cells are preferred to bacterial cells to study the activity of anti-topoisomerase drugs due to their closer genetic and biochemical resemblance to mammalian cells. Inhibition assay of top I and top II by different complexes in mutant yeast strains (JN394, JN394 t_{-1} and JN394 t_{2-5}) known to contain either or both the enzymes is shown in Table 4. Interestingly, there existed no marked difference in the inhibition of topoisomerase activity by any of the complexes tested.

3.6. Anti-microbial activity

A plethora of information on the antibacterial and antifungal activities of natural products obtained from seeds [22], roots [48], plant parts [49], marine bacteria [50], marine animals [51], etc. are available. However, due to complexity in the isolation, identification and synthesis of the natural products, great attention is directed towards synthesis of synthetic compounds active against a wide variety of bacteria and fungi that cause diseases in humans and animals. In this direction, it was felt necessary to test whether any of the synthesized bimetallic complexes (**I**–**Ie**) would prove to be effective in controlling the growth of a few important bacteria and *C. albicans* and the results are shown in Table 5. Surprisingly all the complexes at the conc. of 5 μM did inhibit the growth of Gram-positive and Gram-negative bacteria besides *C. albicans*. It is not known as to why invariably all the complexes did show lesser inhibition with the exception of complex **Ic** against *K. pneumoniae*. As suggested by Chandrasekaran and Venkatesalu [22] this could be due to the permeability of the complexes or resistance mechanism displayed by *K. pneumoniae* against these complexes. However, one interesting aspect that has emerged from our studies is that all the synthesized bimetallic complexes definitely possess anti-microbial activity.

4. Conclusions

Synthesis of six new bimetallic complexes of the type CuCu, CuCo, CuNi, CuZn and CuMn was achieved and the structure is proposed based on various spectroscopical and electro analytical techniques. All the complexes effectively bind to DNA at a concentration ranging from 1×10^{-6} M to 20×10^{-6} M. The enhanced binding capacity of Co and Ni complexes is attributed to their axial binding abilities to purines. The results clearly indicated that among the six 2,2-bipyridyl bimetallic copper(II) complexes, the homo–hetero bimetallic complexes **I**, **Ia** at a concentration of 0.1 μM also partially cleaved the DNA of pBS (SK−) in vitro. Complexes **I** and **Ia** selectively inhibited COX-2 which is a pro-inflammatory marker. Similarly complexes **I** and **Ia** also inhibited both top I and top II enzyme activities at a concentration as low as 5 μM. At a concentration of 5 μM, complexes **I** and **Ia** inhibited the growth of Gram-positive bacteria, Gram-negative bacteria and *C. albicans*. Complexes **I** and **Ia** based on the results of our study hold much promise in the drug discovery program as a potential lead molecule for further cell line studies and its potential use as an anti-inflammatory and anti-microbial candidate molecule.

Table 4
Inhibition[a] of topoisomerase activity by different bimetallic compounds

Cultures (1×10^8 CFU/mL)	Ia	Ib	Ic	Id	Ie	I
Saccharomyces cerevisiae strain 394	13.7	10.1	9.6	12.8	11.8	15.5
S. cerevisiae strain 394 t_{-1}	13.5	10.2	9.2	12.7	11.5	15.4
S. cerevisiae strain 394 t_{2-5}	13.8	10.0	9.8	12.9	11.9	15.7

[a] Values are mean diameter of inhibition zone (mm) and an average of triplicate. **I** [$CuC_{24}H_{22}N_6O_{10}$], **Ia** [$Cu_2C_{32}H_{43}N_8O_3P_4F_{24}$], **Ib** [$CuCoC_{32}H_{39}N_8OP_4F_{24}$], **Ic** [$CuNiC_{32}H_{39}N_8OP_4F_{24}$], **Id** [$CuZnC_{36}H_{55}N_8O_5P_4F_{24}$], and **Ie** [$CuMnC_{34}H_{48}N_8O_4P_4F_{24}$] each at a concentration of 5 μM.

Table 5
Antibacterial and antifungal activities of synthesized bimetallic complexes

Microorganisms	Mean zone of inhibition (mm)[a]						
	I	**Ia**	**Ib**	**Ic**	**Id**	**Ie**	Stds[b]
Gram-positive bacteria							
Bacillus subtilis	25.1	23.7	15.3	12.5	13.1	13.6	23.0
Micrococcus	22.1	20.8	15.2	11.1	13.7	13.5	24.0
Staphylococcus aureus	28.1	25.0	14.0	13.5	16.2	15.3	27.0
Gram-negative bacteria							
Klebsiella pneumoniae	0	3.5	7.2	12.0	10.5	7.4	32.3
Pseudomonas aeruginosa	22.3	18.5	12.2	12.0	10.5	11.4	33.0
Proteus mirabilis	19.7	17.9	11.3	10.9	10.3	15.5	33.0
Shigella flexneri	26.1	23.7	14.5	13.8	13.2	12.5	27.0
Salmonella typhi	25.4	21.1	16.3	13.2	13.7	15.5	25.0
Fungi							
Candida albicans	22.3	17.1	14.4	13.5	12.3	11.0	39.0

[a] Values are mean diameter of inhibition zone (mm) and an average of triplicate.
[b] Standards: antibacterial studies – ciprofloxacin – 5 μg; antifungal studies – cotrimazole – 100 μg. **I** [$CuC_{24}H_{22}N_6O_{10}$], **Ia** [$Cu_2C_{32}H_{43}N_8O_3P_4F_{24}$], **Ib** [$CuCoC_{32}H_{39}N_8OP_4F_{24}$], **Ic** [$CuNiC_{32}H_{39}N_8OP_4F_{24}$], **Id** [$CuZnC_{36}H_{55}N_8O_5P_4F_{24}$], and **Ie** [$CuMnC_{34}H_{48}N_8O_4P_4F_{24}$] each at a concentration of 5 μM.

Acknowledgement

The authors (K. R. R, S. D., M. K) are grateful to the University Grants Commission, New Delhi, India for the award of fellowship. Dr. S. J. is thankful to the Department of Biotechnology, Ministry of Science and Technology, Govt. of India, New Delhi, India (BTA/PR4249/AAQ/03/214/2003) for funding the research project.

References

[1] S. Chan, W.T. Wong, Coord. Chem. Rev. 138 (1995) 219.
[2] K.E. Erkkila, D.T. Odom, J.K. Barton, Chem. Rev. 99 (1999) 2777.
[3] T. Gunnlaugsson, M. Nieuwenhuyzen, C. Nolana, Polyhedron 22 (2003) 3231.
[4] C.V. Kumar, W.B. Tan, P.W. Betts, J. Inorg. Biochem. 68 (1997) 177.
[5] S.J. Franklin, J.K. Barton, Biochemistry 37 (1998) 16093.

[6] C.A. Hastings, J.K. Barton, Biochemistry 38 (1999) 10042.
[7] M.J. Clarke, F. Zhu, D.R. Frasca, Chem. Rev. 99 (1999) 2511.
[8] Z. Afrasiabi, E. Sinn, S. Padhye, S. Dutta, S. Padhye, C. Newton, C.E. Anson, A.K. Powell, J. Inorg. Biochem. 95 (2003) 306.
[9] G.J. Brewer, Curr. Opin. Chem. Biol. 7 (2003) 207.
[10] D.S. Sigman, D.R. Graham, V.D. Aurora, A.M. Stern, J. Biol. Chem. 254 (1979) 12269.
[11] D.S. Sigman, Acc. Chem. Res. 19 (1986) 180.
[12] D.S. Sigman, A. Mazumder, D.M. Perin, Chem. Rev. 93 (1993) 2295.
[13] J.K. Barton, A.C. Raphael, J. Am. Chem. Soc. 106 (1984) 2466.
[14] J.K. Barton, Science 233 (1986) 727.
[15] S. Delaney, M. Pascaly, P.K. Bhattacharya, K. Han, J.K. Barton, Inorg. Chem. 41 (2002) 1966.
[16] M. Pyle, J.K. Barton, Prog. Inorg. Chem. 38 (1990) 413.
[17] G.F. Smith, F.W. Cargle Jr., J. Org. Chem. 12 (1947) 781.
[18] C.V. Kumar, H. Emma, J. Chem. Soc., Chem. Commun. (1992) 470.
[19] E. Nyarko, N. Hanada, A. Habib, M. Tabata, Inorg. Chim. Acta. 357 (2004) 739.
[20] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.
[21] J. Yang, Y.J. Wong, M.S. Yang, Chem. Biol. Interact. 125 (2000) 221.
[22] M. Chandrasekaran, V. Venkatesalu, J. Ethnopharmacol. 91 (1) (2004) 105.
[23] A.B.P. Lever, Inorganic Electronic Spectroscopy, second ed. Elsevier Science Publishers B.V., 1984.
[24] S. Deepalatha, P. Sambasiva Rao, R. Venkatesan, Spectrochim. Acta A Mol. Biomol. Spectros. 64 (4) (2006) 823.
[25] P. Kopel, Z. Travnicek, J. Marek, M. Korabik, J. Mrozinski, Polyhedron 22 (2003) 411.
[26] V.G. Vaidyanathan, B.U. Nair, Eur. J. Inorg. Chem. (2003) 3633.
[27] P. Uma Maheswari, M. Palaniandavar, Inorg. Chim. Acta. (2004) 901.
[28] D. Wettig, D.O. Wood, J.S. Lee, J. Inorg. Biochem. 94 (2003) 94.
[29] L.A. Lipscomb, F.X. Zhou, S.R. Presnell, R.J. Woo, M.E. Peek, R.R. Plaskon, L.D. Williams, Biochemistry 35 (1996) 2818.
[30] C. Liu, J. Zhou, Q. Li, L. Wang, Z. Liao, H. Xu, J. Inorg. Biochem. 75 (1999) 233.
[31] B.K. Keppler (Ed.), Metal Complexes in Cancer Chemotherapy, VCH, Weinheim, 1993.
[32] J.G. Muller, X. Chen, A.C. Dadiz, S.E. Rokita, C.J. Burrows, J. Am. Chem. Soc. 114 (1992) 6407.
[33] X. Chen, S.A. Woodson, C.J. Burrows, S.E. Rokita, Biochemistry 32 (1993) 7610.
[34] C.V. Sastri, D. Eswaramoorthy, L. Giribabu, B.G. Maiya, J. Inorg. Biochem. 94 (2003) 138.
[35] D. Kong, J. Reibenspies, J. Mao, A. Clearfield, A.E. Martell, Inorg. Chim. Acta. 342 (2003) 158.
[36] M. Kizil, E.I. Yilmaz, N. Pirinccioglu, C. Aytekin, Turk. J. Chem. 27 (2003) 539.
[37] S. Dhar, P.A.N. Reddy, A.R. Chakravarty, Dalton Trans. (2004) 697–698.
[38] A.G. Myers, M.E. Kort, M. Hammond, J. Am. Chem. Soc. 119 (13) (1997) 2967.
[39] A. Chin, M.H. Hung, L.M. Stock, J. Org. Chem. 46 (1981) 2203.
[40] J.R. Hwu, C.C. Lin, S.H. Chuang, K.Y. King, T. Su, S. Tsay, Bioorg. Med. Chem. 12 (2004) 2509.
[41] B. Cryer, M. Feldman, Am. J. Med. 104 (1998) 413.
[42] S. Touhey, R.O. Connor, S. Plunkett, A. Maguire, M. Clynes, Eur. J. Cancer 38 (12) (2002) 1661.
[43] B.V. Hille, B.T. Hill, Cancer Chemother. Pharmacol. 42 (1998) 345.
[44] R.J.D. Reid, P. Benedetti, M. Bjornsti, Biochim. Biophys. Acta 1400 (1998) 289.
[45] A. Chen, L.F. Liu, Annu. Rev. Pharmacol. Toxicol. 34 (1994) 191.
[46] S.J. Froelich-Ammon, N. Osheroff, J. Biol. Chem. 270 (1995) 21429.
[47] J. Nitiss, J.C. Wang, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 7501.
[48] M.J. Dobner, S. Schwaiger, I.H. Jenewein, H. Stuppner, J. Ethnopharmacol. 89 (2003) 301.
[49] H. Baydar, O. Sagdic, G. Ozkan, T. Karadogan, Food Control 15 (2004) 169.
[50] D.J. Faulkner, Marine Natural Products, Natural Products Reports, vol. 19, 2002, p. 1.
[51] Y. Venkateswarlu, M.A.F. Biabani, M.V. Reddy, R. Chavakula, J.V. Rao, J. Nat. Prod. 57 (1994) 827.