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Synthesis, spectral investigation and biological interphase of drug-based cytotoxic square pyramidal coordination compounds

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We have synthesized ciprofloxacin-based metal complexes of bipyridine derivatives $[Cu(CFL)(A^n)Cl].2H_2O$ (where CFL = ciprofloxacin and A = bipyridines e.g. A^1 = 4-(4-fluorophenyl)-6-*p*-tolyl-2,2′-bipyridine, A^6 = 4-(4-(benzyloxy)phenyl)-6-(4-bromophenyl)-2,2′-bipyridine, etc.). The ligands and complexes were characterized using analytical (C, H, N elemental analysis, TGA and magnetic measurement) and spectroscopic methods (^{1}H and ^{13}C NMR, FT-IR, fast atom bombardment mass and reflectance spectroscopy). The products were evaluated by screening for DNA interaction activity on herring sperm DNA and studies suggest intercalative mode of DNA binding. The antimicrobial activity was determined in terms of minimum inhibitory concentration. Superoxide dismutase mimic studies were performed using the NADH/PMS/NBT system. The brine shrimp bioassay was also carried out to study the *in vitro* cytotoxic properties of the synthesized metal complexes. Copyright © 2012 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: cytotoxicity; brine shrimp assay; metallonucleases; spectral characterization

Introduction

Substantial progress has been made during the past few decades to develop metal-based small molecules as DNA footprinting as well as therapeutic agents capable of binding and cleaving DNA under physiological conditions.[1,2] Metal complexes, in this context, with their coordination environments and versatile physicochemical properties offer scope for designing and developing highly sensitive diagnostic agents for medicinal applications, as exemplified by chemotherapeutic agents such as cisplatin and bleomycins.[3] Among the metal complexes so far investigated, those containing polypyridyl ligands have attracted much attention by virtue of their propensity to bind nucleic acid under physiological conditions.[4,5]

Synthetic nucleases have important potential applications, ranging from the synthesis of custom-designed artificial restriction enzymes to the development of new antitumor agents.[6,7] Sigman and co-workers developed the first chemical nuclease $[Cu(phen)_2]$ that effectively cleaves B-DNA in the minor groove in the presence of a reducing agent and also exhibits antiviral activity, which inhibits proviral DNA synthesis.[8] In continuation of earlier work,[9] our experimental studies provide information regarding nuclease behaviour of synthesized metal complexes. Various biological parameters (DNA interaction behaviour, cytotoxic assessment) were evaluated for known efficient nuclease behaviour.

Experimental

Materials

All the chemicals and solvents were of reagent grade and used as purchased. Ciprofloxacin hydrochloride was purchased from Bayer AG (Wuppertal, Germany). Cupric chloride dihydrate, *p*-bromoacetophenone, *p*-methylacetophenone, *m*-chlorobenzaldehyde, *m*-bromobenzaldehyde, *p*-fluorobenzaldehyde, *p*-benzyloxybenzaldehyde, acetic acid, ethylenediaminetetraacetic acid (EDTA) and herring sperm DNA (HS DNA) were purchased from Sigma Chemical Co. (India). Ethidium bromide and Luria broth (LB) were purchased from Himedia (India). All reactions were performed in air and chemicals were used as received.

Instrumentation

FT-IR Spectroscopy

FT-IR spectra were recorded on a Shimadzu FTIR-8400S spectrometer (Shimadzu Scientific Instruments, Japan). Spectroscopy IR grade of KBr (S D Fine Chem Ltd, India) were ground with 1.0 wt% of the sample to be analysed. The FT-IR sample chamber was flushed continuously with N_2 prior to data acquisition in the range 4000–400 cm^{-1}.

NMR Analysis

All ^{1}H and ^{13}C NMR were performed in $CDCl_3$ and recorded on a Bruker Avance 400 spectrometer (Germany). ^{1}H NMR spectra

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were collected at 400 MHz using a 8300 Hz spectral width, a relaxation delay of 1.0 s, a pulse width of 90° (12.50 μs), 65 k data points, and $CDCl_3$ (7.27 ppm) as an internal reference. ^{13}C NMR spectra were collected at 100 MHz using a 24 000 Hz spectral width, a relaxation delay of 2.0 s, 65 k data points, a pulse width of 30° (7.5 μs), and $CDCl_3$ (77.23 ppm) as the internal reference.

Fast Atomic Bombardment Mass Spectroscopy (FAB MS)

FAB MS was performed on a Jeol SX 102/Da-600 (Jeol Ltd, India) mass spectrophotometer/data system using argon/xenon (6 kV, 10 mA) as the FAB gas and *m*-nitrobenzyl alcohol as the matrix. The accelerating voltage was 10 kV and spectra were recorded at room temperature.

Reflectance Spectroscopy

Reflectance spectra were recorded on a PerkinElmer Lambda 19 UV/VIS/NIR spectrophotometer (USA) using a slit width of 5.0 nm, smooth bandwidth of 8.0 nm, scan speed of 240.0 nm min^{-1}, and data interval of 1.0 nm.

UV–Visible Spectroscopy

Electronic spectra were recorded on a Shimadzu UV-160A UV–visible spectrophotometer (Shimadzu Scientific Instruments, Japan). Thermal DNA denaturation was performed using an Agilent 8453 UV-Visible spectrophotometer (CA, USA).

Thermogravimetric Analysis (TGA)

Thermal stabilities of compounds under N_2 were examined using a 5000/2960 TGA instrument (New Castle, DE, USA). Samples (5–10 mg) were loaded in alumina pans and ramped to 800°C while heating at 10°C min^{-1}. The N_2 or air flow rate was 60 ml min^{-1}.

Elemental Analysis

C, H and N elemental analyses were performed with a model 240 PerkinElmer elemental analyser.

Metal Estimation

Metal contents of the compounds were analysed gravimetrically and volumetrically[10] after decomposing the organic matter with an acid mixture ($HClO_4$, H_2SO_4, and HNO_3; 1:1.5:2.5).

Magnetic Measurement

Magnetic moments were measured by Gouy's method using mercury tetrathiocyanatocobaltate(II) as the calibrant ($\chi_g = 16.44 \times 10^{-6}$ cgs units at 20°C), with a Citizen balance (India).

Synthesis of Ligands

Ligands A^{1-8} were prepared by reacting the appropriate enone with a respective pyridinium iodide salt.[11] The following procedure is for a typical preparation of ligands, along with their ^1H NMR and ^{13}C NMR assignments as well as analytical data.

4-(4-Fluorophenyl)-6-p-tolyl-2,2′-bipyridine (A^1)

An excess of ammonium acetate (approximately 10 equiv.) was added to a mixture of 3-(4-fluorophenyl)-1-*p*-tolylprop-2-en-1-one (1.5 mmol) and 1-[2-oxo-2-(pyridyl)ethyl]pyridinium iodide (1.5 mmol) in methanol (20 ml) in a 100 ml round-bottom flask. After refluxing with stirring for 5–7 h in air, the reaction mixture was allowed to cool, which led to the formation of greenish-yellow needles of pure product. The solid was filtered off, washed with cold methanol, and dried under vacuum. Yield 50 ± 2%, m.p. 149°C. Anal. Calc. for $C_{23}H_{17}FN_2$ (340.39): C, 81.16; H, 5.03; N, 8.23%. Found: C, 81.29; H, 5.17; N, 8.04%. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.600–8.592 (complex, 1H, $H_{3'}$), 8.412 (d, 1H, $H_{6'}$, $J = 8$ Hz), 8.172–8.162 (complex, 2H, $H_{3,4'}$), 7.840 (d, 2H, $H_{2''',6'''}$, $J = 8$ Hz), 7.796–7.787 (complex, 1H, H_5), 7.675 (d, 2H, $H_{2'',6''}$, $J = 8$ Hz), 7.536 (t, 1H, $H_{5'}$, $J = 5.6$ Hz), 7.442 (d, 2H, $H_{3''',5'''}$, $J = 8$ Hz),7.394 (t, 2H, $H_{3'',5''}$, $J = 6.4$ Hz), 2.321 (s, 3H, Me). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 166.47 ($C_{4''}$), 152.61 (C_2), 151.62 ($C_{2'}$), 150.23 (C_6), 149.22 (C_4), 148.86 ($C_{6'}$), 146.01 ($C_{4'}$), 143.33 ($C_{1''}$), 139.75 ($C_{1'''}$), 135.26 ($C_{4'''}$), 131.27 ($C_{2'',6''}$), 129.30 ($C_{3''',5'''}$), 126.77 ($C_{2''',6'''}$), 125.09 ($C_{3'',5''}$), 124.10 ($C_{5'}$), 122.63 ($C_{3'}$), 115.43 (C_3), 114.81 (C_5), 20.48 (Me).

4-(4-Benzyloxy)phenyl)-6-p-tolyl-2,2′-bipyridine (A^2)

A similar procedure was followed using 3-(4-(benzyloxy)phenyl)-1-*p*-tolylprop-2-en-1-one as the enone. Yield 55 ± 2%, m.p. 151°C. Anal. Calc. for $C_{30}H_{24}N_2O$ (428.52): C, 84.08; H, 5.65; N, 6.54%. Found: C, 83.91; H, 5.76; N, 6.70%. ^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.836 (complex, 3H, $H_{3,3',6'}$), 8.275 (dd, 2H, $H_{2'',6'''}$, $J = 6.4$ and 0.8 Hz), 8.050 (complex, 2H, $H_{5,4'}$), 7.902–7.884 (complex, 2H, $H_{2'',6''}$), 7.626 (dd, 2H, $H_{3''',5'''}$, $J = 3.6$ and 1.2 Hz), 7.528–7.474 (complex, 6H, $H_{5',Bz2,3,4,5,6}$), 7.117 (d, 2H, $H_{3'',5''}$, $J = 6.4$ Hz), 5.343 (s, 2H, OCH_2), 2.262 (s, 3H, Me). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 159.55 ($C_{4''}$), 157.73 (C_2), 155.96 ($C_{2'}$), 153.04 (C_6), 151.70 (C_4), 148.02 ($C_{6'}$), 137.63 ($C_{1'''}$), 136.39 ($C_{4'}$), 135.24 (C_{Bz1}), 133.45 ($C_{1''}$), 129.29 ($C_{4'''}$), 128.01 ($C_{2'',6''}$), 127.21 ($C_{3''',5'''}$), 126.63 ($C_{Bz3,5}$), 124.77 (C_{Bz4}), 124.28 ($C_{2''',6'''}$), 123.88 ($C_{Bz2,6}$), 122.90 ($C_{5'}$), 121.56 ($C_{3'}$), 115.22 (C_3), 114.41 (C_5), 113.44 ($C_{3'',5''}$), 69.77 (OCH_2), 21.54 (Me).

4-(3-Chlorophenyl)-6-p-tolyl-2,2′-bipyridine (A^3)

A similar procedure was followed using 3-(3-chlorophenyl)-1-*p*-tolylprop-2-en-1-one as the enone. Yield 51 ± 2%, m.p. 141°C. Anal. Calc. for $C_{23}H_{17}ClN_2$ (356.85): C, 77.41; H, 4.80; N, 7.85%. Found: C, 77.57; H, 4.93; N, 7.95%.^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.786–8.712 (complex, 3H, $H_{3,3',6'}$), 8.219 (d, 2H, $H_{2''',6'''}$, $J = 8$ Hz), 7.985–7.946 (complex, 2H, $H_{5,4'}$), 7.856 (s, 1H, $H_{2''}$), 7.779 (dd, 1H, $H_{6''}$, $J = 4$ and 1.6 Hz), 7.590–7.563 (complex, 2H, $H_{4'',5''}$), 7.455 (dd, 2H, $H_{3''',5'''}$, $J = 8$ and 1.6 Hz), 7.406 (dt, 1H, $H_{5'}$, $J = 7.6$ and 1.2 Hz), 2.303 (s, 3H, Me). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 157.22 (C_2), 155.83 ($C_{2'}$), 151.93 (C_6), 148.80 (C_4), 145.63 ($C_{6'}$), 142.84 ($C_{1''}$), 140.32 ($C_{1'''}$), 138.86 ($C_{4'}$), 134.99 ($C_{3''}$), 132.04 ($C_{4'''}$), 131.43 ($C_{4'}$), 130.46 ($C_{5''}$), 129.34 ($C_{3''',5'''}$), 128.24 ($C_{2''}$), 127.01 ($C_{6''}$), 125.10 ($C_{2'',6'''}$), 124.61 ($C_{5'}$), 122.27 ($C_{3'}$), 119.32 (C_3), 118.33 (C_5), 23.22 (Me).

4-(3-Bromophenyl)-6-p-tolyl-2,2′-bipyridine (A^4)

A similar procedure was followed using 3-(3-bromophenyl)-1-*p*-tolylprop-2-en-1-one as the enone. Yield 53 ± 2%, m.p. 143°C. Anal. Calc. for $C_{23}H_{17}BrN_2$ (401.30): C, 68.84; H, 4.27; N, 6.98%. Found: C, 68.69; H, 4.16; N, 7.14%.^1H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.788–8.705 (complex, 3H, $H_{3,3',6'}$), 8.272 (d, 2H, $H_{2''',6'''}$, $J = 8$ Hz), 7.985–7.947 (complex, 2H, $H_{5,4'}$), 7.859 (s, 1H, $H_{2''}$), 7.776 (dd, 1H, $H_{6''}$, $J = 5.6$ and 1.6 Hz), 7.591–7.566 (complex, 2H, $H_{4'',5''}$), 7.463 (dd, 2H, $H_{3''',5'''}$, $J = 5.2$ and 1.2 Hz), 7.406 (dt, 1H, $H_{5'}$, $J = 6.8$ and 1.2 Hz), 2.541 (s, 3H, Me). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 159.73 (C_2), 155.29 ($C_{2'}$), 151.49 (C_6), 148.40 (C_4), 145.93 ($C_{6'}$), 143.87 ($C_{1''}$), 142.59 ($C_{1'''}$), 138.40 ($C_{4'}$), 135.88 ($C_{4'''}$), 133.32 ($C_{2''}$), 131.83 ($C_{4''}$), 130.37 ($C_{5''}$), 129.40 ($C_{3''',5'''}$), 128.35 ($C_{2''',6'''}$), 127.57 ($C_{6''}$), 126.98 ($C_{3''}$), 125.10 ($C_{5'}$), 123.65 ($C_{3'}$), 119.51 (C_3), 118.88 (C_5), 22.64 (Me).

6-(4-Bromophenyl)-4-(4-fluorophenyl)-2,2′-bipyridine (A^5)

A similar procedure was followed using 1-(4-bromophenyl)-3-(4-fluorophenyl)prop-2-en-1-one as the enone. Yield 53 ± 2%, m.p. 148°C. Anal. Calc. for $C_{22}H_{14}BrFN_2$ (405.26): C, 65.20; H, 3.48; N, 6.91%. Found: C, 65.05; H, 3.33; N, 6.77%.^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.609–8.597 (complex, 1H, $H_{3'}$), 8.438 (d, 1H, $H_{6'}$, J = 8 Hz), 8.183–8.160 (complex, 2H, $H_{3,4'}$), 7.862 (d, 2H, $H_{2'',6''}$, J = 8 Hz), 7.799–7.788 (complex, 1H, H_5), 7.699 (d, 2H, $H_{2'',6''}$, J = 8 Hz), 7.606 (t, 1H, $H_{5'}$, J = 4.8 Hz), 7.571 (d, 2H, $H_{3'',5''}$, J = 8 Hz), 7.399 (t, 2H, $H_{3'',5''}$, J = 5.6 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 169.22 ($C_{4''}$), 157.65 (C_2), 154.99 ($C_{2'}$), 151.66 (C_6), 150.84 (C_4), 149.04 ($C_{6'}$), 146.98 ($C_{4'}$), 145.46 ($C_{1''}$), 142.10 ($C_{1''}$), 135.65 ($C_{3''}$,$_{5''}$), 133.03 ($C_{2'',6''}$), 129.52 ($C_{2'',6''}$), 125.84 ($C_{4''}$), 124.48 ($C_{3'',5''}$), 124.02 ($C_{5'}$), 123.89 ($C_{3'}$), 119.46 (C_3), 115.76 (C_5).

4-(4-(Benzyloxy)phenyl)-6-(4-bromophenyl)-2,2′-bipyridine (A^6)

A similar procedure was followed using 3-(4-(benzyloxy)phenyl)-1-(4-bromophenyl)prop-2-en-1-one as the enone. Yield 49 ± 2%, m.p. 155°C. Anal. Calc. for $C_{29}H_{21}BrN_2O$ (493.39): C, 70.59; H, 4.29; N, 5.68%. Found: C, 70.71; H, 4.44; N, 5.53%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.849 (complex, 3H, $H_{3,3',6'}$), 8.281 (dd, 2H, $H_{2'',6''}$, J = 11.6 and 1.2 Hz), 8.059 (complex, 2H, $H_{5,4'}$), 7.911–7.894 (complex, 2H, $H_{2'',6''}$), 7.635 (dd, 2H, $H_{3'',5''}$, J = 4 and 1.6 Hz), 7.566–7.479 (complex, 6H, $H_{5',Bz2,3,4,5,6}$), 7.214 (d, 2H, $H_{3',5''}$, J = 6.4 Hz), 5.402 (s, 2H, OCH_2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 160.22 ($C_{4''}$), 157.83 (C_2), 155.18 ($C_{2'}$), 154.64 (C_6), 152.36 (C_4), 146.67 ($C_{6'}$), 138.55 ($C_{1''}$), 137.69 ($C_{4'}$), 136.23 (C_{Bz1}), 135.94 ($C_{1''}$), 128.74 ($C_{3'',5''}$), 128.06 ($C_{2'',6''}$), 127.56 ($C_{Bz3,5}$), 126.91 (C_{Bz4}), 125.99 ($C_{2'',6''}$), 125.57 ($C_{Bz2,6}$), 124.52 ($C_{5'}$), 122.55 ($C_{3'}$), 119.82 (C_3), 116.58 (C_5), 115.79 ($C_{4''}$), 114.34 ($C_{3'',5''}$), 70.94 (OCH_2).

6-(4-Bromophenyl)-4-(3-chlorophenyl)-2,2′-bipyridine (A^7)

A similar procedure was followed using 1-(4-bromophenyl)-3-(3-chlorophenyl)prop-2-en-1-one as the enone. Yield 52 ± 2%, m.p. 146°C. Anal. Calc. for $C_{22}H_{14}BrClN_2$ (421.72): C, 62.66; H, 3.35; N, 6.64%. Found: C, 62.80; H, 3.22; N, 6.76%.^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.811–8.733 (complex, 3H, $H_{3,3',6'}$), 8.298 (d, 2H, $H_{2'',6''}$, J = 8 Hz), 8.112–7.978 (complex, 2H, $H_{5,4'}$), 7.870 (s, 1H, $H_{2''}$), 7.798 (dd, 1H, $H_{6''}$, J = 5.6 and 1.6 Hz), 7.622–7.591 (complex, 2H, $H_{4'',5''}$), 7.557 (dd, 2H, $H_{3'',5''}$, J = 5.2 and 1.2 Hz), 7.442 (dt, 1H, $H_{5'}$, J = 7.2 and 1.2 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 162.26 (C_2), 159.92 ($C_{2'}$), 156.85 (C_6), 151.29 (C_4), 147.58 ($C_{6'}$), 144.82 ($C_{1''}$), 141.47 ($C_{1''}$), 140.49 ($C_{4'}$), 139.94 ($C_{3''}$), 136.95 ($C_{3'',5''}$), 134.66 ($C_{4''}$), 130.82 ($C_{5''}$), 129.70 ($C_{2''}$), 128.09 ($C_{2'',6''}$), 127.78 ($C_{6''}$), 125.89 ($C_{4''}$), 124.36 ($C_{5'}$), 122.50 ($C_{3'}$), 118.02 (C_3), 117.98 (C_5).

4-(3-Bromophenyl)-6-(4-bromophenyl)-2,2′-bipyridine (A^8)

A similar procedure was followed using 3-(3-bromophenyl)-1-(4-bromophenyl)prop-2-en-1-one as the enone. Yield 50 ± 2%, m.p. 150°C. Anal. Calc. for $C_{22}H_{14}Br_2N_2$ (466.17): C, 56.68; H, 3.03; N, 6.01%. Found: C, 56.57; H, 2.90; N, 6.16%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.793–8.712 (complex, 3H, $H_{3,3',6'}$), 8.278 (d, 2H, $H_{2'',6''}$, J = 8 Hz), 7.992–7.952 (complex, 2H, $H_{5,4'}$), 7.868 (s, 1H, $H_{2''}$), 7.788 (dd, 1H, $H_{6''}$, J = 5.6 and 1.6 Hz), 7.607–7.571 (complex, 2H, $H_{4'',5''}$), 7.504 (dd, 2H, $H_{3'',5''}$, J = 4.2 and 0.8 Hz), 7.418 (dt, 1H, $H_{5'}$, J = 6.8 and 1.2 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 163.43 (C_2), 157.61 ($C_{2'}$), 153.90 (C_6), 149.04 (C_4), 147.56 ($C_{6'}$), 144.19 ($C_{1''}$), 141.97 ($C_{1''}$), 138.48 ($C_{4'}$), 136.72 ($C_{3'',5''}$), 131.21 ($C_{2''}$), 129.95 ($C_{4''}$), 128.04 ($C_{5''}$), 127.48 ($C_{2'',6''}$), 126.73 ($C_{6''}$), 124.87 ($C_{3''}$), 124.60 ($C_{4''}$), 123.24 ($C_{5'}$), 122.84 ($C_{3'}$), 119.82 (C_3), 117.69 (C_5).

Synthesis of Metal Complexes

A methanolic solution (10 ml) of $CuCl_2$.2H2O (1.5 mmol) was added to a methanolic solution (20 ml) of bipyridines (1.5 mmol), followed by addition of a previously prepared solution (10 ml) of ciprofloxacin (1.5 mmol) in methanol containing CH_3ONa (1.5 mmol). The pH was adjusted to ~6.8 using 2% CH_3ONa. The resulting solution was refluxed for 2 h in air, on a water bath, followed by concentrating it to half of its volume. A fine amorphous product of green colour was obtained, which was washed with ether–hexane and dried in vacuum desiccators. The physicochemical data (yields, formula weight) are listed in Table 1.

Synthesized Complexes at Biological Interphase

In Vitro Antimicrobial Behaviour

In vitro antimicrobial tests were performed against three Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*) and two Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) bacteria using twofold serial dilution technique, in terms of minimum inhibitory concentration (MIC).[12] An appropriate amount of complexes and sodium salt of ciprofloxacin were dissolved in DMSO and water, respectively to make their final concentration 2000 μM.

Pre-cultures of bacteria were grown by inoculating the bacterial strains in 25 ml of sterile LB in Erlenmeyer flasks followed by shaking overnight at 37 ± 1°C until appropriate growth was achieved. Sterilized stock solutions of compounds were added to Corning tubes having an appropriate volume of sterile 2% LB, which results in the desired concentration of the compound (μM).

Table 1. Physical parameters of compounds

Complex empirical formula	Elemental analysis % found(required)				m.p. (°C)	Yield (%)	Formula weight (g mol^{-1})	μ_{eff} (BM)
	C	H	N	M				
$C_{40}H_{38}ClF_2CuN_5O_5$	59.81 (59.62)	4.56 (4.75)	8.90 (8.69)	7.97 (7.89)	238	62.49	805.76	1.86
$C_{47}H_{45}ClFCuN_5O_6$	62.99 (63.15)	4.84 (5.07)	7.60 (7.83)	7.22 (7.11)	220	61.24	893.89	1.88
$C_{40}H_{38}Cl_2FCuN_5O_5$	58.60 (58.43)	4.55 (4.66)	8.71 (8.52)	7.66 (7.73)	241	62.93	822.22	1.80
$C_{40}H_{38}BrClFCuN_5O_5$	55.52 (55.43)	4.57 (4.42)	7.93 (8.08)	7.26 (7.33)	244	61.86	866.66	1.91
$C_{39}H_{35}BrClF_2CuN_5O_5$	53.64 (53.80)	3.78 (4.05)	8.19 (8.04)	7.39 (7.30)	238	64.22	870.63	1.93
$C_{46}H_{42}BrClFCuN_5O_6$	57.86 (57.63)	4.26 (4.42)	7.52 (7.30)	6.74 (6.63)	251	63.66	958.76	1.91
$C_{39}H_{35}BrCl_2FCuN_5O_5$	52.66 (52.80)	4.20 (3.98)	7.76 (7.89)	7.06 (7.16)	252	61.43	887.08	1.82
$C_{39}H_{35}Br_2ClFCuN_5O_5$	50.39 (50.28)	3.99 (3.79)	7.67 (7.52)	6.72 (6.82)	260	60.19	931.53	1.84

All the implicated Corning tubes were further autoclaved and brought to room temperature using laminar air flow. Each of the strains (10 μl) from pre-cultured bacteria were added to Corning tubes having a previously prepared dilution range of the test compound and were kept shaking at 37°C for 24 h.

MIC is the lowest concentration which inhibits the growth of microorganism, judged by lack of turbidity (clear solution) in the tube. If the dilution inhibits growth, a whole experimental procedure was repeated with the next dilution, i.e. half the concentration of test compound compared with the earlier one. This procedure was repeated until faint turbidity of the inoculum itself was observed and the said concentration was termed the MIC. All these operations were carefully performed under aseptic conditions.

DNA Binding Study by Absorption Titration

The ability of Cu(II) complexes to bind HS DNA herring sperm DNA was measured via DNA-mediated hypochromicity and hyperchromicity of the Cu(II) complex using UV–visible absorbance spectra. Hypochromism and bathochromism from the intercalation mode of binding is due to strong stacking interactions between the aromatic chromophore and the DNA base pair.[13] Selection of an appropriate absorption peak was done by performing spectrophotometric wavelength scans of Cu(II) complexes. The concentration of HS DNA was determined by measuring absorbance at 260 nm and using $1.28 \times 104\ \text{M}^{-1}\ \text{cm}^{-1}$ as the molar extinction coefficient value.[14] HS DNA was added to both the cells (cell containing complex and the reference cell), followed by incubation for 10 min at room temperature. DNA-dependent spectral changes in the peak of test compounds were calculated. This was done specifically to enable direct comparison between the assays, which was required to interpret the results obtained. The intrinsic binding constant, K_b, was determined by making it subject to the following equation:

$$[\text{DNA}]/(\varepsilon_a - \varepsilon_f) = [\text{DNA}]/(\varepsilon_b - \varepsilon_f) + 1/K_b(\varepsilon_b - \varepsilon_f)$$

where [DNA] is the concentration of DNA in base pairs, ε_a is the apparent extinction coefficient, obtained by calculating $A_{obs.}$/[Cu(II)complex], ε_f corresponds to the extinction coefficient of the complex in its free form, and ε_b refers to the extinction coefficient of the complex in the fully bound form. When each set of data, fitted to the above equation, gave a straight line with a slope of $1/(\varepsilon_a - \varepsilon_f)$ and a *y*-intercept of $1/K_b(\varepsilon_b - \varepsilon_f)$. The K_b was determined from the ratio of the slope to intercept.

DNA Binding Study by Hydrodynamic Volume Measurement

An Ubbelohde viscometer maintained at a constant temperature of 27 ± 0.1°C in a thermostatic jacket was used to measure the flow time of DNA in phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 7.2) with an accuracy of 0.01 s and a precision of 0.1 s. The DNA sample used, with an approximate average length of 200 base pairs, was prepared by sonicating to minimize the complexities arising from its flexibility.

Flow time for buffer alone was measured and was termed t_0. By adding a DNA sample of calculated amount, flow time was measured. The solution of complex of known concentration was added to achieved the ratio of lowest [complex]/[DNA]. After mixing and incubating it for 10 min, flow time was measured. Similarly the flow time for the successive ratio was measured. Flow time for each case was measured in triplicate and an average flow time was calculated. The viscosity values were calculated using the equation $\eta = (t - t_0)$, where t_0 is the flow time of buffer alone and t is the flow time for buffer containing DNA. Data are represented in terms of $(\eta/\eta_0)^{1/3}$ versus concentration ratio ([complex]/[DNA]), where η is the viscosity of DNA solution in the presence of complex and η_0 is the viscosity of the solution of DNA alone.

Thermal DNA Denaturation Studies

DNA melting experiments were carried out by monitoring the absorption intensity of herring sperm DNA (100 μM) at 260 nm in the range 25–100°C in 0.5°C min^{-1} increments, both in the absence and presence of the copper(II) complexes. The melting temperature (T_m) of DNA was determined as the midpoint of the optically detected transition curves. The ΔT_m value was defined as the difference between T_m of the free DNA and T_m of the bound DNA.

DNA Cleavage Study by Gel Electrophoresis

Gel electrophoresis of plasmid DNA (pUC19 DNA) was carried out in a TAE buffer (0.04 M Tris–acetate, pH 8, 0.001 M EDTA). The 15 μl reaction mixture contains 100 μg ml^{-1} plasmid DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and 200 μM complex. Reactions were allowed to proceed for 24 h at 37°C. All reactions were quenched by addition of 5 μl loading buffer (0.25% bromophenol blue, 40% sucrose, 0.25% xylene cyanol, and 200 mM EDTA). The aliquots were loaded directly on to 1% agarose gel and electrophoresed at 50 V in 1× TAE buffer. Gel was stained with 0.5 μg ml^{-1} ethidium bromide and was photographed on a UV illuminator. After electrophoresis, the proportion of DNA in each fraction was estimated quantitatively from the intensity of the bands using AlphaDigiDoc™ RT version V.4.0.0 PC-Image software.

Brine Shrimp Assay

Brine shrimp (*Artemia* cysts) eggs were hatched in a shallow rectangular plastic dish (22 × 32 cm), filled with artificial sea water, prepared from commercial salt mixture and double distilled water. An unequal partition was made in the plastic dish with the help of a perforated device.[15] Approximately 50 mg eggs were sprinkled into the large compartment, opened to ordinary light. After 2 days, nauplii were collected using a pipette from the lighted side.

A sample of the test compound was prepared by dissolving 10 mg of each compound in 10 ml DMSO. From these stock solutions, solution was transferred to 18 vials to make final concentrations of 2, 4, 8, 12, 16 and 20 μg ml^{-1} (three sets for each dilution were used for each test sample and the mean of three sets were taken) and three vials were kept as a control having the same amount of DMSO only. When the nauplii were ready, 1 ml sea water and 10 nauplii were added to each vial and the volume was adjusted with sea water to 2.5 ml per vial. After 24 h the number of survivors was counted.[15] Data were analysed by simple logit method to determine the LC_{50} values, in which log of concentration of samples was plotted against percentage of mortality of nauplii.[16]

Superoxide Dismutase (SOD) Mimic Behaviour

A non-enzymatic system made up of 30 μM phenazime methosulphate (PMS), 79 μM reduced nicotinamide adenine dinucleotide (NADH), 75 μM nitro blue tetrazolium (NBT) and phosphate buffer (pH = 7.8) was used to produce superoxide anion ($O_2^{\cdot-}$). The scavenging rate of $O_2^{\cdot-}$ was determined by monitoring the reduction in rate of transformation of NBT to monoformazan

dye under the influence of 0.25–5.0 μM concentration of test compound. The reactions were monitored at 560 nm with a UV–visible spectrophotometer and the rate of absorption change was determined. The percent inhibition (η) of NBT reduction was calculated using following equation:

$$\eta(\text{percent inhibition of NBT reduction}) = (1 - k'/k) \times 100$$

where k' and k present the slopes of the straight line of absorbance values as a function of time in the presence and absence of SOD mimic or a model compound, respectively. IC_{50} value of the complex was determined by plotting the graph of percentage inhibition of NBT reduction against increase in concentration of the complex. The concentration of complex causing 50% inhibition of NBT reduction is reported as IC_{50}.

Results and Discussion

Synthesis

The bipyridine derivatives (A^{1-8}) were prepared by reacting the appropriate enones with pyridinium salt. The product obtained from aldol condensation (enone) was allowed to react with previously prepared pyridinium salts via attack on the active methylene group of pyridinium salt, subsequently undergo ring closure reaction following formation of bipyridines. Metal complexes were prepared in two steps by the template reaction of $CuCl_2.2H_2O$ and bipyridines in a 1:1 ratio, followed by reaction with deprotonated ciprofloxacin solution in equal ratio. These result in stable square pyramidal metal complexes having three coordinate and two covalent bonds. The reaction sequences are outlined in Scheme 1.

Characterization

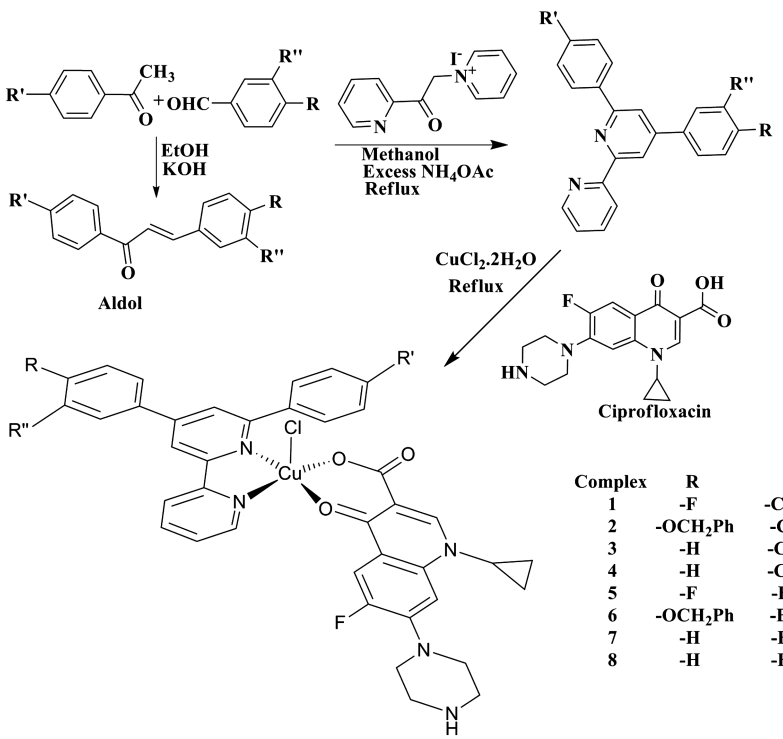
The ligands were characterized using ^1H and ^{13}C NMR spectroscopy and C,H,N elemental analysis. Several analytical techniques, such as FAB MS, IR, reflectance spectroscopy, C,H,N elemental analysis and magnetic measurement, were used to evaluate the structure of the complexes. Elemental analysis data are in good agreement with the proposed structures. Table 1 lists the physicochemical parameters.

IR Spectroscopy

IR spectra of the Cu(II) complexes show major changes as compared to the free ligands (supporting information, Supplementary 1). The absorption bands observed in the case of ciprofloxacin at 1624 and 1340 cm^{-1} are assigned to $\nu(COO)_{asy}$ and $\nu(COO)_{sy}$ respectively. In complexes these bands are observed at 1552–1575 and 1362–1375 cm^{-1}. The frequency separation ($\Delta\nu = \nu COO_{assy} - \nu COO_{sym}$) in investigated complexes is ~200 cm^{-1}, suggesting the unidentate nature of the carboxylato group.[17,18] The sharp band in quinolone at ~3023 cm^{-1} is due to hydrogen bonding,[19] which contributes to ionic resonance structures and peaks observed to the free hydroxyl stretching vibration. This band vanishes completely in the spectra of the complexes, indicating deprotonation of carboxylic proton. The ν(C=O) stretching vibration band in ciprofloxacin appears at 1708 cm^{-1}, while in the complexes these bands shift to 1613–1627 cm^{-1}, suggesting that coordination occurs through the pyridone oxygen atom.[20] These data are further supported by ν(M–O),[21] which appear at ~515 cm^{-1}. The band at ~535 cm^{-1} observed for complexes suggests the *N,N*-donating nature of bipyridines.[22]

Electronic and Magnetic Behaviour

Visible reflectance spectrum of the copper(II) complexes, i.e. d^9 system, were recorded. The complexes exhibit only a broad λ_{max}



Complex	R	R'	R''
1	-F	$-CH_3$	-H
2	$-OCH_2Ph$	$-CH_3$	-H
3	-H	$-CH_3$	-Cl
4	-H	$-CH_3$	-Br
5	-F	-Br	-H
6	$-OCH_2Ph$	-Br	-H
7	-H	-Br	-Cl
8	-H	-Br	-Br

Scheme 1. Reaction scheme for synthesis of ligand and metal complex

at ~645 nm attributed to d–d transitions for Cu(II) atoms in a distorted square pyramidal environment.[23]

The magnetic moment measurement for any geometry in copper(II) complexes generally results in 1.8 BM, which is very close to the spin-only value, i.e. 1.73 BM. The observed values in our case are very close to the spin-only values (Table 1) expected for $s = ½$ system (1.73 BM.), leading to the conclusion that the metal centre in synthesized complexes possesses one unpaired electron.[24]

FAB Mass Spectra

The FAB mass spectrum of the representative complex, [Cu(CFL)(A^1)Cl] (supporting information, Supplementary 2) shows peaks at 136, 137, 154, 289 and 307 *m/z* due to the matrix. The molecular ion peaks observed at *m/z* = 768 and 770 in spectra are assigned to (M) and (M+2) of complex molecule with an intensity ratio of 1:3, indicating presence of chlorine atom. Loss of chlorine gives a fragment ion peak at *m/z* = 733, confirming that chlorine atom is covalently attached to the metal ion.

Biological Evaluation

In Vitro Antimicrobial Screening

The MIC is defined as the lowest concentration of antimicrobial agent showing complete inhibition of growth of microorganisms. The results concerning *in vitro* antibacterial activity (MIC) are presented in Table 2. The compounds show better antimicrobial activity against the five microorganisms than $CuCl_2.2H_2O$. Complex **5** has better antibacterial activity than the ciprofloxacin against tested microorganisms. Complexes **1** and **8** have better antibacterial activity than ciprofloxacin against tested microorganisms, except of **8** against *E. coli*, while all the other complexes are moderate to less bacteriostatic.

The increase in antimicrobial activity of metal complexes can be the result of chelate effects, the nature of the ligands, the nature of the ion neutralizing the complex, and nuclearity of the metal centre in the compounds.[25] All complexes show higher antibacterial activity against *S. aureus* than ciprofloxacin–copper(II) complexes reported by Drevensek *et al.*[26] All complexes show higher antibacterial activity than fluoroquinolone drug-based metal complexes, [Mg(R-oflo)(S-oflo)$(H_2O)_2$].$2H_2O$ and [Mg(S-oflo)$_2$$(H_2O)_2$].$2H_2O$ against *P. aeruginosa*.[27] Complexes **1**, **5**, and **8** have higher antibacterial activity against *E. coli*, *P. aeruginosa* and *S. aureus* than some copper(II) complexes reported by Efthimiadou *et al.*[28]

DNA Interaction Studies by Absorbance Titration

A representative absorption titration curve is shown in Supplementary 3 (supporting information). The changes observed in the UV spectra of the complexes after mixing with DNA, i.e. hypochromism and bathochromism (~4 nm) indicate that the interaction of complexes with DNA takes place by direct formation of a new complex with double-helical DNA.[29,30] The extent of the binding strength of complexes was determined quantitatively by calculating the intrinsic binding constants K_b of the complexes by monitoring the change in absorbance at various concentration of DNA. From the plot of [DNA]/($\varepsilon_a - \varepsilon_f$) versus [DNA], (supporting information, inset Supplementary 3) the K_b value of complexes were determined and were found to be in the range 1.10–1.77 $\times 10^4$ M^{-1} (Table 3).

Comparing the intrinsic binding constant of complexes with those of DNA-intercalative $[Ru(bpy)_2ppd]^{+2}$ (1.18×10^4 M^{-1}) complex,[31] we can deduce that all of the complex binding is moderated to DNA by intercalation. The K_b value of complexes is greater than Co(II) complexes of terpyridines reported by Indumathy *et al.*[32] and Cu(II) complexes of type [Cu(phen)$_2$$Cl_2$],[33] while comparable to ruthenium complexes reported by Tan *et al.*[34] These spectral characteristics obviously suggest that the complexes in our paper interact with DNA most likely through a mode that

Table 3. Binding constants, IC_{50}, ΔT_m and LC_{50} values of compounds

Complex	Binding constant K_b (M^{-1})	ΔT_m (°C)	IC_{50}[a] (μM)	LC_{50}[a] (μM)
Ciprofloxacin	2.6×10^3	-	164 (±2.08)	214 (±1.15)
1	1.3×10^4	4.8	1.2 (±0.03)	6.2 (±0.16)
2	1.2×10^4	4.2	1.3 (±0.04)	13.4 (±0.19)
3	1.2×10^4	5.1	1.3 (±0.02)	9.0 (±0.13)
4	1.1×10^4	5.0	1.4 (±0.05)	9.2 (±0.17)
5	1.8×10^4	5.6	0.6 (±0.01)	6.0 (±0.15)
6	1.4×10^4	5.2	0.9 (±0.02)	12.5 (±0.18)
7	1.4×10^4	5.4	1.0 (±0.01)	9.0 (±0.16)
8	1.4×10^4	5.3	1.0 (±0.03)	9.8 (±0.11)

[a]Mean of at least three determinations.

Table 2. MIC[a] data of compounds (μM)

	S. aureus	*B. subtilis*	*S. marcescens*	*P. aeruginosa*	*E. coli*
$CuCl_2.2H_2O$	2698	2815	2756	2404	3402
Ciprofloxacin	1.6 (±0.06)	1.1 (±0.08)	1.6 (±0.08)	1.4 (±0.07)	1.4 (±0.07)
[Cu(CFL)(A^1)Cl].$2H_2O$ (1)	0.8 (±0.05)	1.1 (±0.04)	1.0 (±0.06)	1.3 (±0.09)	1.2 (±0.03)
[Cu(CFL)(A^2)Cl].$2H_2O$ (2)	1.4 (±0.04)	1.6 (±0.07)	1.5 (±0.03)	1.7 (±0.06)	1.8 (±0.05)
[Cu(CFL)(A^3)Cl].$2H_2O$ (3)	1.4 (±0.01)	1.2 (±0.03)	1.6 (±0.09)	1.7 (±0.05)	1.6 (±0.04)
[Cu(CFL)(A^4)Cl].$2H_2O$ (4)	1.3 (±0.06)	1.4 (±0.07)	1.5 (±0.04)	1.6 (±0.03)	1.7 (±0.07)
[Cu(CFL)(A^5)Cl].$2H_2O$ (5)	0.5 (±0.03)	0.4 (±0.01)	0.7 (±0.04)	0.7 (±0.02)	0.6 (±0.02)
[Cu(CFL)(A^6)Cl].$2H_2O$ (6)	1.1 (±0.08)	1.2 (±0.08)	1.0 (±0.06)	0.9 (±0.04)	1.1 (±0.02)
[Cu(CFL)(A^7)Cl].$2H_2O$ (7)	1.1 (±0.07)	1.0 (±0.06)	1.2 (±0.06)	1.1 (±0.05)	1.2 (±0.01)
[Cu(CFL)(A^8)Cl].$2H_2O$ (8)	0.7 (±0.02)	0.8 (±0.02)	0.9 (±0.03)	1.0 (±0.03)	1.6 (±0.04)

[a]Mean of at least three determinations.

involves a stacking interaction between the aromatic chromophore and the base pairs of DNA.

Thermal DNA Denaturation Study

The binding of metal complexes to herring sperm DNA was studied by measuring changes in the melting temperature (T_m) of DNA that characterizes the transition from a double-stranded nucleic acid to a single-stranded one. The temperature at which half of a DNA sample has melted is known as the melting temperature and is strongly related to the stability of the double-helical structure. A change in T_m may be observed when the complexes interact with DNA.

The melting curves for herring sperm DNA in the absence and presence of the complexes are illustrated in Supplementary 4 (supporting information) and Table 3. The T_m value for the free herring sperm DNA is 82.8°C. A small change in the DNA melting temperature (ΔT_m) on addition of all the complexes to herring sperm DNA was observed. The ΔT_m values (4.2–5.6°C) suggest that the complexes may interact with DNA by intercalatiion.[35,36]

DNA Binding Study by Viscosity Measurement

Viscometric titration experiments, regarded as a reliable tool in the absence of crystallographic data, were also carried out to explore the propensity of DNA binding by the complexes.[37] A significant increase in DNA viscosity on addition of any external species can result only in intercalation, as intercalation leads to separation of DNA bases and hence to increases in the DNA effective size.[38] A plot of $(\eta/\eta 0)^{1/3}$ versus [complex]/[DNA] gives a measure of the viscosity changes (Fig. 1). A marginal increase in the relative viscosity was observed on addition of the present complexes to DNA solution, suggesting the mainly intercalation nature of the complexes.

Gel Electrophoresis: Photo Quantization Technique

When plasmid DNA was subjected to electrophoresis after interaction, upon illumination of gel (supporting information, Supplementary 5) the fastest migration was observed for supercoiled (SC) form I, whereas the slowest moving was open circular (OC) form II and the intermediate moving form was the linear (L) form III generated on cleavage of OC. Figure 2 shows a bar diagram for the data of plasmid cleavage (supporting information, Supplementary 6). The different DNA cleavage efficiency of the complexes, metal salt and drugs is due to the difference

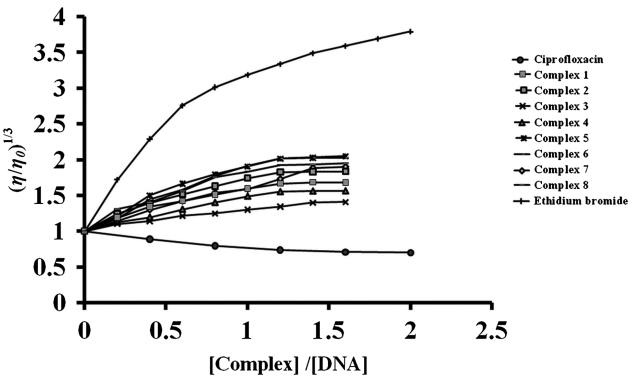


Figure 1. Effect on relative viscosity of DNA under the influence of increasing amount of compounds at 27 ± 0.1°C in 5 mM Tris–HCl buffer (pH 7.2) as a medium

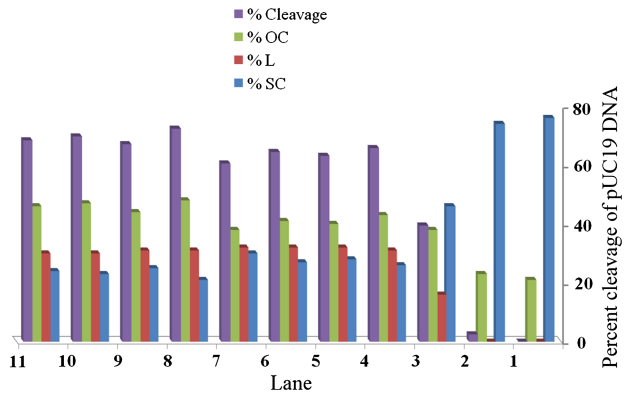


Figure 2. Bar diagram for interaction of pUC19 DNA (100 μg ml^{-1}) with series of copper(II) complexes (200 μM): 1, DNA control; 2, $CuCl_2.2H_2O$ + pUC19; 3, ciprofloxacin + pUC19; 4, 1 + pUC19; 5, 2 + pUC19; 6, 3 + pUC19; 7, 4 + pUC19; 8, 5 + pUC19; 9, 6 + pUC19; 10, 7 + pUC19; 10, 8 + pUC19

in binding affinity of the complexes to DNA and the structural dissimilarities of ligands. The data show higher cleavage ability of metal complexes than of metal salt and ciprofloxacin.

Cytotoxicity

Brine shrimp lethality bioassay is a recent development in the assay of bioactive compounds, which indicates cytotoxicity as well as a wide range of pharmacological activities (such as anticancer, antiviral, insecticidal, pesticidal, AIDS) of the compounds.[15,39] All the synthesized compounds were screened for their cytotoxicity (brine shrimp bioassay) using the protocol of Meyer *et al.*[15] From the data recorded in Table 3, it is evident that compounds **1** and **5** displayed potent cytotoxic activity against *Artemia* cysts, while the other compounds were moderately active in this assay.

SOD-Like Activity: Decomposition of Reactive Oxygen Species

The NADH/PMS/NBT system was used to generate the superoxide radical artificially in order to check SOD-like behaviour of the complexes. The percentage inhibition of formazan formation at various concentrations of complexes as a function of time was determined by measuring the absorbance at 560 nm and plotting to obtain a straight line obeying the equation $Y = mX + C$ (supporting information, Supplementary 7); with an increase in concentration of the tested complexes a decrease in slope (m) was observed. Percentage inhibition of the reduction rate of NBT was plotted against the concentration of the complex (supporting information, Supplementary 8).

Compounds exhibit SOD-like activity at biological pH, with their IC_{50} values ranging from 0.60 to 1.35 μM (Table 3), which is higher than Cu(II) chloride (0.45 μM). These higher values may be due to the strong ligand field created by the ligands, which opposes the interaction of the copper(II) complexes with the superoxide radical. The IC_{50} values of our compounds are better than complexes of type $Cu(stz)py_3Cl$ (1.31), $Cu(Hstz)_2(MeOH)Cl_2$ (2.51) and $Cu(Hstz)_2(EtOH)Cl_2$ (5.17) reported by Cassanova *et al.*[40]

Conclusions

Reflectance spectroscopy suggests that ciprofloxacin-based copper(II) complexes of bipyridine derivatives possess distorted square pyramidal geometry. The binding properties of copper

(II) complexes to herring sperm DNA have been studied by viscosity measurement, absorption titration and thermal DNA denaturation. The hypochromism and bathochromism (~4 nm) observed in absorption titration is consistent with the intercalation mode of metal complex interaction with DNA. The increase in the relative viscosity of DNA in the presence of metal complexes suggests a classical intercalation binding mode. The increase in melting temperature of DNA in the presence of compounds by ~5°C also supports this conclusion. The cleavage of plasmid pUC19 DNA by compounds as observed in gel electrophoresis study suggests sufficient interaction of compounds with DNA. The SOD mimic study suggests that copper(II) complexes can effectively scavenge superoxide anion radicals at their vacant coordination side. Cytoxicity assessment shows a potent lethality (LC_{50}) of metal complexes against brine shrimp.

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