Cytotoxic, DNA binding, DNA cleavage and antibacterial studies of ruthenium–fluoroquinolone complexes

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Abstract. Six new Ru(II) and Ru(III) complexes have been synthesized and characterized by elemental analysis, LC-MS, electronic spectra, IR spectra and magnetic moment measurements. DNA-binding properties of Ru complexes have been studied by means of absorption spectrophotometry and viscosity measurements as well as their HS DNA cleavage properties by means of agarose gel electrophoresis. The experimental results show that all the complexes can bind to DNA via partial intercalative mode. The K_b values of complexes were found in the range 2.14×10^4 to 2.70×10^5 M^{-1}. All the complexes show excellent efficiency of cleaving DNA than respective fluoroquinolones. Brine shrimp lethality bioassay has been performed to check the cytotoxic activity. The IC$_{50}$ values of the complexes are in the range of 6.27 to 16.05 µg mL^{-1}.

Keywords. Ruthenium; fluoroquinolone; LC$_{50}$; partial intercalation; HS DNA.

1. Introduction

It is well-known that deoxyribonucleic acid (DNA) plays an important role in the life process since it contains all the genetic information for cellular function. However, DNA molecules are prone to be damaged under various conditions like interactions with some molecules. This damage may cause various pathological changes in living organisms, which is due to their possible application as new therapeutic agents and their photochemical properties which make them potential probes of DNA structure and conformation.[1–3] The binding interaction of transition metal complexes with DNA is of interest for both therapeutic and scientific reasons.[4] Many transition metal complexes are known to bind to DNA via both covalent and non-covalent interactions. In covalent binding the labile ligand of the complexes is replaced by a nitrogen base of DNA. On the other hand, the non-covalent DNA interactions include intercalative, electrostatic and groove (surface) binding of cationic metal complexes along outside of DNA helix, major or minor groove.

Metals are considered essential to a human body being an integral part of an organic structure in performing physiologically important and vital functions, in the body.[5] It seems that the role of metal ions is imperative for the way of function of fluoroquinolones. The synthesis and characterization of new metal complexes with fluoroquinolones are of great importance for better understanding of the drug–metal ion interactions.[6] It was suggested that the reactions of metal ions with fluoroquinolones were essential for the activity of these antimicrobial agents, and the metal ions (magnesium, copper, and iron) may bridge the binding of the quinolone to DNA gyrase or of bacterial DNA directly.[7,8] In recent years, a lot of work has been done on the interaction of the quinolones pefloxacin, ofloxacin and sparfloxacin with diverse metal ions.[9–17]

In continuation of our previous work,[18] this paper mainly focuses on exploring the trend in DNA-binding affinities of six complexes and the important differences in some related properties. Understanding the features that contribute to recognition of DNA by small ligands or metal complexes is crucial for the development of drugs targeted at DNA. We hope the results will be of value in further understanding DNA binding, and the efficiency of DNA recognition and cleavage by Ru(II) and Ru(III) complexes.

2. Experimental

2.1 *Reagent*

All chemicals and solvents used were of analytical grade. Pefloxacin, ofloxacin and sparfloxacin were generously supplied by Bayer AG (Wuppertal, Germany). Ruthenium trichloride was purchased from Chemport

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(Mumbai, India). Ethidium bromide, bromophenol blue, agarose, xylene cyanol FF and Luria Broth (LB) were purchased from Himedia (India). Acetic acid and EDTA were purchased from Sd fine Chemicals (India). The starting complex [RuCl$_3$(PPh$_3$)$_3$] was prepared by reported method.[19]

2.2 *Physical measurement*

The elemental analysis (C, H and N) of the synthesized complexes has been performed with a model 240 Perkin Elmer elemental analyzer. Room temperature magnetic measurement of the complexes has been carried out using a Gouy magnetic balance. The Gouy tube has been calibrated using mercury(II)tetrathiocyanatocobaltate(II) as the calibrant ($\chi_g = 16.44 \times 10^{-6}$ cgs units at 20°C). The electronic spectra have been recorded on a UV-160A UV–Visible spectrophotometer, Shimadzu, Kyoto (Japan). Infrared spectra have been recorded on a FT–IR ABB Bomen MB 3000 spectrophotometer as KBr pellets in the range 4000–400 cm^{-1}. The LC–MS have been recorded using Thermo Scientific mass spectrophotometer (USA).The minimum inhibitory concentration (MIC) study has been performed by means of laminar air flow cabinet Toshiba, Delhi (India).

2.3 *Synthesis of complexes*

2.3a [Ru(PFL)(PPh$_3$)$_2$Cl$_2$](1): The [RuCl$_3$(PPh$_3$)$_3$] (0.1 mmol) in toluene and a methanolic solution of the pefloxacin (0.1 mmol) in presence of CH$_3$ONa has been mixed in 1:1 molar ratio and refluxed for 4–5 h. Colour of the solution was changed after the addition of drug which indicates the formation of complex. The resulting solution has been concentrated to small volume on a rotary evaporator and the product has been separated by the addition of small amount of pet-ether (60–80°C). The compound that separated has been filtered, washed with toluene followed by ether, dried *in vacuo* over anhydrous CaCl$_2$, then recrystallized from 1:2 (v:v) chloroform-pet ether (60–80°C) mixture (scheme 1). Yield: 57.9%, m.p.: >300°C, μ$_{eff}$: 1.96 B.M. Anal. Calcd. for: C$_{53}$H$_{49}$Cl$_2$FN$_3$O$_3$P$_2$Ru (1028.90): C, 61.87; H, 4.80; N, 4.08. Found: C, 61.70; H, 4.59; N, 3.90%. UV–Vis λ (nm) (ε, M^{-1} cm^{-1}): as solid: 620, 340, 284, 278; as solution: 614(244), 336(8297), 281(19646), 274(19829), m/z = 1028.27.

2.3b [Ru(OFL)(PPh$_3$)$_2$Cl$_2$](2): Complex **2** has been synthesized using ofloxacin by above method. Yield: 52.1%, m.p.: 263°C, μ$_{eff}$: 1.91 B.M. Anal. Calcd. for: C$_{53}$H$_{47}$Cl$_2$FN$_3$O$_3$P$_2$Ru (1026.88): C, 61.99; H, 4.61; N, 4.09. Found: C, 61.85; H, 4.48; N, 3.87%. UV–Vis λ (nm) (ε, M^{-1} cm^{-1}): as solid: 620, 304, 279, 272; as solution: 616(305), 299(18913), 273.5(11622), 266(10829), m/z = 1026.39.

**2.3c [Ru(SPF)(PPh$_3$)$_2$Cl$_2$](3):** Complex **3** has been synthesized using sparfloxacin by above method. Yield: 51.7%, m.p.: 271°C, μ$_{eff}$: 1.89 B.M. Anal. Calcd. for: C$_{55}$H$_{51}$Cl$_2$F$_2$N$_4$O$_3$P$_2$Ru (1087.94): C, 60.92; H, 4.72; N, 5.15. Found: C, 60.71; H, 4.56; N, 4.94. UV–Vis λ (nm) (ε, M^{-1} cm^{-1}): as solid: 628, 348, 297, 274; as solution: 623(335), 344.5(7626), 290(12019), 276(11683), m/z = 1087.25.

2.3d [Ru(PFL)(PPh$_3$)$_3$Cl](4): The [RuCl$_3$(PPh$_3$)$_3$] (0.1 mmol in 20 mL toluene) and a methanolic solution of the pefloxacin (0.1 mmol) in presence of CH$_3$ONa, methanolic solution of triethylamine (0.1 mmol) and methanolic solution of lithium chloride (0.4 mmol) have been mixed and refluxed for 4–5 h. The resulting solution has been concentrated to small volume on a rotary evaporator and the product was separated by the addition of small amount of pet-ether (60–80°C). The compound that separated has been filtered, washed with toluene followed by ether, dried *in vacuo* over anhydrous CaCl$_2$, then recrystallized from 1:2 (v:v) chloroform-pet ether (60–80°C) mixture (scheme 1).

Yield: 53.8%, m.p.: 285°C, Anal. Calcd. for: C$_{71}$H$_{64}$ClFN$_3$O$_3$P$_3$Ru (1255.73): C, 67.91; H, 5.14; N, 3.35. Found: C, 67.70; H, 4.96; N, 3.21%. UV–Vis λ (nm) (ε, M^{-1} cm^{-1}): as solid: 595, 310, 270; as solution: 591(884), 305(20530), 263(14734), m/z = 1255.23.

2.3e [Ru(OFL)(PPh$_3$)$_3$Cl](5): Complex **5** has been synthesized using ofloxacin by above method. Yield: 57.6%, m.p.: >300°C, Anal. Calcd. for: C$_{72}$H$_{64}$ClFN$_3$O$_4$P$_3$Ru (1283.30): C, 67.36; H, 5.03; N, 3.27. Found: C, 67.21; H, 4.86; N, 3.11%. UV–Vis λ (nm) (ε, M^{-1} cm^{-1}): as solid: 609, 344, 290; as solution: 605(213), 338(6284), 286(13209), m/z = 1283.42.

2.3f [Ru(SPF)(PPh$_3$)$_3$Cl](6): Complex **6** has been synthesized using sparfloxacin by above method. Yield: 55.6%, m.p.: 266°C, Anal. Calcd. for: C$_{73}$H$_{66}$ClF$_2$N$_4$O$_3$P$_3$Ru (1314.77): C, 66.69; H, 5.06; N, 4.26. Found: C, 66.50; H, 4.86; N, 4.09%. UV–Vis λ (nm) (ε, M^{-1} cm^{-1}): as solid: 600, 313, 272; as solution: 596(1098), 310.5(21629), 266(12599), m/z = 1314.29.
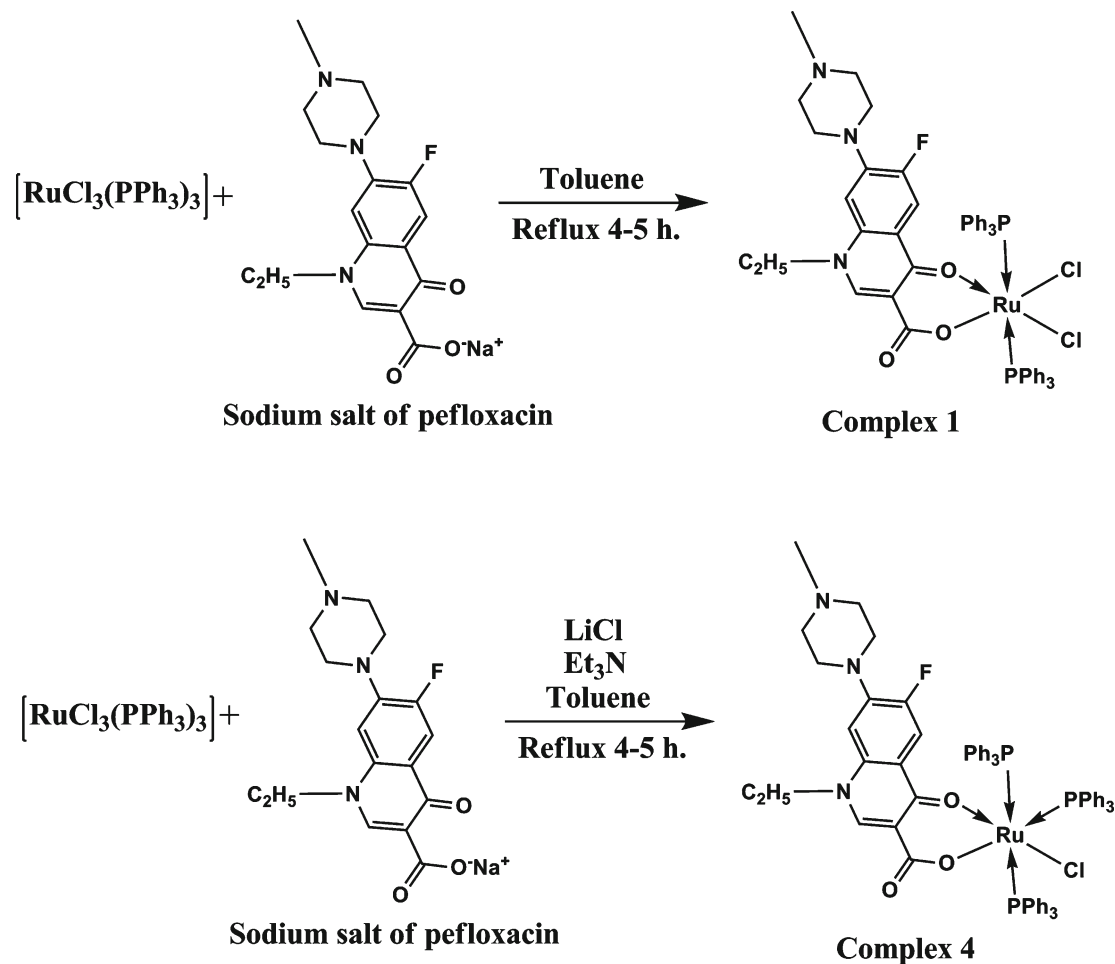
Scheme 1. Synthesis of [Ru(PFL)(PPh$_3$)$_2$Cl$_2$] (**1**) and [Ru(PFL)(PPh$_3$)$_3$Cl] (**4**).

2.4 *In vitro-antimicrobial screening*

To study the antibacterial activity of the compounds, we have used various microorganisms i.e., *Escherichia coli* (MTCC 433), *Pseudomonas aeruginosa* (MTCC P09), *Serratia marcescens* (MTCC 7103), *Bacillus subtilis* (MTCC 7193) and *Staphylococcus aureus* (MTCC 3160). Screening has been performed by determining the minimum inhibitory concentration (MIC) using LB as a medium. Cultures for Gram$^{(+ve)}$ and Gram$^{(-ve)}$ microorganisms were incubated at 37°C. Since the compounds are water insoluble, samples have been dissolved in DMSO. A control test without active ingredient has also been performed.[20] The MIC has been determined using two-fold serial dilutions in liquid media containing the test compound. A preculture of bacteria has been grown in LB overnight at the optimal temperature for each species. We monitored bacterial growth by measuring the turbidity of the culture after 18 h. If a certain concentration of a compound inhibited the bacterial growth, half the concentration of compound was then tested. This procedure has been carried out until we get a concentration where the bacteria grew normally. The lowest concentration which inhibits bacterial growth has been determined as the MIC value. All equipments and culture media used were sterile.

The bactericidal action of all compounds has been evaluated against same microorganisms. The inoculum was prepared by diluting an overnight culture of microorganisms grown in LB, to obtain 10^6 viable bacteria/mL. Bacteria have been exposed to various concentrations of compounds. Control tubes without compound have been included in each run. The final volume was 1 mL. Cultures have been incubated at 37°C for 2 h. The 100 µL bacterial culture from each dilution has been taken and spread over previously prepared agar plate. Then the plates were incubated for 24 h. The number of colonies present on the plates have been counted. The number of colonies was in the range of 30–300.

2.5 *DNA interaction study*

2.5a *UV–Vis spectra of the complexes in the presence of buffered HS DNA solution*: The absorption titrations of Ru(II) and Ru(III) complexes in the buffer have been performed by using a fixed complex concentration to which increments of the nucleic acid stock solution was done. Concentration of complex solutions employed was 20 μM. While measuring the absorption spectra, equal amount of DNA has been added to both complex solution and reference solution to eliminate the absorbance of DNA itself. After addition of equivalent amount of DNA to reference cell, incubation for 10 min at room temperature has been provided, followed by absorbance measurement. DNA mediated hypochromism (decrease in absorbance) or hyperchromism (increase in absorbance) for synthesized compounds has been calculated. The intrinsic binding constant K_b has been determined by making it subject in the following equation:[21]

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

where ε_a, ε_f and ε_b corresponds to A_{obsd}/[Ru], the extinction coefficient for the free ruthenium complex, and the extinction coefficient for the ruthenium complex when fully bound to DNA, respectively. In plots of [DNA]/(ε_a- ε_f) versus [DNA], K_b is given by the ratio of slope to the intercept.

2.5b *Viscosity measurements*: Viscosity measurements have been carried out using an Ubbelohde viscometer, immersed in a thermostatic water-bath that maintained at a constant temperature at $27 \pm 0.1°$C. The compounds have been titrated into the HS DNA solution which presented in the viscometer. The flow time of each sample has been measured by a digital stopwatch for three times, and an average one was calculated. Data are presented as $(\eta/\eta_0)^{1/3}$ vs. binding ratio,[19] where η and η_0 are the viscosity of HS DNA in the presence or absence of complex, respectively. Viscosity values have been calculated from the observed flow time of HS DNA containing solutions corrected from the flow time of buffer alone (t_0), $\eta \infty (t - t_0)$.[22]

2.5c *Gel electrophoresis technique*: For the gel electrophoresis experiment, a total volume of 15 μL containing 300 μg/mL of pUC19 DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) has been treated with different complexes (200 μM). The mixture has been incubated for 24 h at 37°C. Then the samples have been analysed on the basis of their charge and size difference on a 1% agarose gel bed consisting of 0.5 μg/mL of ethidium bromide at 50 V, after quenching the reaction with 5 μL loading buffer (40% sucrose, 0.2% bromophenol blue). The whole bed has been immersed in 1X TAE buffer (0.04 M Tris-Acetate, pH 8, 0.001 M EDTA). The bands have been visualized using UV light, then photographed. An estimation of intensity of the DNA bands has been done using AlphaDigiDoc™RT. Version V.4.0.0 PC–Image software gel documentation system.

2.6 *Brine shrimp assay*

Cytotoxicity of the compounds has been tested by using brine shrimp lethality bioassay. The method of Meyer *et al.*[23] was adopted for this purpose. Artificial seawater has been prepared with commercial salt mixture and double distilled water. Brine shrimp (*Artemia cysts*) eggs have been hatched in a shallow rectangular plastic dish (22 × 32 cm), filled with artificial seawater. An unequal partition has been made in the plastic dish with the help of a perforated device. Approximately 50 mg of eggs have been sprinkled into the hatching chamber and has been opened to ordinary light. After hatching, active nauplii free from egg shells have been collected from brighter portion of the hatching chamber and were used for the assay. A sample of the test compound has been prepared by dissolving 10 mg of each compound in 10 mL of DMSO. Choudhary *et al.*[24] suggested that the compound should be prepared by dissolving in DMSO in the suggested maximum volume range of 2% to prevent possible false effects originated from DMSO's toxicity to the experimental results. Solutions have been transferred to 18 vials from the stock solutions in such an amount to make final concentration 4, 8, 12, 16 and 20 μg mL^{-1} (three sets for each dilutions were used for each test sample and mean of three sets were taken). Three vials have been kept as control having same amount of DMSO only. When the nauplii were ready, 1 mL of seawater and 10 nauplii have been added to each vial (30 nauplii/dilution) and the volume has been adjusted with artificial seawater to 2.5 mL per vial. After 24 h. the number of survivors has been counted.[25] Data have been analysed by simple method to determine the LC_{50} values, in which log of concentration of samples have been plotted against percentage of mortality of nauplii.

3. Results and discussion

3.1 *Magnetic moments and electronic spectra*

Magnetic susceptibility of the synthesized complexes was measured using Gouy magnetic balance at 300 K.

The magnetic moment value for complexes **1–3** lie in the range of 1.89–1.96 BM, which confirms the presence of single unpaired electron in a low-spin $4d^5$ configuration for ruthenium(III) ion in octahedral environment.[26] While the magnetic moment value for complexes **4–6** are found to be zero which confirms the absence of unpaired electron in a lowspin d^6– configuration for Ru(II) ion in octahedral environment.[27] So, from the magnetic moment values, it is proved that in case of complexes **1–3**, the ruthenium is in +3 oxidation state, while in case of complexes **4–6**, the ruthenium is in +2 oxidation state.

The solution of complexes is prepared in DMSO for recording UV-Visible spectra. The electronic spectra of complexes **1–3** showed bands in the 268–623 nm regions. The band in the 614–623 nm region have been assigned to the d–d transition, which is in conformity with assignments made for the similar ruthenium(III) complexes.[28–30] Other bands in the 344–268 nm region have been assigned to the charge transfer transitions. In general, the electronic spectra of all the complexes are characteristic of an octahedral environment around ruthenium(III) ions.

Complexes **4–6** are diamagnetic indicating the presence of ruthenium in +2 oxidation state in all the complexes. The electronic spectra of complexes **4–6** dissolved in DMSO, consists of various bands between 263 and 605 nm regions. The bands in the region 591–611 nm are assigned as d–d transition. The other bands in the region of 338–263 nm are probably due to charge transfer transitions. The nature of the electronic spectra are similar to those observed for other octahedral ruthenium(II) complexes.[31]

3.2 *IR spectra*

In table 1 the most characteristic absorptions of IR spectra of the complexes are listed. In the IR spectra of complexes **1–6** the absorption of the $\nu(C=O)_{carb}$ of the free quinolone ligands at 1708–1730 cm^{-1} has disappeared and has been replaced by two very strong characteristic bands in the range of 1558–1583 cm^{-1} and 1335–1381 cm^{-1} which can be assigned as asymmetric, $\nu(CO_2)_{asym}$, and symmetric, $\nu(CO_2)_{sym}$, stretching $\nu(O–C–O)$ vibrations, respectively. The difference $\Delta\nu = \nu(CO_2)_{asym} - \nu(CO_2)_{sym}$ is a useful characteristic for determining the coordination mode of the carboxylato group of the ligands. For complexes **1–6**, the Δ values fall in the range of 201–247 cm^{-1} indicating a monodentate coordination mode of the carboxylato group.[32,33] Finally, the pyridone stretch $\nu(C=O)_p$ is shifted from 1624–1642 cm^{-1} in the free ligands to 1620–1628 cm^{-1} upon bonding. The overall changes of the IR spectra of the complexes suggest that fluoroquinolones are coordinated to the metal via the pyridone oxygen and one carboxylate oxygen.[34–37]

3.3 *Mass spectra*

Figure 1 represents the mass spectrum of complex **1**. The proposed mass fragmentation pattern of complex **1** has been given in Supplementary material. The mass spectrum of complex **1** shows molecular ion peak at m/z = 1028.27, 1030.21 and 1032.24 are due to the presence of two chlorine atoms and it also confirms that chlorine atoms are attached to metal atom through covalent bond. The two peaks observed at m/z = 993.30 and 995 are due to loss of chlorine atom in the complex **1**. The peaks observed at m/z = 696.19, 698.16 and 700.11 are due to loss of the pefloxacin. These peaks also confirm the presence of two chlorine atoms. The peaks observed at m/z = 333.07 and 262.19 are observed due to pefloxacin and triphenylphosphine moieties, respectively.

Figure of mass spectrum and proposed mass fragmentation pattern of complex **4** has been provided in Supplementary material. The mass spectrum of complex **4** shows molecular ion peak at 1255.23 and 1257.29 are due to the presence of one chlorine atom and it also confirms that chlorine atom is attached to metal atom through covalent bond. The peak observed at m/z = 1220.34 is due to loss of the chlorine atom in complex **4**. The peak observed at m/z = 923.06 is observed due to loss of pefloxacin. The peaks observed at m/z = 333.28 and 262.38 are observed due to pefloxacin and triphenylphosphine moieties, respectively.

Following important points confirmed that the formation of Ru complexes having different oxidation states.

(i) Ru(III) complexes are reddish brown in colour while, Ru(II) complexes are dark green.
(ii) Ru(III) complexes are air stable while, Ru(II) complexes are air unstable.
(iii) Ru(III) complexes are paramagnetic while, Ru(II) complexes are diamagnetic in nature.
(iv) Ru(III) complexes have lower mass/charge ratio while, Ru(II) complexes have higher mass/charge ratio.

3.4 *In vitro-antimicrobial screening*

All the fluoroquinolones and their ruthenium complexes were screened *in vitro* for their growth inhibitory activity against the pathogenic bacteria *E. coli*,

Table 1. Change in IR bands for interaction of fluoroquinolones with Ru(II) and Ru(III) in addition to triphenylphosphines (4000–400 cm^{-1}).

Compounds	ν(C=O) cm^{-1} pyridone	ν(COO)$_{asym}$ cm^{-1}	ν(COO)$_{sym}$ cm^{-1}	$\Delta \nu$ cm^{-1}	Bands due to PPh$_3$
Pefloxacin	1632	1716^a	–	–	–
Ofloxacin	1634	1728	–	–	–
Sparfloxacin	1643	1714	–	–	–
[Ru(PFL)(PPh$_3$)$_2$Cl$_2$]	1628	1582	1381	201	1450, 1080, 694
[Ru(OFL)(PPh$_3$)$_2$Cl$_2$]	1620	1582	1335	247	1443, 1049, 694
[Ru(SPF)(PPh$_3$)$_2$Cl$_2$]	1628	1558	1355	203	1435, 1095, 694
[Ru(PFL)(PPh$_3$)$_3$Cl]	1628	1580	1342	238	1435, 1065, 694
[Ru(OFL)(PPh$_3$)$_3$Cl]	1628	1583	1370	213	1435, 1065, 694
[Ru(SPF)(PPh$_3$)$_3$Cl]	1628	1578	1348	230	1435, 1065, 694

^{a} as ν(COOH)

P. aeruginosa, *S. marcescens*, *B. subtilis* and *S. aureus*. The results indicate that the complexes exhibit considerable activity compared to free fluoroquinolones against the same microbes under identical experimental conditions and the toxicity of ruthenium chelate increases on increasing the concentration. *In vitro* antibacterial activity data of the pefloxacin, ofloxacin, sparfloxacin and synthesized complexes (**1–6**) are given in table 2. All the Ru(III) complexes (**1–3**) are active against all the microorganisms than those of respective fluoroquinolones. In case of Ru(II) complexes, complex **6** is more active against all the microorganisms than that of sparfloxacin. Complex **4** and complex **5** are less active against *S. aureus* and *P. aeruginosa* than pefloxacin and ofloxacin, respectively. Both these complexes are active than pefloxacin and ofloxacin against *E. coli*, *S. marcescens* and *B. subtilis*. Another interesting finding is Ru(III) complexes show better activity than Ru(II) complexes and free fluoroquinolones.

The possible mode of increased toxicity of the ruthenium complexes compared to that of the free ligands may be explained in terms of Tweedy's chelation theory.[38] Chelation considerably reduces the polarity of
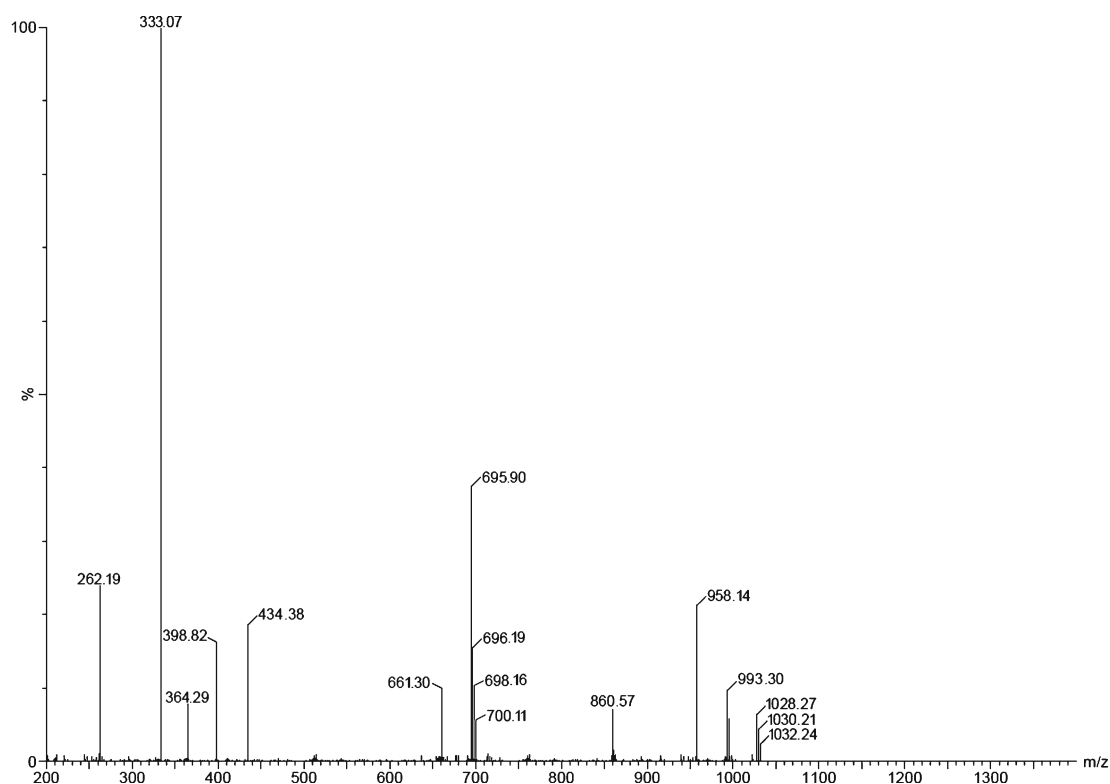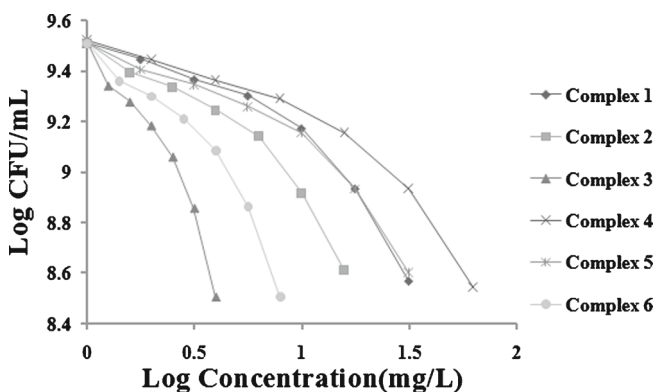

Figure 1. LC-mass spectrum of complex **1**, i.e., [Ru(PFL)(PPh$_3$)$_2$Cl$_2$].

Table 2. Bacteriostatic concentration of fluoroquinolones and complexes (μM).

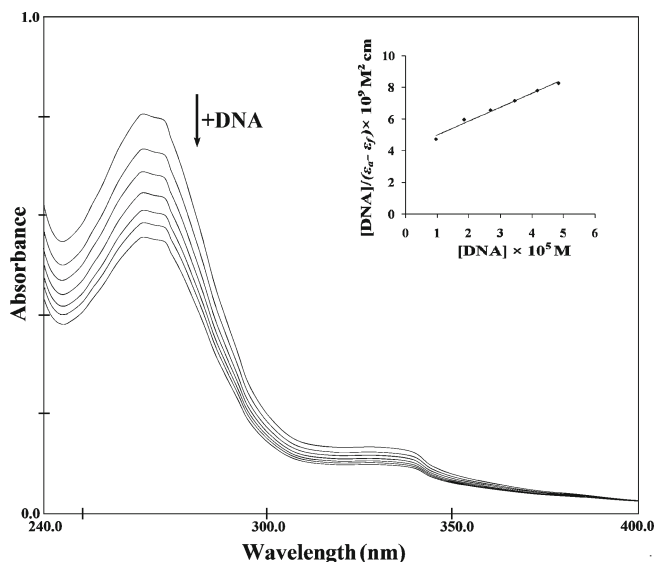| Compounds	Gram-positive		Gram-negative		
	S. aureus	*B. subtilis*	*S. marcescens*	*P. aeruginosa*	*E. coli*
Pefloxacin	1.9	2.2	4.8	5.4	2.44
Ofloxacin	1.7	1.24	1.52	2.0	1.24
Sparfloxacin	1.2	1.9	1.4	1.46	1.2
[Ru(PFL)(PPh$_3$)$_2$Cl$_2$]	1.7	1.85	3.4	5.2	1.8
[Ru(OFL)(PPh$_3$)$_2$Cl$_2$]	1.5	0.8	1.1	1.8	0.9
[Ru(SPF)(PPh$_3$)$_2$Cl$_2$]	0.7	1.4	0.6	1.0	0.8
[Ru(PFL)(PPh$_3$)$_3$Cl]	2.2	2.1	3.8	5.7	2.2
[Ru(OFL)(PPh$_3$)$_3$Cl]	1.9	1.0	1.2	2.2	1.1
[Ru(SPF)(PPh$_3$)$_3$Cl]	1.0	1.7	0.8	1.3	1.0

the metal ion because of partial sharing of its positive charge with donor groups and possible π-electron delocalization over the whole chelate ring. Such a chelation could enhance the lipophililic character of the central metal atom, which subsequently favours it permeation through the lipid layers of cell membrane[39] and blocking the metal binding sites on enzymes of microorganism. The variation in the effectiveness of different compound against different organisms depends either on the impermeability of the cells of the microbes or differences in ribosomes of microbial cells.

In addition to our study regarding MIC, we performed bactericidal activity in terms of CFU/mL of metal complexes against same microorganisms (two Gram$^{(+ve)}$ and three Gram$^{(-ve)}$). Results reveal that decrease in number of colonies with increasing concentration of compounds. The results are shown in figure 2 for all the complexes against *S. aureus*. The number of colonies counted in this technique is in the range 30–300.

3.5 *DNA interaction study*

3.5a *UV–Vis spectra of the complexes in the presence of buffered HS DNA solution*: In general, metal complexes can bind to DNA via both covalent and non-covalent interactions.[40–42] These different modes of binding can be monitored by following the changes in the wavelength and intensity of absorption of a particular peak exhibited by the unbound complex. A decrease in absorbance (hypochromism) or an increase in absorbance (hyperchromicity) upon addition of DNA to a compound in solution is indicative of an interaction between these molecules.[43] An absorption spectrum of complexes with Herring Sperm DNA was recorded for a constant concentration of complexes with varying concentration of DNA to obtain different DNA:complex

Figure 2. Relationship between concentration and bactericidal activity of all complexes against *S. aureus*.

Figure 3. Electronic absorption spectra of [Ru(PFL)(PPh$_3$)$_2$Cl$_2$] in phosphate buffer(Na$_2$HPO$_4$ /NaH$_2$PO$_4$, pH 7.2) in the absence and presence of increasing amount of DNA. Inset: Plot of [DNA]/($\varepsilon_a - \varepsilon_f$) vs. [DNA]. Arrow shows the absorbance change upon increasing DNA concentrations.
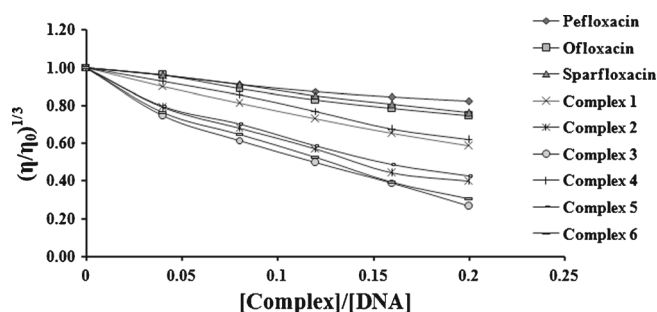
Figure 4. Effect on relative viscosity of DNA under the influence of increasing amount of complexes at $27 \pm 0.1°C$ in phosphate buffer (Na_2HPO_4 /NaH_2PO_4, pH 7.2).

mixing ratio. A representative titration curve is shown in figure 3. The extent of the binding strength of complexes was quantitatively determined by calculating intrinsic binding constant K_b) of the complexes by monitoring the change in absorbance at various concentrations of DNA. From the plot of $[DNA]/(\varepsilon_a - \varepsilon_f)$ versus [DNA] (inset, figure 4).The K_b values of complexes were found in the range 2.14×10^4 to 2.70×10^5 M^{-1} and represented in table 3. The K_b) values of all the complexes are higher than free fluoroquinolones. The K_b) values of all the complexes are lower than [Ru(PEF)Cl$_2$(H$_2$O)$_2$].5H$_2$O (5.00×10^7 M^{-1}),[14] [Ni(sf)$_2$(phen)] (1.13×10^6 M^{-1}),[44] Ni(sf)$_2$(py)$_2$ (3.87×10^7 M^{-1}),[45] [Ni(sf)$_2$(bipy)] 7.75×10^5,[46] [Cu(flmq)(phen)Cl] (2.39×10^5) and [Cu(flmq)$_2$(py)$_2$] (8.12×10^5)[47] and comparable to [Ru(cipro)$_3$].4H$_2$O (2.50×10^4 M^{-1}) [12], [Ni(sf)$_2$(bipyam)] 2.23×10^4.[46] Therefore, the above results indicate that the complexes may bind with DNA either via classical or via partial intercalative mode. The binding mode is further confirmed by viscosity measurements. For a particular drug, Ru(III) complexes have higher binding constant values than Ru(II) complexes. This observation suggest that Ru(III) complexes can bind with DNA better than Ru(II) complexes.

3.5b *Viscosity measurements*: As a means for further clarifying the binding of the ruthenium complexes, viscosity measurements were carried out on HS DNA by varying the concentration of the added complex. Spectroscopic data are necessary, but not sufficient to support a binding mode. Hydrodynamic measurements that are sensitive to length increase (for example, viscosity, sedimentation) are regarded as the most critical tests of binding in solution in the absence of crystallographic structure data. A classical intercalation model demands that the DNA helix lengthens as base pairs are separated to accommodate the binding ligand, which leads to an increase in the viscosity of DNA.[48] However, a partial and/or non-classical intercalation of ligand may bend (or link) the DNA helix, resulting in decreasing its effective length and, concomitantly, its viscosity.[49] The effect of increasing amount of complexes on the relative viscosity of HS DNA is shown in figure 3. For all complexes, relative viscosity of HS DNA solution is decreases upon increasing the concentration ratio of complex to HS DNA. This result is similar to the previously reported [Ru(dmp)$_2$(MCMIP)](ClO$_4$)$_2$,[50] [Ru(dmb)$_2$(pdpt)](ClO$_4$)$_2$.H$_2$O.[51] The partial intercalation from the minor/major groove may act as a 'wedge' to pry one side of a base-pair stack apart, as observed for the Δ-[Ru(phen)$_3$]$^{2+}$,[52,53] but does not fully separate the stack as required by the classical intercalation mode. This would cause a static bend or kink in the helix and decrease the viscosity of HS DNA. The viscosity of Ru(III) complexes is decreasing more than Ru(II) complexes which proves that Ru(III) complexes bind to HS DNA more strongly than Ru(II) complexes. Considering the results of spectroscopic and viscosity measurements synthetically, we suggest that complexes could bind to HS DNA by partial intercalation.

3.5c *Gel electrophoresis technique*: The potential of the present complexes to cleavage DNA was studied by gel electrophoresis using supercoiled pUC19 DNA.

Table 3. The binding constant (K_b) values of compounds.

Compounds	K_b (M^{-1})
Pefloxacin	2.31×10^3
Ofloxacin	2.56×10^3
Sparfloxacin	2.93×10^3
[Ru(PFL)(PPh$_3$)$_2$Cl$_2$]	2.14×10^4
[Ru(OFL)(PPh$_3$)$_2$Cl$_2$]	5.19×10^4
[Ru(SPF)(PPh$_3$)$_2$Cl$_2$]	2.70×10^5
[Ru(PFL)(PPh$_3$)$_3$Cl]	8.42×10^3
[Ru(OFL)(PPh$_3$)$_3$Cl]	1.52×10^4
[Ru(SPF)(PPh$_3$)$_3$Cl]	8.16×10^4

Table 4. Complex mediated DNA cleavage data by gel electrophoresis.

Lane	Compound	Form I (SC)	Form II (OC)	Form III (LC)	% Cleavage
1	DNA	86	14	–	–
2	RuCl$_3$	83	17	–	–
3	Pefloxacin	59	24	17	31.39
4	Ofloxacin	75	15	10	12.79
5	Sparfloxacin	52	29	19	39.53
6	[Ru(PFL)(PPh$_3$)$_2$Cl$_2$](1)	33	51	16	61.62
7	[Ru(OFL)(PPh$_3$)$_2$Cl$_2$](2)	35	49	16	59.30
8	[Ru(SPF)(PPh$_3$)$_2$Cl$_2$](3)	27	49	24	68.60
9	[Ru(PFL)(PPh$_3$)$_3$Cl](4)	37	46	17	56.97
10	[Ru(OFL)(PPh$_3$)$_3$Cl](5)	39	47	14	54.65
11	[Ru(SPF)(PPh$_3$)$_3$Cl](6)	31	48	21	63.95

DNA cleavage is controlled by relaxation of supercoiled form of pUC19 DNA into nicked circular and linear form. When circular plasmid DNA is submitted to electrophoresis, the fastest migration will be observed for the supercoiled form (Form I). If one strand is cleaved, the supercoiled form (SC) will relax to produce a slower-moving open circular form (OC, Form II). If both strands are cleaved, a linear form (L) (Form III) will be generated that migrates in between SC form and OC form.[54] The data of the percentage cleavage of DNA are presented in table 4. As shown in figure 5, no obvious cleavage was observed for the control in which metal complex was absent (DNA alone). From the data, it is clear that complexes can cleave DNA more efficiently than the metal salt and respective free fluoroquinolones. The data show that cleavage efficiency of complex **3** is the highest, while complex **5** has the lowest cleavage efficiency. The Ru(III) complexes can cleave the DNA better than Ru(II) complexes. The different cleaving efficiency may be ascribed to the different DNA-binding affinity of Ru(II) and Ru(III) complexes.

3.6 *Brine shrimp assay*

The Brine shrimp lethality bioassay has been chosen to assess the *in vitro* cytotoxic effects of the compounds, as it is inexpensive, reliable and quick method for the purpose.[23] The technique is easily mastered, of little cost, and utilizes small amount of test material. Since its introduction, this *in vivo* lethality test has been successfully employed for providing a frontline screen that can be backed up by more specific and more sophisticated bioassays once the active compounds have been isolated. Results for the lethality were noted in term of deaths of nauplii. The degree of lethality was found to be directly proportional to the concentration of the compounds. In other words, mortality increased gradually with the increase in concentration of the test samples. A plot of Log of sample's concentration versus percentage of mortality showed a linear correlation. From the graph, the LC$_{50}$ values of the compounds were calculated and they were found in the range from 6.27 to 16.05 µg mL^{-1}. From the data reported in table 5, complex **3** is the most potent amongst all the compounds.
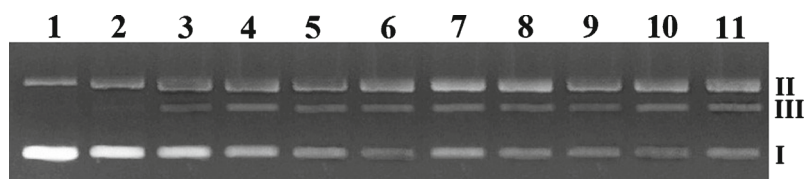

Figure 5. Gel electrophoresis diagram showing the cleavage of SC pUC19 DNA with series of copper(II) complex, incubated at 37°C, using 1% agarose gel, at 50 mV for 1.5 h. Lane 1, DNA control; Lane 2, DNA + RuCl$_3$; Lane 3, DNA + Pefloxacin; Lane 4, DNA + Ofloxacin; Lane 5, DNA + Sparfloxacin; Lane 6, DNA + [Ru(PFL)(PPh$_3$)$_2$Cl$_2$]; Lane 7, DNA + [Ru(OFL)(PPh$_3$)$_2$Cl$_2$]; Lane 8, DNA + [Ru(SPF)(PPh$_3$)$_2$Cl$_2$]; Lane 9, DNA [Ru(PFL)(PPh$_3$)$_3$Cl]; Lane 10, DNA + [Ru(OFL)(PPh$_3$)$_3$Cl]; Lane 11 DNA + [Ru(SPF)(PPh$_3$)$_3$Cl].

Table 5. Effect of compounds on brine shrimp lethality bioassay.

Compound	LC_{50} ($\mu g\ mL^{-1}$)
Pefloxacin	218.77
Ofloxacin	245.47
Sparfloxacin	177.82
$[Ru(PFL)(PPh_3)_2Cl_2]$	15.77
$[Ru(OFL)(PPh_3)_2Cl_2]$	10.57
$[Ru(SPF)(PPh_3)_2Cl_2]$	6.27
$[Ru(PFL)(PPh_3)_3Cl]$	16.05
$[Ru(OFL)(PPh_3)_3Cl]$	11.73
$[Ru(SPF)(PPh_3)_3Cl]$	6.72

Complex **4** is the least potent amongst the synthesized ones. The order of potency of compounds is **3 > 6 > 2 > 5 > 1 > 4**. We have also performed the experiment in case of fluoroquinolones. The LC_{50} values for fluoroquinolones were in the range 177.82 to 245.57 μg mL^{-1}. From these results we can say that, all the complexes show good cytotoxic activity than the respective fluoroquinolones. Another interesting finding is for a particular drug, Ru(III) complexes have higher cytotoxic effect than Ru(II) complexes. The shrimp lethality assay is considered as a useful tool for preliminary assessment of toxicity. Further investigations are required to explore the exact mechanism of their cytotoxic properties, which may be helpful to explore new type of potent cytotoxic agents with the hope of adding new and alternative chemotherapeutic agents in clinical implications.

4. Conclusion

In summary, Ru(III) and Ru(II) complexes have been synthesized and characterized. Their DNA-binding behaviour has been examined by absorption spectroscopy and viscosity measurements. Results supported that Ru(III) and Ru(II) complexes can partially intercalate into DNA base pairs. The present results should be of value for the further understanding of the binding nature of ruthenium complexes to DNA, as well as laying the foundation for the rational design of novel probes for DNA. All the complexes bring about cleavage of plasmid DNA. DNA cleaving ability of complex **3** is higher compared to the rest of the complexes, metal salt and free fluoroquinolones. The compounds synthesized in the present study have shown good cytotoxic activity when screened using Brine Shrimp Lethality Assay. The most important conclusion from this article is that Ru(III) complexes are superior to Ru(II) complexes.

Supplementary information

Structures of fluoroquinolones and complexes, figures of colony forming units for microorganisms like *B. subtilis*, *S. marcescens*, *P. aeruginosa* and *E. coli* are given in supplementary materials (see www.ias.ac.in/chemsci).

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