Original Article

CYTOTOXICITY AND ANTIMICROBIAL ACTIVITY OF MONO-, DI- AND TRINUCLEAR RUTHENIUM(II) POLYPYRIDINE COMPLEXES

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ABSTRACT

Objectives: To evaluate the *in vitro* cytotoxicity, antibacterial and antifungal activity of the synthesized mono-, di- and trinuclear ruthenium(II) polypyridine complexes.

Methods: A series of synthesized ruthenium(II) complexes, R1, R2 and R3, are screened for *in vitro* antiproliferative activity against HepG2 cancer cell line using 96-well plate method. An assay of antimicrobial activity was performed by disc diffusion method. In addition an assay of an antifungal was performed by broth micro-dilution method.

Results: The cytotoxicity of complexes revealed IC_{50} values of 14.52 (R3), 19.53 (R2) and 22.32 μM (R1) against HepG2 cell line in a dose dependent manner. All the complexes inhibited moderately the growth of Gram positive bacteria (G^+) such as *Staphylococcus aureus (MRSA), Eubacterium lentum,* and *Bacillus subtillis*, quite meagerly the growth of the Gram negative bacterium (G^-), *Enterobacter aerogenes,* but did not inhibit at all the growth of *Erwinia amylovora (MTCC 2760)* and showed a slight antifungal activity.

Conclusion: From this study, we could suggest that the systematic increase in number of imidazole moiety along with expanding cloud of conjugated π-electron system of ruthenium(II) polypyridine complexes is responsible for the antiproliferative activity which increases in the order, R1 < R2 < R3 against HepG2 cancer cells. Consequently, the complexes show good antimicrobial activity against Gram positive bacteria, but show poor or no effect against Gram negative bacteria and exhibit a little antifungal activity.

Keywords: HepG2 cancer cells, Cytotoxicity, MTT assay, Antibacterial activity, Antifungal activity, Ruthenium polypyridyl complexes.

INTRODUCTION

Transition metal complexes have occupied the highest place in the field of medical science which deals with antimicrobial [1, 2], anti-cancer activities [3-6], DNA binding ability [7] and chemiluminescence [8]. Ruthenium is one of the transition metals which possesses several favorable properties suited to rationalize anticancer drug design due to a high coordination number as that of platinum, which could provide additional coordination sites to the metal so that it may potentially be used to fine-tune the properties of the complexes [9]. Consequently, researchers have now developed ruthenium complexes well suited towards pharmacological applications as promising anticancer and antimicrobial agents. Two ruthenium drugs under clinical trials, namely NAMI-A and KP1019, have afforded great research interest in anticancer study [9, 10] to overcome the chief limitations of the platinum based drugs [11-13]. Based upon the versatile nature of transition metal complexes and their recent success as anticancer agents [14-16], there has been growing interest in their use as antimicrobial agents as well, and, in particular, ruthenium complexes have been widely examined [17-22]. One of the greatest threats of microbes is that their growing resistance to antimicrobials have lead to the development of new antimicrobial drugs every season [23, 24]. Multidrug resistant bacteria, such as, methicillin-resistant Staphylococcus aureus (MRSA) or extended spectrum beta lactamase (ESBL) producing *Escherichia coli* are critical issues [25]. They have lead in a continuous look for new types of antibacterial compounds. Well-known examples of bacteria that create significant problems are *Staphylococcus aureus, Eubacterium lentum, Bacillus subtilis, Enterobacter aerogenes,* and *Erwinia amylovora (*MTCC 2760).

More than six decades ago, Dwyer and co-workers [26, 27] first illustrated the excellent antibacterial activity of the ruthenium(II) polypyridyl complexes against drug-sensitive strains. However, these complexes were significantly less active against the corresponding current drug-resistant strains. Recently, inert ruthenium complexes (which can only reversibly associate with biological receptors) have been investigated as antimicrobial agents. The unique properties of the ruthenium(II) polypyridyl complexes are that they do not change their structure under physiological conditions and are stable in strong acids and bases [21]. In comparison, inert multinuclear transition metal complexes hold greater potential than inert mononuclear complexes [2, 28]. In addition, recent studies show that multinuclear complexes seem to maintain their activity against drug-resistant bacteria such as MRSA. Consequently, in this study, we have examined the cytotoxicity against HepG2 (Human hepatocellular liver carcinoma cell) and antimicrobial activity of an extensive range of mono-, di- and trinuclear inert polypyridyl ruthenium(II) complexes against three Gram positive, two Gram negative bacteria and six fungi.

Experimental section

Materials and reagents

The complexes [{Ru(phen)₂}(L1)](ClO₄)₂ (R1) (where L1 = 2-benzyloxy-1-naphthyl(1H-imidazo-2-yl[4,5-*f*][1,10] phenanthroline)), [{Ru (phen)₂}₂(L2)](ClO₄)₄ (R2) (where L2 = 1,4-Bi(2-oxymethyl-1-yl(1H-imidazo-2-yl[4,5-*f*][1,10] phenanthroline) naphthyl)benzene and [{Ru(phen)₂}₃(L3)] (ClO₄)₆ (R3) (where L3 = 2,4,6-Trimethyl-1,3,5-tris(2-oxymethyl-1-yl(1H-imidazo-2-yl[4,5-*f*][1,10] phenanthroline) naphthyl)benzene) were synthesized according to method described in the literature [29].

Cell lines and cell culture

The HepG2, a perpetual cell line (Human hepatocellular liver carcinoma cell line), was obtained from the National Center for Cell Science (Pune, India) and grown in Dulbecco's Modified Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100U/ml)/streptomycin (100μg/ml), 2 mM glutamine and 1 mM sodium pyruvate. HepG2 cells were cultured as adherent monolayers and maintained at 37 °C in a humidified atmosphere of

5% CO₂ and 95% air in 100% relative humidity and they were harvested after brief trypsinization.

Microorganisms

Staphylococcus aureus (MRSA), Eubacteriumlentum, Bacillus subtillis, Enterobacteraerogenes, and *Erwinia amylovora (MTCC 2760)* were used for the experiment. All cultures were obtained from IMTECH, Chandigarh, India.

Preparation of inoculums

The bacterial inoculums were prepared by growing the cells in Mueller Hinton Agar (MHA), (Hi-media) for 24 h at 37 °C. These cell suspensions were diluted with sterile MHA to provide initial cell counts of about 10^8 CFU/ml.

Antifungal activity

The following test fungal strains were used for the experiment: *Aspergillus flavus, Botrytis cinerea, Curvularia lunata* (46/01), *Aspergillus niger* MTCC 1344, *Trichophyton rubrum* (57/01) and *T. mentagrophytes* (66/01).

Preparation of fungal spore

The filamentous fungi were grown on SDA slants at 28 °C for 10 days. The spores were collected using sterile double distilled water and stored in refrigerator until used.

Antifungal assays using the broth micro dilution method

Antifungal activity was performed according to the standard reference method. [30] The extracts were dissolved in water with 2% DMSO (concentration of the active compound was 2 mg/ml). The initial test concentration was serially diluted two fold in 96 well plates. Each well was inoculated with 5 µl of suspension containing approximately 10^4 spore/ml. The antifungal agent, fluconazole was used as positive control and a MIC was determined as the lowest

concentration showing no visible fungal growth after incubation time.

Cell growth inhibition study using the MTT assay

The cytotoxicity of the synthesized complexes was measured by using the MTT assay. According to earlier reported procedures, the cell viability was measured by its ability to transform tetrazolium to a purple formazan dye of MTT [31, 32]. Initially, HepG2 cells were plated in 96-well culture clusters (Costar) to grow overnight at a density of 6×10^3 cells/well. When growing at 30% confluence, the HepG2 cells were treated with the test complexes such as R1, R2 and R3 in different concentrations 25µM, 50µM, 75µM and 100µM for 24 and 48 h. After adding 20µL/well of MTT solution (MTT working solution, 5 mg/mL phosphate buffered saline) and being incubated for 5 h, the medium was aspirated and replaced with 100 µL/well of DMSO to dissolve the formazan.

The color intensity of the formazan solution, which reflects the cell growth condition, was quantified at 490 nm using an enzyme-linked immunosorbent assay reader (SpectraMax; Molecular Devices, Sunnyvale, CA). Data were collected from triplicate independent experiments and expressed as mean ± standard deviation.

Disc diffusion method

Petri plates were prepared with 20 ml of sterile Mueller Hinton Agar (MHA) (Hi-media, Mumbai). The test cultures were swabbed on top of the solidified media and allowed to dry for 10 min. The complexes were dissolved in DMSO and 25 µl of the complexes, R1, R2 and R3, were loaded per disc (6 mm diam.). The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for diffusion. Negative control was prepared using respective solvent. Streptomycin (10µg /disc) was used as positive control. The plates were incubated for 24 h at 37 °C for bacteria and 48 h at 30 °C for yeast. The zone of inhibitions was recorded in millimeters [33]. All such experiments were performed duplicate.
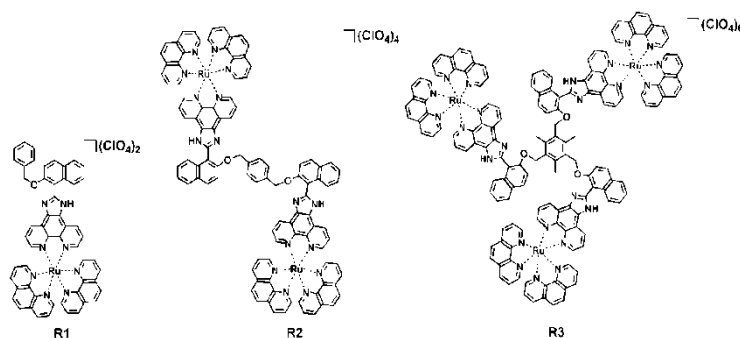

Fig. 1: Structure of series of oligo-homonuclear Ru(II) polypyridine complexes

RESULTS AND DISCUSSION

Synthesis of ruthenium complexes

The ruthenium(II) complexes were prepared by multistep process. At first the organic precursors were prepared by appending 2-hydroxy-1-naphthaldehyde, individually, with benzyl chloride, 1,4-bis (bromomethyl) benzene and with 2,4,6-tris (bromomethyl) mesitylene using a suitable base and by refluxing in suitable solvents. The ruthenium precursor complex was prepared by the addition of phenanthroline and lithium chloride to ruthenium dichloride and stirred to 80 °C in DMF. Once all the precursors were made ready, the respective ligands were prepared by treating the purified organic precursors with 1,10-phenanthroline-5,6-dione and ammonium acetate in glacial acetic acid followed by dilution with water and neutralization with aqueous ammonia.

The ligands, thus prepared when treated with ruthenium precursor complex formed the oligo-homonuclear ruthenium(II) complexes, R1, R2 and R3 and its structure is depicted in fig. 1.

In vitro cytotoxicity study

Experiments were designed to investigate the *in vitro* cytotoxicity of R1, R2 and R3 complexes against HepG2 cancer cell line for the time period of 24 and 48 h. All the complexes exhibit dose-dependent growth inhibitory effect against the tested cell line and the resulting IC_{50} values for the tested compounds are shown in table 1. Complexes exhibit a broad spectrum of inhibition on HepG2 cancer cell with IC_{50} values ranging from 14 to 32 µM revealing cytotoxic effects of the complexes on the cell line and its bar diagram is shown in fig. 2. The complex R3 is especially susceptible to HepG2 cancer cell with lower IC_{50} than other complexes displaying its superior antiproliferative activity. This remarkable cytotoxicity effect improves systematically from R1 to R3 as the multiplicity of π-system [34] of ruthenium(II) complexes increases while IC_{50} values change in the reverse order. In addition, the cytotoxicity effect of these new ruthenium complexes can also be explained by the preference of the non-leaving N-donor containing imidazoles as useful ligands for tuning the physicochemical properties of the metal ions, along with the other coordinated species, because of their

hydrogen bonding properties. This effective hydrogen bonding character increases with increasing number of imidazole moiety in the oligohomonuclear ruthenium(II) complexes [35-41] and as a result, it shows a sequence of increasing antiproliferative activity from R1 to R3.

Table 1: IC$_{50}$ (µM) values of the ruthenium(II) complexes against HepG2 cancer cell line

S. No.	Complex	24 h	48 h
1.	R1	31.40±0.81	22.32±0.69
2.	R2	26.11±0.64	19.53±0.98
3.	R3	21.62±0.83	14.52±0.91

Antimicrobial activity

All the complexes exhibited varying antimicrobial activity towards most of the selected microbes (table 2) and were found to be dose dependent.

Fig. 2: Bar diagram representation of IC$_{50}$ values of complexes R1, R2 and R3 against the HepG2 cancer cell line after 24 and 48 h incubation

All the complexes inhibited moderately the growth of Gram positive bacteria (G+) such as *Staphylococcus aureus (MRSA), Eubacterium lentum,* and *Bacillus subtillis*, quite meagerly the growth of the Gram negative bacterium (G⁻) *Enterobacter aerogenes* but did not inhibit at all the growth of *Erwinia amylovora (MTCC 2760).*

Table 2: Antibacterial activity of ruthenium(II) complexes, R1, R2 and R3.

Bacteria	Gram reaction	Streptomycin (10µg/disc) (mm)	R1 (mm)	R2 (mm)	R3 (mm)
Staphylococcus aureus (MRSA)	G+	26.5±0.892	20.2±0.618	22.5±0.783	23.6±0.640
Eubacterium lentum	G+	28.6±0.945	18.3±0.721	22.3±0.691	26.5±0.758
Bacillus subtillis	G+	14.2±0.917	12.1±0.849	13.4±0.708	12.3±0.969
Enterobacter aerogenes	G⁻	22.5±0.851	11.5±0.733	13.2±0.592	14.3±0.689
*Erwinia amylovora (*MTCC 2760*)*	G⁻	16.3±0.984	0	0	0

In exhibiting the antimicrobial activity, imidazole moiety also plays a key role because of its effective surface activity behavior. The imidazole moiety has the ability to interact directly with the double lipid layer of the membrane structure, probably by binding with the unsaturated fatty acid part of the phospholipid components of the membrane [42]. It is due to the choice of the non-leaving the nature of N-donor containing imidazoles as useful ligands for tuning the physicochemical properties of the metal ions along with its coordinated species and their surface activity behaviors. This effective surface activity character increases with increasing imidazole moiety in the oligohomonuclear ruthenium(II) complexes and as a result, it shows the increasing antimicrobial activity in the order R1 < R2 < R3. Similar to the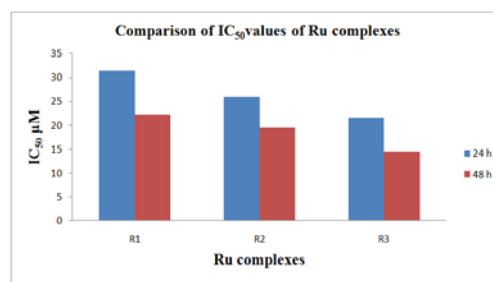 antibacterial activity, all three complexes exhibit different levels of antifungal activity against the six fungi. According to the results, listed in the table 3, the newly synthesized series of ruthenium complexes had promising activities against fungi. This would suggest that the chelation could facilitate the ability of a complex to cross a cell membrane and this can be explained by Tweedy's chelation theory [43]**.** According to the theory, chelation considerably reduces the polarity of the metal ion because of partial sharing of its positive charge with donor groups and possible π-electron delocalization over the whole chelate ring. As a result of chelation, one could enhance the lipophilic character of the metal atom, which subsequently favors its permeation through the lipid layers of cell membrane and blocking the metal binding sites on enzymes of microorganism.

Table 3: Antifungal activity of ruthenium(II) complexes, R1, R2 and R3 (MIC, µg/ml)

Tested fungi	R1 (µg/ml)	R2 (µg/ml)	R3 (µg/ml)	Fl (µg/ml)
Curvularia lunata 46/01	125	31	62	250
T. rubrum 57/01	250	125	125	250
T. mentagrophytes 66/01	250	250	125	250
Botrytis cinerea	250	125	250	1000
Asprgillus flavus	250	250	250	2000
Aspergillus niger MTCC 1344	250	250	250	2000

The other possible mode of action may be explained by Overtone's concept [44]. According to Overtone's concept of cell permeability, the lipid membrane that surrounds the cell favours the passage of only the lipid-soluble materials. It has been observed that different compounds exhibit microbial activity of little variation against bacterial and fungal species. This difference in activity depends either on the impermeability of the cells of the microbes which, in case of Gram +ve, is single layered and in the case of –ve it is multilayered structure [45], or differences in the ribosomes of microbial cells [46]. Also, it has been reported that Gram-negative bacteria have a thin cell wall consisting of a few layers of peptidoglycan surrounded by a second lipid membrane containing lipopolysaccharides and lipoproteins and the Gram-positive bacteria possess a thick cell wall containing many layers of peptidoglycan and teichoic acids. These differences in cell wall structure can produce differences in antibacterial susceptibility and therefore some antibiotics can kill only Gram-positive bacteria and are ineffective against Gram-negative pathogens [47]. This is possible because the cell wall is essential for the survival of bacteria and some antibiotics are able to kill bacteria by inhibiting any one step in the synthesis of peptidoglycan [48]. Therefore the overall results show that the studied complexes are effective against Gram-positive and ineffective against Gram-negative bacteria.

CONCLUSION

A series of mono-, di- and trinuclear ruthenium (II) polypyridyl complexes are thus prepared and their cytotoxicity and antimicrobial activities are studied. From these studies, it could be suggested that the systematic increase of imidazole moiety along with expanding cloud of conjugated π-electron system of ruthenium(II) polypyridine complexes is responsible for the antiproliferative activity against HepG2 cancer cells which increases

in the order R1 < R2 < R3. In addition, the complexes show good antimicrobial activity against Gram positive bacteria, but poor or no effect against Gram negative bacteria and exhibit a little antifungal activity.

CONFLICT OF INTERESTS

Declared None

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