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Short communication

Cytotoxic and genotoxic effects of $[Ru(phi)_3]^{2+}$ evaluated by Ames/*Salmonella* and MTT methods

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ABSTRACT

In this work, we synthesized and evaluated the cytotoxic effect of $[Ru(phi)_3]^{2+}$, on rat C6 glioma cell line. Cell viability was determined by assay with 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The mutagenicity of $[Ru(phi)_3]^{2+}$ was studied in vitro by using two strains of *Salmonella typhimurium* with frameshift mutation (TA98) and base-pair substitution mutation (TA100) were used in plate incorporation assay in the absence of metabolic activation. According to the results, the Ru compound is not toxic but mutagenic, and it shows cytotoxic effect towards C6 rat glioma cells in100 μM.

1. Introduction

In recent years, the attention on ruthenium compounds has increased in the view of cytotoxic and mutagenic effects of these compounds. Octahedral complexes of bidentate ligands with a kinetically inert metal ion, such as $[Ru(phen)_3]^{2+}$ that bind non-covalently to DNA have been of interest in the last several years [1–9]. It is reported that tris(phenanthroline) complexes of ruthenium(II) display enantiomeric selectivity in binding to DNA [10–13].

There are some examples of metal complexes containing the 9,10-phenanthrenequinone diimine (phi) ligand in the literature [14–25]. It was reported that the ability of octahedral complexes possessing quinone diimine ligands to inhibit transcription by stabilization of the DNA duplex structure was investigated. The study of A. M. Pyle and J.K. Barton in 1987 was guide for us [18]; they synthesized $[Ru(phi)_3]C1_2$ complex and reported that the ligand is planar with a large hydrophobic surface that extends far away from the metal; imine protons on the ligand can hydrogen bond with Lewis base substrates or templates and, the phi ligand therefore provides a rich proton source near the metal center in addition to the substantial electron acceptor capability.

Such an interest stems from the tremendous importance of $[Ru(phi)_3]^{2+}$ in the synthesis of biologically active compounds and the use of $[Ru(phi)_3]^{2+}$ themselves as pharmaceutical agents [25,26]. Ruthenium complexes based on ruthenium have been proposed as potential antitumor activity [27]. In vitro studies have been confirming the binding of Ru compounds to the DNA, and cytotoxic activity tests in cultured cells indicate a direct correlation between this activity and binding to the DNA was reported [28]. The *Salmonella*/microsome test system and MTT are widely used to detect the mutagenicity and cytotoxicity of various chemicals.

2. Chemistry

The synthetic route of the compound is outlined in Scheme 1. We have used the literature methods for the synthesis of $[Ru(phi)_3]CI_2$ [18,29]. 9,10-Phenanthrenequinone bis ((trimethylsily1)-imine) was synthesized from 9,10-phenanthrenequinone and sodium bis(trimethylsily1)amide. It's important that the reaction temperature is not greater than 65 °C. The phenanthrenequinone diimine ligand (phi) was chelated in situ by combining the silylated imine ligand with an ethanolic solution of metal chloride.

3. Activity

The cytotoxic effect of $[Ru(phi)_3]CI_2$, on rat C6 glioma cell line was evaluated. Cell viability was determined by assay with

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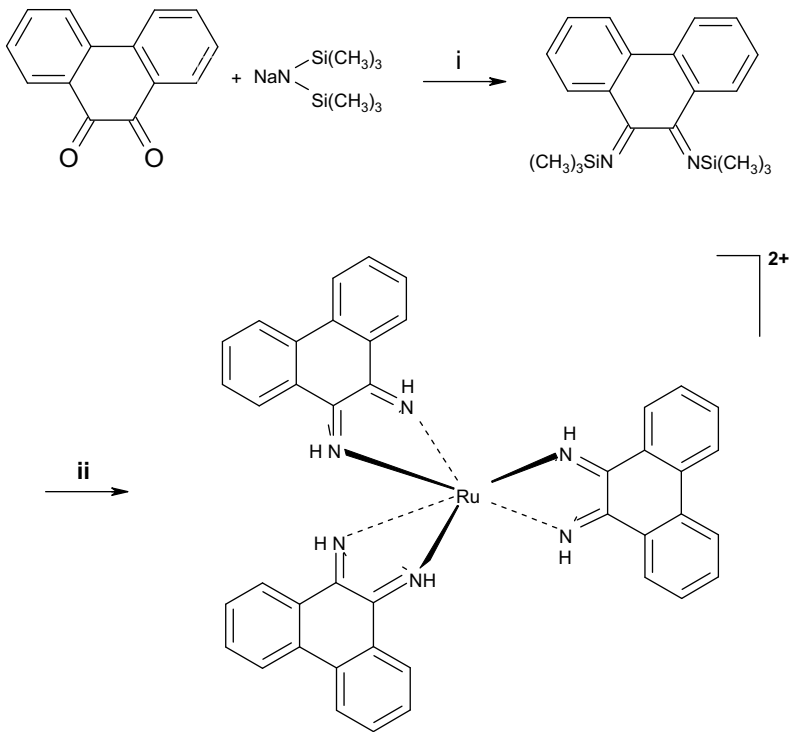

Scheme 1. Synthetic route of $[Ru(phi)_3]^{2+}$; *i*: Ar, benzene, reflux, $SiCl(CH_3)_3$, *ii*: Ar, EtOH/benzene, $RuCl_3$.

3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The mutagenicity of $[Ru(phi)_3]^{2+}$ was studied in vitro by using two strains of *Salmonella typhimurium* with frameshift mutation (TA98) and base-pair substitution mutation (TA100) were used in plate incorporation assay in the absence of metabolic activation.

4. Results, discussion and conclusion

4.1. Activity

4.1.1. Mutagenicity of $[Ru(phi)_3]^{2+}$

Mutagenicity results of $[Ru(phi)_3]^{2+}$compound were displayed in Table 1, Figs. 1 and 2. In order to assess the mutagenic effects of $[Ru(phi)_3]^{2+}$, induction or suppression of revertant colonies was examined in *S. typhimurium* TA98 and TA100.

4.1.2. Cytotoxic activity by MTT

It was observed that by means of MTT methods applied on rat glioma cell line C6 $[Ru(phi)_3]^{2+}$ caused a decrease in the

Table 1
Mutagenicity mean number of revertants induced by $[Ru(phi)_3]^{2+}$, spontaneous control, different concentrations of $[Ru(phi)_3]^{2+}$, positive controls: TA98: NPD (4-nitro-*o*-phenylenediamine) (20 μg/plate) ; TA100: AZS (sodium azide) (1.5 μg/plate) and solvent control (DMSO) values in *Salmonella* plate incorporation test using TA98 and TA100 tester strains

Dose level (μg/plate)	TA98	TA100
Control	38 ± 6	135 ± 14
5000	92 ± 4	275 ± 15
2500	72 ± 5	229 ± 15
1000	62 ± 3	185 ± 10
1000	47 ± 6	154 ± 11
10	39 ± 2	144 ± 6
1	33 ± 3	133 ± 8
0.1	27 ± 3	112 ± 4
NPD, 20	720 ± 265	–
AZS, 1.5	–	795 ± 74
DMSO	28 ± 2	122 ± 11

Mean and ± S.D of three plates (Dunnett's test, $p < 0.05$).

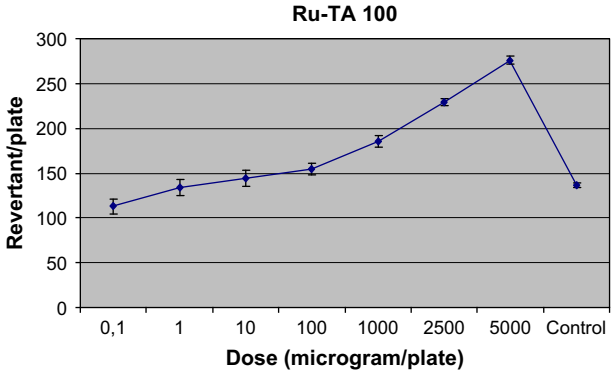

Fig. 1. Mutagenicity of $[Ru(phi)_3]^{2+}$ with *S. typhimurium* TA100 tester strain without metabolic activation (S9) (Dunnett's test, $p < 0.05$).

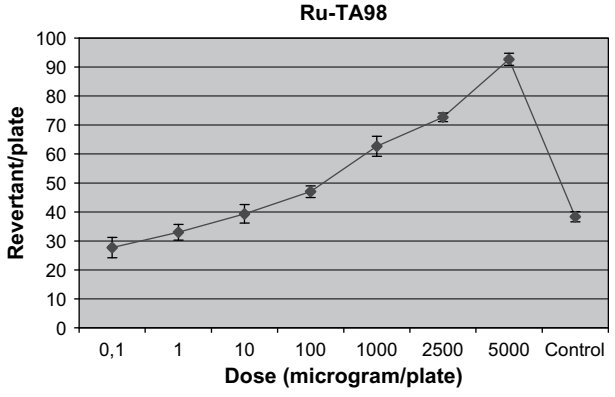

Fig. 2. Mutagenicity of $[Ru(phi)_3]^{2+}$ with *S. typhimurium* TA98 tester strain without metabolic activation (S9) (Dunnett's test, $p < 0.05$).

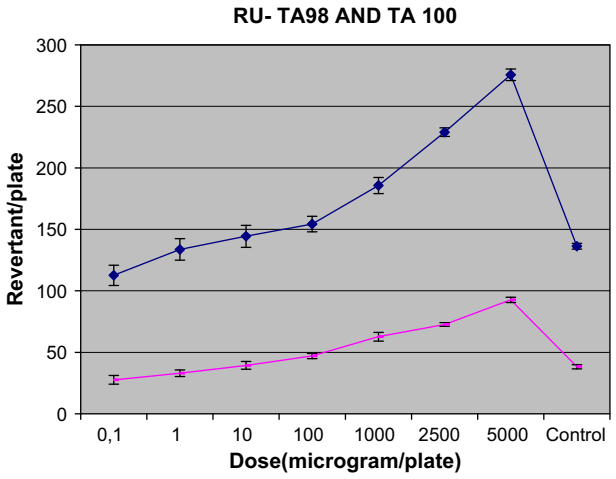

Fig. 3. Mutagenicity of $[Ru(phi)_3]^{2+}$ with *S. typhimurium* TA98 and TA100 tester strains without metabolic activation (S9) (Dunnett's test, $p < 0.05$).

mitochondrial activity at the rate of 43% concentration 100 μM at 24 h in the second day 100 μM 50%, in the third day 100 μM 52%. The results are shown in Figs. 3 and 4.

4.1.3. Ames test

The doses of 0.1 μg/plate, 1 μg/plate, 10 μg/plate, 100 μg/plate of ruthenium compounds were not found to be mutagenically ineffective on TA98 and TA100 strains. The number of the revertant colonies did not exceed the spontaneous values. However, ruthenium compounds at the doses of 1000 μg/plate, 2500 μg/plate, 5000 μg/plate were mutagenically effective on TA98 and TA100 strains depending on the doses.

4.1.4. MTT assay

The $[Ru(phi)_3]^{2+}$ was examined on rat glioma cells (C6) using the MTT assay, a colorimetric determination of cell viability during in vitro treatment with a drug [35]. There appears to be a correlation between cytotoxicity and DNA binding for the representative ruthenium amine anticancer compounds, *cis*-$[Cl_2(NH_3)_4$-Ru(III)$]Cl_2$ and (HIm)[*trans*-$[(Im)_2Cl_4Ru(III)]$ in cell cultures [31]. Also consistent with DNA binding in vivo, a number of ammine, amine and heterocyclic complexes of ruthenium exhibit: inhibition of DNA replication [36], mutagenic activity and induction of the SOS repair mechanism [36], binding to nuclear DNA [37–40], and reduction of RNA synthesis [38–40]. EDTA type complexes of Ru have shown anticancer activity, apparently through DNA binding [39,40].

5. Experimental

5.1. Chemistry

5.1.1. Materials and measurements

$RuCl_3$, 9,10-phenanthrenequinone (Aldrich) and sodium bis (trimethylsily1)amide (Fluka) were purchased from Aldrich organics. All the reagents were used without further purification. The compounds were checked for purity by TLC on silica gel 60 F_{254} (Merck). All melting points (m.p.) were determined in open capillaries on a Gallenkamp apparatus and are uncorrected. Elemental analyses were performed on a CHNS-O Carlo Erba EA 1108 elemental analyser. The infrared spectra were recorded as KBr discs on a Shimadzu IR-470 infrared spectrophotometer in the 4000–600 cm^{-1} region. ^1H NMR spectra (δ, ppm, Hz) were recorded on a Bruker DPX300 spectrometer with $(CD_3)_2SO$ as solvent at room temperature, and tetramethylsilane (TMS) as the internal standard. FAB-MS spectra were recorded on MS-FAB$^+$, VG Quattro mass spectrometer.

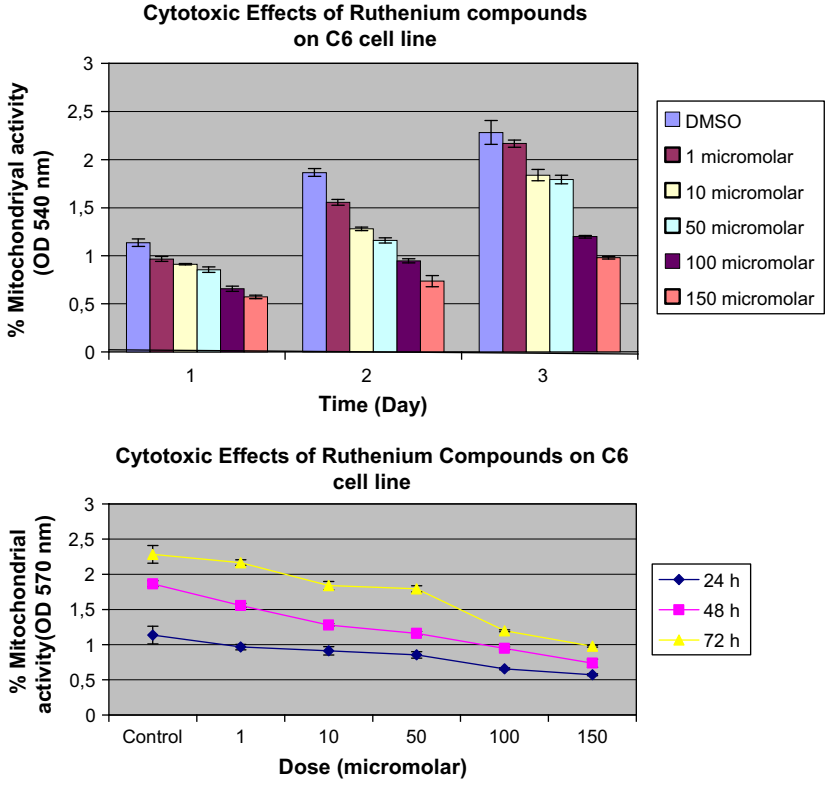

Fig. 4. Dose-dependent action of $[Ru(phi)_3]^{2+}$ as inhibitors of C6 glioma cell line. C6 cell lines were incubated in the presence of Ru and after 24, 48, 72 h at 37 °C, the cellular viability was assessed by the MTT colorimetric method. Data show mean ± S.D of four independent experiments. Analysis of variance (ANOVA); Tukey's *b*; $p < 0{,}001$.

5.1.2. Synthesis of [Ru(phi)$_3$]Cl$_2$

A 1.025-g sample of 9,10-phenanthrenequinone bis-(trimethylsily1)imine (2.9 mmol) dissolved in 75 mL benzene was added to a vigorously stirring suspension of Ru(DMSO)$_4$Cl$_2$ (0.355 g, 0.73 mmol) in 25 mL of EtOH and 75 mL of benzene. All solvents were dried and distilled under nitrogen before use. This mixture was heated at 65 °C for 1 h until a rich purple solution was generated. The reaction vessel was then opened to the air. After the crude reaction mixture was filtered, it was cooled and evaporated to a small volume. [Ru(phi)$_3$]Cl$_2$ was precipitated with diethyl ether and collected on a frit. Solid [Ru(phi)$_3$]Cl$_2$ was washed with acetone. After several diethyl ether precipitations from ethanol solutions, [Ru(phi)$_3$]Cl$_2$ was washed with H_20 to give a final purple yield of 49%. Samples were often further purified by cellulose column chromatography [18].

[Ru(phi)$_3$]$^{2+}$; IR data (KBr disc ν_{max}/cm^{-1}): 3390–3124 (NH), 1582–1474 cm^{-1} (C═C and C═N). ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 7.56–7.98 (9H, m), 8.06–9.84 (15H, m), 13.92–14.2 (6H, br s). For Ru(phi)$_3$C1$_2$·H$_2$0 Anal. Calculated: C, 62.38; H, 3.99; N, 10.39. Found: C, 62.24; H, 4.32; N, 10.05. MS-FAB$^+$: *m*/*z*: 719 [M].

5.2. Activity

5.2.1. Chemicals and tester strains

Nutrient broth and bacto agar were obtained from Oxoid, sodium azide (SAZ) purchased from Merck, biotin, ampicillin trihydrate, histidine and dimethyl sulfoxide (DMSO) were purchased from Sigma.

S. typhimurium strains TA98 and TA100 were kindly supplied by Professor B.N. Ames (University of California, Berkeley, C.A. USA). The confirmation of genetic signs of test strains, the control and the protection of the genetic signs of TA98 and TA100 used in the study were done according to the method of Maron and Ames [30].

5.2.2. Ames cytotoxicity test

The dose of test compounds to be used in the mutation assays was selected in cytotoxicity assay according to Mortelmans and Zeiger. Samples were diluted 10^{-5} fold in DMSO 0.1 mL of different concentration of sample was added to 2 mL top agar, with fresh overnight culture. The top agar was poured onto nutrient agar plates and assessment of cytotoxicity was made after 48 h incubation at 37 °C [31]. Five different concentrations of samples were used for mutagenicity testing.

5.2.3. Salmonella mutagenicity test

In this test histidine-requiring mutants of *S. typhimurium* were used without metabolic activation. Each test strain has a characteristic spontaneous mutant frequency. Spontaneous revertant control value of TA98 is 20–50 and also TA100 is 75–200 colonies.

Mutagenicity determination in the Ames *Salmonella* test requires the evaluation of characteristics of the final population on the minimal glucose agar plate after the 48 h incubation, 0.1 mL of *Salmonella* fresh overnight culture, 0.1 mL of sample, were added to 2 mL of top agar containing 0.2 mL histidine/biotin were added to minimal glucose agar plate under sterile conditions. The tube was mixed by vortexing the tube for about 2 s at low speed and directly poured onto a minimal glucose agar plate. Plates were in triplicate. The plates were inverted and incubated at 37 °C for 48 h [30]. Samples were dissolved in DMSO. Also DMSO was used for solvent control. Positive control chemicals were sodium azide for TA98 and 2-aminoflourene for TA100 without metabolic activation. After 48 h incubation, histidine dependent bacteria were grown with direct proportion to the dose of the sample. Results evaluated as described by Zeiger and colleagues [32].

5.2.4. Cell culture and chemicals

The rat glioma cell line C6 was used for this study. C6 was cultured from 10% fetal bovine serum, Dulbecco's modified Eagle's medium, nutrient mixture Ham's F-12 (Sigma), and 1% penicillin–streptomycin.

5.2.5. MTT assay

For the study of cell viability, 5×10^3 cells were plated in each well of a flat-bottom 96-well culture plate and incubated for 24 h after 24, 48, 72 h exposure to [Ru(phi)$_3$]$^{2+}$ (1, 10, 50, 100, 250 μM doses), the cells were treated with 10 μL MTT and incubated for 3 h at 37 °C in a humidified incubator containing 5% CO_2. To dissolve the cells, we used 100 μL of DMSO and measured the resulting solutions absorbance at 570 nm by microplate reader (Elisa Biotek). The experiments were run 3 times during which 8 samples from each of the doses were examined each time. The viability was determined as the percentage of absorbance of [Ru(phi)$_3$]$^{2+}$-treated cultures compared with those of untreated control and DMSO controlled cultures [33,34].

5.2.6. Statistical analysis

Ames results were compared by analysis of variance followed by a Dunnett's test to compare the treated groups to the control group. MTT values were compared by ANOVA, followed by Tukey's *b* tests for the determination of statistical differences.

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