

Synthesis and biological evaluation of novel mononuclear Ru(II) compounds as potential antiviral and cytotoxic agents

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ABSTRACT

Ruthenium (II) complexes of the type $[Ru(S)_2(K)]^{2+}$, where S=1,10-phenanthroline and K=2-NO₂-phenyl thiosemicarbazone (Compound R₁) and 2-OH-phenyl thiosemicarbazone (Compound R₂) were evaluated for broad antiviral activity testing against a variety of viruses including Varicella Zoster Virus, Herpes Simplex Virus-1&2, Vaccinia Virus and Cytomegalovirus. Cytotoxic evaluation of these compounds were investigated against human embryonic lung cells (HEL), human epithelial cells (HeLa), African green monkey kidney cells (Vero cells), Crandell-Rees feline kidney cells (CRFK) and Madin Darby canine kidney cells (MDCK) using MTS assay. Among the compounds tested, R₁ showed inhibitory activity against cytomegalovirus (EC₅₀: 7.3-11 μM), while R₂ was found to show modest antiviral activity with EC₅₀ values of 27 and 69 μM for feline corona virus and feline herpes virus, respectively. The ruthenium compound R₁ showed more cytotoxic activity against HeLa (MCC: 4μM) and MDCK (CC₅₀: 4.9 μM) cell lines than against HEL, Vero and CRFK cells (CC₅₀ values of ≥ 100 μM). These results suggest that the test Ru(II) compounds might be the potential antiviral agents with good cytostatic potentiality to various cell cultures.

Keywords: Ruthenium, Thiosemicarbazone, Antiviral, Cytotoxicity, MTS assay.

1. INTRODUCTION

Viruses are the smallest infectious agents, consisting essentially of nucleic acid (either DNA or RNA) enclosed in a protein coat or capsid. They are obligate intracellular parasites and thus depend upon the host cell for their replication. Viral infections are very common and responsible for a variety of diseases ranging from the common cold to rabies and AIDS. In contrast to the enormous number of anti-bacterial drugs, much lesser effective antiviral drugs are available. One of the most important reasons for the lack of success in developing antiviral drugs is due to the nature of the viruses, which totally depend upon the host cell for their multiplication and survival. Accordingly many compounds that may cause the death of viruses are also very likely to injure the host cells that harbour them. Although several compounds have potent antiviral activity both in vitro and in vivo at present only a limited number of synthetic compounds and α -interferon have been approved by the FDA for antiviral therapy in humans [1].

However, none of these drugs are without toxicities and hence there is a demand for new antiviral agents, which needs all possible approaches towards the development of new antiviral drugs for the therapy of viral infections for which at present no clinically useful drugs or vaccines are available. Research on drugs based on ruthenium complexes is a fast developing field in medicine, especially in the development of chemotherapeutic agents with minimal side effects and immunity to acquisition of drug resistance [2].

One particular group of Schiff bases as chelating agents that have been investigated over the past decade are the thiosemicarbazones (TSCs). TSCs are potent inhibitors of the enzyme ribonucleotide reductase and are capable of impairing the DNA synthesis and repair [3]. Thiosemicarbazone derivatives have a great importance in chemistry and biology due to their antiprotozoal [4], antibacterial [5], antiviral [6], antifungal [7] and antineoplastic [8] activities. The more electrophilic ruthenium complexes are always less toxic than their corresponding ligands [9]. It has been observed that the presence of certain bulky groups at position N⁴ of the thiosemicarbazone moiety greatly enhances the activity [10]. The synthesis of ruthenium complexes with thiosemicarbazone ligands has been receiving considerable attention due to the pharmacological properties of both complexes and ligands [11]. The thiosemicarbazone ligands usually coordinate to ruthenium through oxygen, nitrogen and sulfur donor atoms in their (N, S) bidentate form [12].

Many of the biological properties have been attributed to ruthenium complexes including anticancer activity [13-18], antinociceptive [19, 20], antioxidant [21, 22], antitubercular [23], antimalarial [24, 25] and antimicrobial activities [26, 27]. Ruthenium-based anticancer chemotherapies are making significant advances in clinical trials. For example, the two ruthenium (III) compounds namely, imidazolium [trans-tetrachloro [1H-imidazole) (S-dimethylsulfoxide) ruthenate (III)] (NAMI-A) [28] and indazolium [trans-tetrachlorobis (1H-

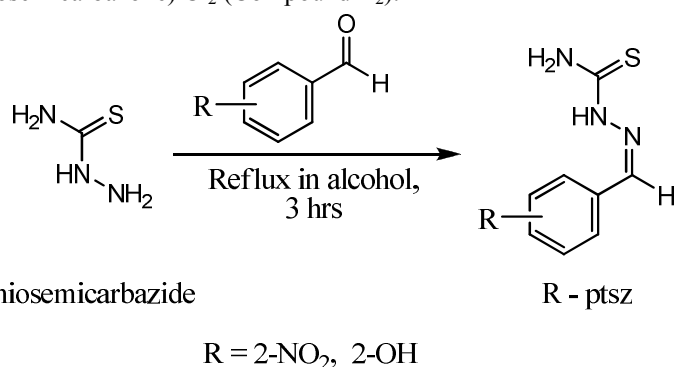
indazole) ruthenate (III)] (KP1019 or FFC14A) [29] as antimetastatic drugs have successfully completed Phase 1 clinical trials and are scheduled to enter Phase 2 trials in the near future.

The two ruthenium complexes of the type thiosemicarbazones (TSCs) with the general formula $[\text{Ru}(\text{S})_2(\text{K})]^{2+}$, where S=1,10-phenanthroline and K=2-NO₂-phenyl

thiosemicarbazone (ptsz) and 2-OH-ptsz, have been studied for in vitro antiviral activity against various viruses including varicella zoster virus (VZV), herpes simplex virus-1 and-2 (HSV-1&2), vaccinia virus and cytomegalovirus (CMV) and cytotoxic activity against HEL, HeLa, Vero Cells, CRFK and MDCK cell lines using the MTT assay.

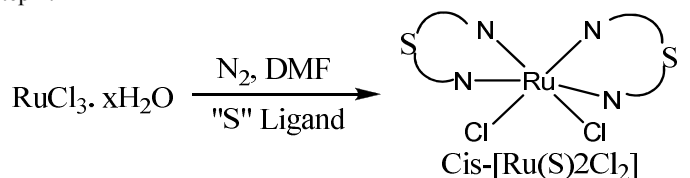
2. EXPERIMENTAL SECTION

The ruthenium complexes R₁ & R₂ were prepared using the synthetic strategy described in Schemes 1-2. The synthesis began by preparation of ptsz ligands². The next step was performed by commercially available ruthenium trichloride with 1,10-phenanthroline. The final ruthenium complexes were synthesized by treating $[\text{Ru}(\text{S})_2\text{Cl}_2]$ with phenyl thiosemicarbazone ligands to provide the corresponded ruthenium complexes Ru (1,10-phenanthroline)₂ (2-nitro phenyl thiosemicarbazone) Cl₂ (Compound R₁) and Ru (1,10-phenanthroline)₂ (2-hydroxy phenyl thiosemicarbazone) Cl₂ (Compound R₂).

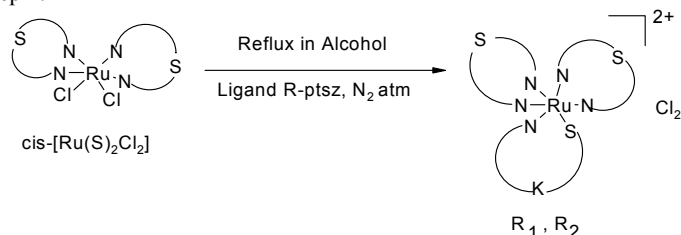


Scheme 1. Synthesis of Nitrogen & Sulphur bearing ligands. (34)

Step 1:



Step 2:



Scheme 2. Synthesis of Ruthenium Complexes (R₁&R₂), where S= 1, 10 phenanthroline, K=2-NO₂-ptsz/2-OH-ptsz, R₁=Ru (1,10-phenanthroline)₂ (2-nitro phenyl thiosemicarbazone) Cl₂, R₂=Ru (1,10-phenanthroline)₂ (2-hydroxy phenyl thiosemicarbazone) Cl₂

2.1. Chemistry.

2.1.1. Preparation of Complexes.

The solvents of AR grade were obtained from S.D Fine Chem., Mumbai, and E. Merck, Mumbai. The reagents (puriss grade) were obtained from Fluka and E. Merck.

Hydrated ruthenium trichloride was purchased from Loba Chemie, Mumbai, and used as received. UV-visible spectra were on a Jasco spectrophotometer. FTIR spectra were recorded in KBr powder on a Jasco V410 FTIR spectrometer by the diffuse reflectance technique. ¹H-NMR spectra were measured in CDCl₃ and DMSO-d₆ on a Bruker Ultraspec 500 MHz/AMX 400 MHz/300 MHz spectrometer. FAB mass spectra were recorded on a JEOL JMS600 spectrum with mNBA matrix.

2.1.2. Preparation of substituted phenyl thiosemicarbazones (R-ptsz)³ (Scheme-1).

A mixture of substituted benzaldehyde (1 mmol) and thiosemicarbazide (1 mmol) in 100 mL of ethanol was refluxed for 3hrs and left Cytotoxicity, MTS assay, overnight. The solid was filtered, dried and purified by recrystallization from alcohol.

2-NO₂-phenyl thiosemicarbazone (2-NO₂-ptsz):

Yield 84%, M.P. 256-258°C (lit.,256°C). IR (KBr) cm⁻¹: 3417-3380 (NH₂& N-H), 3136 (C-H), 2901 (C-H), 1370 (C=S). Calcd. for C₈H₈O₂N₄S: C, 42.85; H, 3.57; N, 25.00. Found C, 42.69; H, 3.52; N, 24.94%. λ_{max} nm (MeOH): 230, 307, 375. ¹H-NMR (DMSO-d₆): δ= 10.36 (1H, s), 9.02 (1H, s), 8.04 (2H, d), 7.83 (2H, d), 7.24 (2H, d, J= 8.6 Hz).

2-OH-phenyl thiosemicarbazone (2-OH-ptsz):

Yield 56%, M.P. 234-235°C (lit.,235°C). IR (KBr) cm⁻¹: 3500-3200 (O-H), 3469-3320 (NH₂ & N-H), 3133 (C-H), 1610 (N-H), 1328 (C=S). Calcd. for C₈H₉N₃OS: C, 49.21; H, 4.64; N, 21.52. Found C, 49.20; H, 4.62; N, 21.28%. λ_{max} nm (MeOH): 242, 321, 398. ¹H-NMR (DMSO-d₆): δ= 12.6 (1H, s), 11.24 (1H, s), 8.07 (1H, s), 7.99 (1H, s), 7.89 (1H, s, -OH), 7.73 (2H, d, J=8.6 Hz), 6.95 (2H, d, J=8.6 Hz).

2.1.3. Preparation of cis-[bis(S)dichlororuthenium(II)] cis-[Ru(S)₂Cl₂]⁴ (Step-1).

RuCl₃ · H₂O, 1g (2.5 mmol) and Ligand S (5 mmol) were refluxed in 50 mL DMF for 3hrs under nitrogen atmosphere. The reddish brown solution slowly turned purple and the product precipitated in the reaction mixture. The solution was cooled overnight at 0°C. A fine microcrystalline mass was filtered off. The residue was repeatedly washed with 30% LiCl solution and finally recrystallized. The product was dried and stored in a vacuum desiccator over P₂O₅ for further use (Yield 75%).

2.1.4. Preparation of [Ru(S)₂(K)Cl₂] (Where S=1,10-phenanthroline; K=2-NO₂-ptsz, 2-OH-ptsz) (Step-2).

To the black microcrystalline cis-[bis(S)dichloroRu(II)] cis-[Ru(S)₂Cl₂] (2 mmol) excess of ligand (2-NO₂-ptsz, 2-OH-ptsz) (2.5 mmol) was added and refluxed in ethanol under nitrogen atmosphere. The initial colored solution slowly changed to

brownish orange at the end of the reaction, which was verified by TLC on silica plates. Then the excess of ethanol was distilled off and this solution was added onto silica gel (60-120 mesh). The product was purified by column chromatography by using silica gel as stationary phase and chloroform-methanol as mobile phase.

Compound R₁ [Ru(phen)₂(2-NO₂-ptsz)Cl₂]:

Yield 44%, black crystals, IR (KBr) cm⁻¹: 3417-3380 (NH₂&N-H), 3136 (C-H) 2958 (C-H), 1370 (C=S). Calcd. for C₃₂H₂₄Cl₂N₈O₂Ru₁S₁: C, 50.79; H, 3.17; N, 14.81. Found C, 50.65; H, 3.15; N, 14.73%. ¹H-NMR (DMSO-d₆): δ ppm: 12.49 (1H, s), 9.04 (1H, s), 8.58 (3H, t), 8.36 (d, J=4.9 Hz, 2H), 8.26 (d, J=8.4 Hz, 2H), 8.06 (3H, t), 8.01 (d, J=5.0 Hz, 2H), 7.96 (3H, m), 7.74 (3H, t), 7.62 (d, 2H, NH₂), 6.23 (2H, d). FAB-MS (m-NBA): 756 [Ru(phen)₂(2-NO₂-ptsz)]²⁺(Cl₂)⁻; 685 [Ru(phen)₂(2-NO₂-ptsz)]²⁺; 505 [Ru(phen)(2-NO₂-ptsz)]²⁺; 462 [Ru(phen)₂]

Compound R₂ [Ru(phen)₂(2-OH-ptsz)Cl₂]:

Yield 46%, black crystals, IR (KBr) cm⁻¹: 3510-3200 (O-H), 3402-3329 (NH₂&N-H), 3036 (C-H), 1611 (N-H), 1328 (C=S). Calcd. for C₃₂H₂₅Cl₂N₇ORu₁S₁: C, 52.81; H, 3.43; N, 13.48. Found C, 52.26; H, 3.39; N, 13.32%. ¹H-NMR (DMSO-d₆): δ ppm: 10.02 (d, J=5.1 Hz, 1H), 9.03 (1H, s), 8.91 (d, J=4.9 Hz, 1H), 8.84 (t, J=8.6 Hz, 2H), 8.63 (d, J=8.4 Hz, 1H), 8.49 (d, J=8.4 Hz, 1H), 8.34-8.20 (6H, m), 8.15-8.08 (2H, m), 7.91 (d, J=5.0 Hz, 1H), 7.81-7.75 (2H, m), 7.68-7.64 (s, 1H, O-H), 7.49-7.45 (1H, m), 6.91 (s, 2H, br, NH₂), 6.73 (d, J=14.6 Hz, 2H), 6.13 (1H, s). FAB-MS (m-NBA): 727 [Ru(phen)₂(2-OH-ptsz)]²⁺(Cl₂)⁻; 656

[Ru(phen)₂(2-OH-ptsz)]²⁺; 475 [Ru(phen)(2-OH-ptsz)]²⁺; 462 [Ru(phen)₂].

2.2. Biological Activities.

Ruthenium compounds (R₁&R₂) were evaluated for their antiviral and cytostatic activity in HEL, HeLa, Vero, CRFK and MDCK cells according to well-established procedures ⁵].

2.2.1. Antiviral assay.

Different cell types were seeded at a density of 5x10³ cells per well in 96-well cell culture plates in suitable medium. Following 24 hrs incubation at 37°C and 5% CO₂, medium was removed and 5-fold serial dilutions of the test ruthenium compounds were added in a total volume of 100 μL, after which the virus inoculum was added to each well. This inoculum resulted in a greater than 90% destruction of the cell monolayer after 3-5 days of incubation at 37°C depending on the nature of the virus. The cytopathicity was determined microscopically or in the presence of a MTS solution that was added to each well. Following two hours of incubation at 37°C, the optical density of each well was then read at 498 nm in a microplate reader to measure the reduction of the MTS dye by cellular dehydrogenases (20 μL MTS for 3hrs at 37°C) into a water soluble colored formazan product. The 50% effective concentration (EC₅₀) was defined as the concentration of compound of which 50% cell viability was protected from the virus-induced cytopathic effect (CPE). The results are shown in Tables 2-4.

Table 2. Antiviral activity of ruthenium compounds against CMV, VZV, HSV, VV and VSV in HEL cell cultures.

Compounds	Effective concentration EC ₅₀ (μM) ^a									
	HEL cell cultures									
	CMV		VZV		HSV-1 (KOS)	HSV-2 (G)	HSV-1 KOS ACV ^r (TK ^r)	Vaccinia virus	Vesicular stomatitis	
	AD-169	Davis	TK+VZV	TK-VZV						
R ₁	11	7.3	>20	>20	>20	>20	>20	>20	>20	
R ₂	>20	>100	>20	>100	>100	>100	>100	>100	>100	
Ganciclovir	7.9	7.9	-	-	0.02	0.01	4	>100	>100	
Cidofovir	0.95	1.3	-	-	2	1	0.9	22	>250	
Acyclovir	-	-	2.1	143	0.4	0.2	22	>250	>250	
Brivudin	-	-	0.019	104	0.04	112	50	4	>250	

^aEffective concentration required to reduce virus-induced cytopathicity by 50%.
CMV: Cytomegalovirus, VZV: Varicella-Zoster virus, HSV: Herpes simplex virus

Table 3. Antiviral activity of ruthenium compounds in HeLa and Vero cell cultures.

Compounds	Effective concentration EC ₅₀ (μM) ^a							
	HeLa cell culture				Verocell culture			
	Vesicular stomatitis virus	Coxsackie virus B4	Respiratory syncytial virus	Para-influenza-3 virus	Reovirus-1	Sindbis virus	Coxsackie virus B4	Punta Toro virus
R ₁	>0.8	>0.8	>0.8	>20	>20	>20	>20	>20
R ₂	>20	>20	>20	>20	>20	>20	>20	>20
DS-5000	1	>100	0.8	>100	>100	>100	>100	>100
(S)-DHPA	>250	>250	>250	>250	>250	>250	>250	>250
Ribavirin	4	112	4	85	146	>250	250	25

^aEffective concentration required to reduce virus-induced cytopathicity by 50%.

Table 4. Antiviral activity of ruthenium compounds against feline corona and feline herpes virus, and influenza viruses.

Compounds	Effective concentration EC ₅₀ (μM) ^a							
	CRFK cell culture		MDCK cell culture					
	Feline Corona Virus	Feline Herpes Virus	Influenza A virus (H ₁ N ₁ subtype)		Influenza A virus (H ₃ N ₂ subtype)		Influenza B virus	
			Visual CPE score	MTS	Visual CPE score	MTS	Visual CPE score	MTS
R ₁	>100	>100	>4	>4	>4	>4	>4	>4
R ₂	27	69	>100	>100	>100	>100	>100	>100
HHA	4.2	11	-	-	-	-	-	-
UDA	1.6	1.2	-	-	-	-	-	-
Ganciclovir	>100	1.4	-	-	-	-	-	-
Ribavirin	-	-	2	3.1	2	2.2	0.8	2.7
Amantadine	-	-	5	3.2	0.9	0.8	>200	>200
Rimantadine	-	-	>200	>200	0.4	0.6	>200	>200

2.2.2. Cytotoxic assay.

MDCK cells were seeded at a density of 5x10³ cells per well of a 96-well plate in suitable media. Twenty four hours later, serial dilutions of the test ruthenium compounds were added. Cells were allowed to proliferate for 3 days at 37°C, after which the cell

number was determined by means of the MTS method. The 50% cytostatic concentration (CC₅₀) was defined as the compound concentration that inhibited the proliferation of exponentially growing cells by 50%. Results are shown in Table 1.

Table 1. Cytotoxicity of Ruthenium compounds.

Compounds	MCC(μM) ^a			CC (μM) ^b	
	HEL	HeLa	Vero	CRFK	MDCK
R ₁	100	4	100	>100	4.9
R ₂	≥100	100	100	>100	>100
Ganciclovir	>350	-	-	>100	-
Cidofovir	>300	-	-	-	-
Brivudin	>300	-	-	-	-
DS-5000	-	>100	>100	-	-
(S)-DHPA	-	>250	>250	-	-
Ribavirin	-	>250	>250	-	-
HHA	-	-	-	>100	-
UDA	-	-	-	26.5	-
Amantadine	-	-	-	-	>200
Oseltamivircarb oxylate	-	-	-	-	>100

^aMinimum cytotoxic concentration (MCC) that causes a microscopically detectable alteration of cell morphology.

^bCytostatic concentration (CC) required to reduce cell growth by 50%.

HEL: Human Embryonic Lung cells, HeLa: Human epithelial cervix carcinoma cells, Vero: African green monkey kidney cells, CRFK: Crandell-Rees Feline Kidney cells, MDCK: Madin Darby Canine Kidney cells

3. RESULTS SECTION

The compounds of the newly synthesized ruthenium complexes were confirmed by UV, FT-IR, ¹H NMR, Mass spectroscopy and C, H, N analysis. In the UV spectra all the ruthenium complexes showed broad and intense visible bands between 340 and 510 nm due to metal to ligand charge transfer transition (MLCT). In the UV region the bands at 280 and 310 nm were assigned to 1,10-phenanthroline ligand π-π* charge transfer transitions. The IR spectras contained the absorption bands

revealing the existence of the NH₂, C=N, C=S gps. The ¹H-NMR spectra of the complex, [Ru(phen)₂(2-NO₂-ptsz)Cl₂] showed 24 resonance peaks (δ 10.03-6.13). The mass spectra of the Complex R₁ gave the anticipated molecular ion peak and main fragmentation peaks, which were in accordance with the title complexes.

Both antiviral activity and cytotoxicity were determined by means of microscopical reading or the MTS method. For each

compound, the 50% effective concentration (EC₅₀) and the minimum cytotoxic concentration (MCC) or the 50% cytostatic concentration (CC₅₀) were determined. Among the tested ruthenium compounds, R₁ afforded more cytotoxic activity against HeLa (MCC of 4 μM) and MDCK (CC₅₀ of 4.9 μM) cell lines than against HEL, Vero and CRFK cells, whereas compound R₂ was found to show less toxicity to the above cells even at concentrations up to 100 μM.

The compounds were also tested for *in vitro* antiviral activity against CMV, VZV, (HSV-1 and -2), influenza virus (A and B), feline corona virus and feline herpes virus, vesicular stomatitis, vaccinia virus, Coxsackie virus B4, respiratory syncytial virus, reovirus-1, Sindbis virus and Punta Toro virus in different cell cultures. From the obtained results it was found that the compound R₁ afforded antiviral activity against both CMV

strains in HEL cell cultures (EC₅₀: 2.3-11 μM). In these assays, the anti-CMV activity of the established cidofovir (EC₅₀: 0.95-1.3 μM) and ganciclovir (EC₅₀: 7.9 μM) was also determined. Thus, the R₁ compound was about equally active against CMV as ganciclovir. The other viruses were not affected (minimal antiviral effective concentration ≤5-fold lower than the minimal cytotoxic concentration) by the R₁ and R₂ compounds, except compound R₂ that was also found to show a modest antiviral activity against feline corona and feline herpes virus with EC₅₀ values of 27 and 69 μM, respectively. It should, however, be noticed that compound R₁ is more cytotoxic than ganciclovir and cidofovir, but may be the rational basis for further exploration of novel related derivatives. The results suggest that the test Ru(II) compounds might be the potential antiviral agents with moderate cytostatic potentiality to various cell cultures.

4. CONCLUSIONS

The present study involves the synthesis of two Ruthenium complexes by reaction with RuCl₃ with thiosemicarbazones. The complexes were purified by column chromatography and characterized by FT-IR, NMR and Mass spectra. The complexes were screened for antiviral activity against various stains of DNA

and RNA viruses and exhibited moderate antiviral activity and compared with reference compounds. Among the two complexes, R₁ could be a potential antiviral agent with good cytostatic potentiality to various cell cultures.

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6. ACKNOWLEDGEMENTS

The authors acknowledge the kind help of Mr. A. Madhukar Reddy, Secretary and Correspondent, S.R. College of Pharmacy, Warangal, India for providing laboratory facilities for the present research work and the technical assistance of Mrs. Leentje Persoons, Mrs. Lies Van den Heurck, Mr. Steven Carmans and Mrs. Anita Camps was greatly appreciated. The antiviral/cytostatic assays were supported by the KU Leuven (GOA 10/14).

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