ORIGINAL ARTICLE

Antinociceptive and antitumor activity of novel synthetic mononuclear Ruthenium (II) compounds

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Background: From the thousands of years, metal compounds have been used in medicine for treatment of various diseases including various types of cancers. Ruthenium was seen as a promising metal due to its similar kinetics to platinum and its lower toxicity. Therefore, we aimed to evaluate the newer mononuclear ruthenium (II) compounds for antinociceptive and antitumor activities. **Materials and Methods:** Ruthenium (II) compounds were evaluated for antinociceptive and antitumor activity using the various *in vitro* and *in vivo* models. The compounds were injected to mice at concentrations of 1 and 2 mg kg^{-1} intraperitoneally and were screened for antinociceptive activity, and the antiproliferative effect was evaluated against murine leukemia cells (L1210), human T-lymphocyte cells (CEM) and human cervix carcinoma cells (HeLa) using MTT assay. **Results:** The results for antitumor activity clearly indicated that compound R_1 was potent cytotoxic agent than R_2 with IC_{50} values ranging from 4-6 µM for R_1, whereas IC_{50} values for compound R_2 ranging from 65-103 µM. The compounds have shown a significant anti-inflammatory effect in carrageenan and dextran models but do not having the central analgesic activity, this indicating that the antinociceptive activity is related to the peripheral nervous system. The results for 5-Lipoxygenase (5-LOX) activity showed that both $R_1$and R_2 compounds were found to be significant 5-LOX inhibitory activity with IC_{50}values of 14.35 µg ml^{-1} and 29.24 µg ml^{-1} respectively. **Conclusion:** These findings concluded that the new ruthenium compounds might be the promising antiproliferative agents as these compounds showing significant 5-LOX inhibitory activity and potential agents in the management of pain related disorders.

Key words: 5-LOX, antinociception, MTT, ruthenium compounds

INTRODUCTION

Although research into metal-based drugs began in the early 1900s, metal compounds have been used in medicine for thousands of years that stimulated research into inorganic medicinal chemistry worldwide. Metal complexes of ruthenium containing nitrogen and oxygen donor ligands are found to be effective catalysts for oxidation, reduction, hydrolysis and other organic transformation.[1] Ruthenium was seen as a promising metal due to its similar kinetics to platinum comparable to cellular division processes[2] and its lower toxicity thought to be due to its ability to mimic iron and therefore bind to biomolecules such as serum albumin and transferrin.[3]

The ruthenium compounds are important tools in inorganic chemistry[4,5] because they possesses multiple applications by inclusion of biologically-active ligands into organometallic complexes, this results in a "metal drug synergism" in which the metal acts as a carrier and stabilizer for the drug until it reaches its target.[6] At the same time, the organic drug carries and protects the metal, preventing side reactions in its transit toward a second target of biological action.[7]

The inflammatory response is a physiological characteristic of vascularized tissues.[8] Increased vascular permeability seen in the inflammatory reaction leads to exudation of fluid rich in plasma proteins including immunoglobulins (antibodies), coagulation factors[9] and cells[10] into the injured tissues with subsequent edema at the site. Nitric oxide (NO) and PGs are involved in inflammation and other related disorders. NO localized in high amounts in inflamed tissues has been shown to induce pain locally and enhances central, as well as peripheral nociception.[11,12] The arachidonic acid metabolizing enzymes cyclooxygenase-2 (COX-2) and 5 lipoxygenase (5-LOX) are involved in the biosynthesis of various proinflammatory lipid mediators. LOX enzyme involved in the pathogenesis of various cancers including colon, lung, breast, prostate, pancreas, bone, brain, and mesothelioma.[13] Experimental studies have demonstrated that 5-lipoxygenase inhibitors have antitumoral properties and 5-lipoxygenase inhibition

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is a rational therapy for certain cancers[14] and for adjuvant approaches to cancer therapy.

Many of biological properties have been attributed to ruthenium complexes, for example, antitumor activity,[15-24] antinociceptive activity,[25,26] the attenuation of reperfusion damage and infarct size[27] and covalent binding to biomolecules.[28]

In view of the above facts, we have designed a novel range of Ru (II) complexes namely, Ru (1, 10-phenanthroline)$_2$ (2-nitro-phenyl thiosemicarbazone) Cl_2 (Compound R_1) and Ru(1, 10-phenanthroline)$_2$ (2-hydroxy-phenyl thiosemicarbazone) Cl_2 (Compound R_2) in relation to their ability to produce antinociception in various models, like carrageenan, dextran induced acute paw edema, hot plate method and acetic acid induced writhing.

The compounds were also investigated for *in vitro* 5-LOX and cytotoxicity activity and its correlation to antiproliferative activity. The present study was clearly concluded that the substitution of the central metal atom of the complexes with different ligands provides major alterations among the observed biological activities.

MATERIALS AND METHODS

Chemicals and drugs

Acetic acid, 5-LOX enzyme kit, Diclofenac sodium, Pentazocine, Indomethacin, Carrageenan, dextran, MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Test substances

The test ruthenium compounds were coded as R_1 and R_2 and were dissolved in less than 1% dimethyl sulfoxide for the various experimental procedures.[29,30]

Experimental animals

Male Swiss albino mice (Mahaveer enterprises, Hyderabad) of average body weight of 25 ± 5 g were used in this investigation. The animals were housed in colony cages in a room where the congenial temperature was 27°C ± 1°C, relative humidity of 45-55%, and 12 h light and dark cycles were maintained. All the animals were acclimatized for a week before the experiment and supplied with a standard animal feed. The study protocol was approved by institutional animal ethics committee (IAEC NO: 1047/ac/07CPCSEA).

In vivo studies

Hot plate test

The hot-plate test was used to measure the response latency according to the method described previously by Eddy and Leimbach,[31] with some modifications. Groups of mice (n = 6) were treated with ruthenium compounds, R_1 (1 and 2 mg kg^{-1}, i.p), R_2 (1 and 2 mg kg^{-1}, i.p), 10 mg $kg^{-1}$of Pentazocine i.p. (positive control), and saline (normal control, 0.9%). Mice were placed on the hot plate which was kept at 56° ± 1°C and the reaction time was noted by observing either the licking of the hind paws or jumping from hot surface at an intervals of 0, 30, 60, 90,120 and 150 min after drug treatment and the time between placement and the first licking of the paws or jumping from hot surface was recorded as the response latency. Thirty seconds cut-off time was imposed in order to avoid any tissue damage and subsequent induction of hypernociception.

Writhing test

The writhing test was carried out as described by Koster *et al.*,[32] with few modifications. Groups of mice (n = 6) were treated intraperitoneally with vehicle (10 ml kg^{-1}) or compounds R_1 and R_2 at doses of 1 and 2 mg.$kg^{-1}$and Diclofenac (20 mg kg^{-1}). Writhing was induced by an i.p. injection of 1% acetic acid solution (1 ml 100 g^{-1} body weight), 15 min after treatment. After injection of the acetic acid solution, the number of writhings (abdominal constrictions) was cumulatively counted over 15-25 min for nociception evaluation.

Significant reductions in number of writhes by drug treatment as compared to control animals were considered as a positive analgesic response. The percent inhibition of writhing was calculated.[33]

$$\% \text{ Inhibition} = WC - WT \times 100$$

Where, WC is the mean number of writhes in control group and WT is the number of writhes in test group. Compounds with > 70% inhibition shows maximum analgesic activity and those with < 70% inhibition, minimum activity.

Carrageenan-induced acute paw edema

The acute anti-inflammatory effect was evaluated by carrageenan induced rat paw edema according to the method of Winter *et al.*[34] Edema was induced by injection of 1% suspension of carrageenan in 0.9% sterile saline solution into the right plantar region of the rat. The test compounds (1 and 2 mg kg^{-1}, i.p), Indomethacin (10 mg kg^{-1}, i.p), were administrated 1 h before injection of carrageenan. The paw volume was measured initially and then at 1, 2, 3, 4 and 6 h after the carrageenan injection by plethysmographic method[35] using Ugo-basile plethysmometer. The inhibitory activity was calculated according to Olajide *et al.*[36]

$$\text{Percentage inhibition} = \frac{(Ct - Co)\text{ control} - (Ct - Co)\text{ treated}}{(Ct - Co)\text{ control}} \times 100$$

Where, *Ct* is displacement volume at *t* time after carrageenan administration and *Co* is displacement volume before carrageenan administration.

Dextran induced paw edema

The paw edema was induced in the right hind paw of rats by sub plantar injection of 0.1 ml of freshly prepared 1% dextran solution. Paw volume was measured at 0, 45, and 90 min after dextran injection. The test compounds were administered in the same above and the percentage of inhibition was calculated.[37]

In vitro studies

5-lipoxygenase enzyme assay

5-Lipoxygenase (5-LOX) enzyme inhibitory activity was measured using the method of Reddanna *et al*,[38] modified by Ulusu *et al*.[39] The assay mixture contained 80 mM linoleic acid and 10 µl potato enzyme 5-LOX in 50 mM phosphate buffer (pH 6.3). The reaction was initiated by the addition of enzyme buffer mix to linoleic acid and the enzyme activity was monitored as the increase in absorbance at 234 nm. The inhibitory potential of the test substances was measured by incubating various concentrations of test substances for 2 min before addition of linoleic acid. Percentage inhibition was calculated by comparing slope or increase in absorbance of test substances with that of control enzyme activity.

Cytotoxicity assay

Cytotoxic activity of the ruthenium compounds were determined using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) reduction assay.[40] Culturing the different cancer cells in a 96-well microtiter plate and add 100 µl of test ruthenium compounds. Incubating them with 20 µl of MTT solution for 2 h. Eighty microliter of lysis buffer was added to each well and the plate was placed on a shaker for overnight. The absorbance was recorded on the plate reader at 562 nm. The absorbance of the test compounds was compared with that of DMSO control to get the % inhibition.

Statistical analysis

Results were expressed as mean ± SEM. The statistical significance of the observed data was determined by One Way ANOVA followed by Dunnet's test.

RESULTS

Hot plate test

In the hot plate test, both the ruthenium compounds (R_1 and R_2) at different doses have not increased the response latency compared to pentazocine, which was shown a significant ($P < 0.001$) increase in response time of paw licking and jumping response from hot surface. Results were shown in Table 1.

Writhing test

The antinociceptive action of test compounds R_1 and R_2 were compared with the diclofenac sodium in the writhing test. The % reductions in the number of abdominal constrictions/writhings were 63.49 and 76.08% for R_1 compound and 51.65 and 66.78% for R_2 compound at 1 and 2 mg kg^{-1} respectively, compared to diclofenac (88.82%). These results suggested that the R_1 compound at 2 mg kg^{-1} had a maximum activity than lower dose and both the doses of R_2. Results were shown in Table 2.

Carrageenan-induced paw edema

In carrageenan induced paw edema model, both the test compounds at concentrations 1 and 2 mg kg^{-1} inhibited the edema formation and the percentage inhibition values were ranging from 66 to 70 for R_1 and 53 to 64 for compound R_2. The inhibitory effect was gradually reduced with increased time but it was found to be increased significantly ($P < 0.001$) in sixth hour. This indicates antinociceptive effect was found to be maximum in early phase due to significant inhibition of histamine/serotonin release and it was also maximum in late phase due to inhibitory activity on prostaglandin synthesis. Results were shown in Table 3.

Dextran induced paw edema

In dextran induced inflammatory model, compound R_1 at 45 min inhibited the dextran induced paw edema by 64.75% and 74.33%, ($P < 0.001$) at the concentration of 1 and 2 mg.kg^{-1} respectively. At 90 min, there was decrease in the percentage of inhibition ($P < 0.01$) by 51.89% and 55.67% respectively. Likewise, 1 and 2 mg.kg^{-1} of R_2 treated groups showed 42.04% and 52.31% inhibition at 45 min and 40.91%

Table 1: Hot plate method

Compounds	Reaction time (seconds)					
	0 min	30 min	60 min	90 min	120 min	150 min
Control	9.16±0.02	9.18±0.02	9.21±0.04	9.22±0.01	9.20±0.01	8.91±0.12
R_1 (1 mg kg^{-1})	8.78±1.02	9.95±1.78	10.57±1.32	9.21±2.01	11.33±3.21	13.33±3.21
R_1 (2 mg kg^{-1})	9.33±2.10	7.13±4.58	12.6±9.86	11.6±4.93	14.73±1.73	9.66±3.78
R_2 (1 mg kg^{-1})	8.66±1.15	8.33±1.52	10.43±2	13.75±1	10.33±1.52	14.66±5.13
R_2 (2 mg kg^{-1})	8.78±1.21	12.33±6.80	9.66±4.04	8.66±1.15	9.33±1.15	11.33±2.51
Pentazocine (10 mg kg^{-1})	9.21±0.01	25.17±0.08*	27.25±0.02*	28.08±0.02*	29.15±0.05*	26.12±1.1*

Values are expressed as mean±SEM. (*n*=6); significance at **P*<0.001 as compared to control

and 52.31% inhibition at 90 min, respectively. Results were shown in Table 4.

5-Lipoxygenase enzyme assay

The test compounds (R_1 and R_2) exhibited a dose dependent 5-lipoxygenase inhibitory activity with IC_{50} values of 14.35 µg ml^{-1} and 29.24 µg ml^{-1} respectively. The test compounds exhibited moderate 5-LOX inhibitory activity, when compared with known standard Nordihydroguaiaretic acid (NDGA) (IC_{50} value of 3.82 µg ml^{-1}).

MTT assay

In vitro cytotoxic profile of the test ruthenium compounds against various cell lines, murine leukemia cells (L1210), human T-lymphocyte cells (CEM) and human cervix carcinoma cells (HeLa) was given in Table 5. The results revealed that the test compounds exhibited dose dependant inhibition of the cell line growth and the compound R_1 was found to be significant cytotoxic agent with IC_{50} values ranging from 4-6 µM, whereas IC_{50} values for compound R_2 ranging from 65-103 µM. This clearly indicates that compound R_1 was showing potent cytotoxic agent than R_2, compared to positive control, Cisplatin.

DISCUSSION

Inflammation is a complex pathophysiological process that has been mediated by a variety of signaling molecules produced by leucocytes, macrophages and mast cells.[41] However, inflammation that is unchecked leads to chronic inflammatory disorders. Although, there are several steroidal and nonsteroidal anti-inflammatory drugs (NSAID's) available to manage inflammatory hyperalgesia, causes undesired and serious side effects. Opioids induce constipation and nausea, whereas NSAIDs possess significant side effects on the gastrointestinal, cardiovascular and renal systems.[42] Therefore, development of new and less toxic drugs is still needed.

Ascending pathways of spinal cord that relays nociceptive information from the periphery to supraspinal central nervous system sites has been studied as potential targets for antinociceptive studies.[43,44] Acetic acid induced writhing and hot plate test are the models of pain that mainly involve peripheral[45] and central[46] mechanisms, respectively.

The hot-plate test is a neurogenic model that produces two kinds of behavioral responses, called paw licking and jumping. Both of these are considered to be supraspinally-integrated responses.[47] Compounds R_1 and R_2 were evaluated in the hot-plate thermal nociception model, which is suitable method for the centrally but not of peripherally acting analgesic drugs. Data obtained from above results concluded that none of these test ruthenium compounds have shown significant central analgesic activity as compared to the pentazocine.

Writhing is a stretch, torsion (twist/rotate) to one side, drawing up of a hind leg, retraction of abdomen and opisthotonus. The acetic acid writhing model was a more convenient assay for nociceptive screening, because the intensity of response depends on the interaction of several factors, neurotransmitters and neuromodulators that determine nociception, such as kinines, acetylcholine, substance *P* and prostaglandins.[48] Since, acetic acid induced writhing can be considered as a model of prostaglandin synthesis sensitive response,[49] the enhanced analgesic effect of ruthenium compounds may be due to inhibition of the synthesis of arachidonic acid metabolites via inhibiting COX. As the test compounds showing significant reduction in acetic acid induced writhings, it was found that antinociceptive activity of test compounds was mediated by both neurogenic and/or inflammatory pain involving peripheral nervous mechanisms.

Carrageenan induced rat hind paw edema is the standard experimental procedure of acute inflammation and it has been widely used for the discovery and evaluation of newer anti-inflammatory drugs with high degree of reproducibility.[34] Carrageenan-induced paw edema in rats

Table 2: Anti-nociceptive activity of test ruthenium compounds on acetic acid induced writhings

Compounds	Number of writhings	% inhibition
Control	58.16±5.35	–
R_1 (1 mg kg^{-1})	21.23±2.76*	63.49
R_1 (2 mg kg^{-1})	13.91±1.98**	76.08
R_2 (1 mg kg^{-1})	28.12±1.09*	51.65
R_2 (2 mg kg^{-1})	19.32±2.01**	66.78
Diclofenac (20 mg kg^{-1})	6.5±0.42**	88.82

Values are expressed as mean±SEM. (*n*=6); significant difference from controls, *P<0.01 and **P<0.001

Table 3: Anti-inflammatory activity of R_1 and R_2 on carrageenan induced rat paw edema

Compounds	% inhibition of paw volume				
	1 h	2 h	3 h	4 h	6 h
R_1 (1 mg kg^{-1})	66.36±9.13***	64.91±9.38***	45.69±14.2**	30.56±7.19*	48.05±11.9**
R_1 (2 mg kg^{-1})	70.33±16.9***	68.87±13.0***	60.01±9.48***	56.51±10.7***	75.81±9.02***
R_2 (1 mg kg^{-1})	53.67±1.73***	58.35±6.16***	36.16±5.5*	32.07±4.31*	41.91±5.7*
R_2 (2 mg kg^{-1})	64.12±11.3***	61.64±8.0***	54.87±6.02***	43.12±9.7*	59.75±6.46**
Indomethacin (10 mg kg^{-1})	59.49±22.3***	53.50±12.9**	45.03±18.3*	60.04±19.6**	72.37±9.80***

Values are expressed as mean±SEM. (*n*=6); significance at *P<0.05, **P<0.01, ***P<0.001 as compared to control

Table 4: Effect of ruthenium compounds on dextran induced paw edema

Compounds	% inhibition of paw volume	
	45 min	90 min
R_1 (1 mg kg^{-1})	64.75±9.54***	51.89±18.7**
R_1 (2 mg kg^{-1})	74.33±13.9***	55.67±23.4**
R_2 (1 mg kg^{-1})	42.04±4.13*	40.91±9.7*
R_2 (2 mg kg^{-1})	52.31±10.1**	50.34±13.5*
Indomethacin (10 mg kg^{-1})	66.18±27.0***	57.25±22.9**

Values are expressed as mean±SEM. (*n*=6); significance at **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to control

Table 5: Inhibitory effects of compounds R_1 and R_2 on the proliferation of murine leukemia cells (L1210), human T-lymphocyte cells (CEM) and human cervix carcinoma cells (HeLa)

Compounds	IC_{50}(μM)		
	L1210	CEM	HeLa
R_1	17±0.1**	6.2±2.2**	4.6±0.6**
R_2	103±7*	69±0.1*	65±2*
Cisplatin	1.2±0.02***	0.51±0.1***	2.2±1.78***

Values are expressed as mean±SEM. Significance at **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to control

is believed to be bi-phasic in response.[50] The first phase of inflammation is due to the release of histamine and serotonin and the second phase is due to the release of bradykinins, protease, prostaglandins and lysosomes.[51] Moreover, carrageenan-induced paw edema is more effectively controlled with arachidonate cyclo-oxygenase inhibitors than arachidonate lipo-oxygenase inhibitors.[52] The results of carrageenan experiment showed maximum activity at first hour after the ruthenium administration. This explains the inhibition of first phase of inflammation. In addition, the antiedemic effect of test compounds was also significantly maintained in late phase of edema development. This may be due to the inhibition of cyclooxygenase enzymes that are involved in the formation of prostaglandins.

Dextran induced paw edema is considered as a consequence of histamine and serotonin liberation from the mast cells.[53] In this study, the dextran-mediated inflammation was reduced probably as a result of antihistaminic effect of the test compounds, which may be due to the inhibition of mast cell degranulation.

Unlike opoids, the ruthenium compounds had a significant effect on the inflammatory phase of the carrageenan and dextran without significant activity in the hot plate test. This suggests that the ruthenium complexes have no analgesic activity in the central nervous system, this indicating that its antinociceptive activity is related to the peripheral nervous system.

5-Lipoxygenase plays an essential role in the biosynthesis of leukotrienes (LTs), proinflammatory mediators which are mainly released from myeloid cells. Indeed, LTB4 inhibits apoptosis[54] and has been shown to be procarcinogenic in several studies.[55] Suppression of leukotrienes and prostaglandin synthesis by interfering with the 5-LOX and COX pathways represent an efficient pharmacological approach for the treatment of inflammatory diseases.[56] Based on the results obtained, the anti-inflammatory activity is due to inhibition of inflammatory mediators by interfering with LOX pathways and might be due to COX inhibitory effect. These data raised the possibility that 5-LOX inhibition may function as stand for cancer chemotherapies alone or combination with COX inhibitors.[57]

The main biological target for nitro compounds is DNA due to their conversion into an electrophilic nitrogen species, giving rise to concern regarding their mutagenic and carcinogenic property.[58] Our results revealed that the compound containing-NO_2 group, showing more potent biological activities than-OH group containing ligand.

CONCLUSION

Based on earlier published findings the working mechanism of their anti-inflammatory activity by either blocking prostaglandins and/or through antioxidant activity may not be ruled out. Future studies will provide new insight into the anti-inflammatory activity of ruthenium compounds and possible mechanism of action, which eventually lead to development of a new class of anti-inflammatory agents.

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