Anti-HIV and cytotoxic ruthenium(II) complexes containing flavones: Biochemical evaluation in mice

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Ru(II) polypyridyl complexes containing 3-hydroxyflavone derivatives as coligands were screened for anti-HIV and cytotoxic activities against eleven tumor cell lines. In order to check the effect of flavones containing Ru(II) complexes \textit{in vivo} on a mammal, a representative complex Ru(LMDMS\textsubscript{0.5}H\textsubscript{2}O (LH-3-Hydroxy-4'-benzyloxyflavone; M\textsuperscript{2+}) was orally administered to adult male mice. Its effects on protein content and LDH were studied in different tissues of the animal. The compound got absorbed and retained in the blood between 1-3 hr after feeding. As compared to the normal and DMSO control sets, tissue specific significant reversible changes in the protein content as well as in LDH activity were observed between 1-4 hr of treatment. However, on polyacrylamide gel electrophoresis, except some tissue specific transitory alterations, expression patterns of five LDH isozymes were unchanged after feeding the compound. The present results suggested that in addition to its potent cytotoxic and anti-HIV effects on cell lines \textit{in vitro}, M\textsuperscript{2+} inhibited LDH activity, but reversibly with a little effect on biosynthetic status of the enzyme in mice.

Keywords: Anti-HIV, Cytotoxicity, Flavones, Ruthenium(II) complexes
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After the discovery of cisplatin as anticancer drug\textsuperscript{1}, the search for other metal ions that could complex with biomolecules and exhibit carcinostatic properties is continued. In this context, ruthenium complexes, because of their interaction with DNA\textsuperscript{2}, have been found to be the best target of the researchers. Several Ru(II) polypyridyl complexes have currently been reported as potential cytotoxic and anti-HIV agents\textsuperscript{3-6}. These bioactivities have been well correlated with the specific modifications of the coordinating ligands also. Therefore, we thought worthwhile to use flavones as co-ligand of Ru(II) complexes owing to their known bioactivities\textsuperscript{7} beside their bidentate coordinating nature.

Significant increase in glycolytic efficiency has been reported to be one of the major characteristics of the neoplastic cells\textsuperscript{8,9}. Lactate dehydrogenase (LDH: EC 1.1.1.27) is a key enzyme of tissue glycolysis in mammals. The enzyme has been reported increased significantly in serum of a variety of cancer patients\textsuperscript{10-12}. Increased level of LDH in malignant tissues signifies unusually high rate of glucose consumption as a prerequisite for selective growth of the tumor\textsuperscript{8,13}. Recently, few drugs targeted to inhibit LDH activity have also been observed to regress a select group of tumors\textsuperscript{13-15}. These findings suggest that LDH can be used as a diagnostic marker as well as a biochemical target of chemotherapy against some of the tumors.

Most of the new anticancer compounds are routinely tested on cell lines. Such experiments may provide precise information, however, it is difficult to correlate the similar effects in the whole organism. In the present study, a new ruthenium Ru(II) complex containing flavones has been synthesized\textsuperscript{7}. After having seen its cytotoxic and anti-HIV effects \textit{in vitro}, physiological acceptability of the compound and its effect \textit{in vivo} on LDH profiles have been studied with a view to assay bioactivities of the complex at organism level.

Materials and Methods

Male AKR adult mice maintained under laboratory conditions were used for the present experiment. All the chemicals used were of analytical grade. Npyruvate, NADH and acrylamide were purchased from Sigma Chemical Co., USA. Microanalysis of C, H and N (performed on a Carlo Erba Elemental Analyzer 1108 and FAB-mass data using JEOL SX-102 mass spectrometer) were carried out at Central Drug Research Institute, Lucknow, India. IR (KBr
pellets) and UV/Vis data were obtained using a JASCO FTIR (5300) spectrometer and Shimadzu 1601 spectrophotometer respectively. $^1$H NMR (CDCl$_3$) was recorded on a JEOL FX 90Q spectrometer.

**Synthesis of Ru(II) complexes containing flavones**—Ligands 3-hydroxy-4'-benzyloxy flavone (LH) and 3-hydroxy-4'-hydroxy flavone (L'H) were prepared as reported earlier.$^{16}$ However, their complexes (Tables 1, 2) with [Ru(bpy)$_3$Cl$_2$] and [Ru(phen)$_2$Cl$_2$]$^{17}$ were prepared and characterized by us recently.$^{7,18}$ Based on the spectroscopic (IR, $^1$H NMR, UV/Vis), elemental analysis and FAB-mass data, proposed structure of the complex is shown in Fig. 1.

**Assay of cytotoxic activity**—Complexes were assayed for their cytotoxicity and anti-HIV properties at the School of Pharmacy, University of North Carolina, USA following a procedure as reported earlier.$^4$ All stock cultures were grown in T-25 flask containing 4 ml of RPMI-1640 medium supplemented with 25 mM of HEPES, 0.25% (w/v) of sodium bicarbonate, 10% (v/v) of fetal bovine serum and 100 µg/ml of kanamycin. Freshly trypsinized cells were seeded with DMSO diluted compounds in a 96-well microtitre plates at 2500-10,000 cells per well. The final DMSO concentration in the well ($\leq 2\%$ v/v) was assayed separately and found to show no effect on IC$_{50}$ of the cell growth in culture.

**Assay of anti-HIV activity**—Inhibition of HIV replication was studied according to the established protocols.$^5$ T-cell line (H9) was maintained in continuous culture with the complete medium (RPMI-1640 with 10% fetal calf serum supplemented with L-glutamine at 5% CO$_2$ and 37°C). Stepwise detailed protocol followed was the same as described recently,$^4$ along with the complex M.$^3$ AZT was also assayed during each experiment as a positive control. Aliquots from normal culture of H9 cells received normal medium in place of HIV and were used as MOCK infected cells. IC$_{50}$ and EC$_{50}$ against MOCK infected H9 cells was estimated and calculated as therapeutic index (TI; ratio of IC$_{50}$ to EC$_{50}$).

**Experimental protocol**—Complex [Ru(LH)(DMSO)$_2$]$_2$H$_2$O (M) was dissolved in DMSO (50 mg/ml) and diluted in triple distilled water (TDW), so that 500 µl of diluted sample contained 12 mg of M. Oral administration of the samples was done through a syringe fitted with a thick and suitably bent needle, so that the compound reaches directly to the digestive tract. Mice were divided into three separate experimental groups with 4-5 animals in each set. The first group of (normal) mice were given 500 µl of TDW; and second group was given orally the same volume of DMSO diluted in TDW (DMSO control). The third group (experimental group; 18-20 mice) was administered orally with 500 µl of test sample containing 12 mg (non-lethal concentration) of the complex. Oral administrations were done at around 11-12 AM to avoid the effect of circadian rhythm (if any). Thereafter, animals were left in their respective cages with the supply of water ad libitum.

Animals of first (control) and second (DMSO treated; control) groups were sacrificed after 4 hr. However, animals (3-4) from third group were sacrificed after 1, 2, 3 and 4 hr of oral administration of the compound. Blood samples were collected in heparinized tubes and the serum collected was used to measure the presence of M.$^3$ Liver, kidney, brain, heart and intestine were quickly removed and washed in normal saline (0.9%, NaCl). Tissue extracts were prepared in 10 volume of 0.02 M Tris-Cl (pH 7.4) using Potter-Elvehjem homogenizer in a cold room maintained at 4°-6°C. The homogenate was centrifuged at 18,000×g and the supernatant collected was used for measuring protein as well as for the studies on LDH.

**Assay of LDH**—LDH was assayed following the method of Kornberg.$^{19}$ Reaction mixture consisted of 0.02 M, Tris-Cl (pH 7.4); 1 mM, Na-pyruvate; and 0.2 mM, NADH. The reaction was started with the addition of suitably diluted enzyme extract and decrease in OD/min was observed at 340 nm in a JASCO 7800 spectrophotometer. Unit of the enzyme
was defined as utilization of μmole of NADH min⁻¹ at 25°C. The activity was expressed as units g⁻¹ wet wt of tissue and units mg⁻¹ protein. Protein content in all the samples was measured using folin method of Lowry et al.²⁰

Monitoring of [Ru(L)₆(DMSO)₃]SH₂O complex—M⁵⁺ was observed to show λₘₐₓ at 440 nm. For monitoring M⁵⁺, suitably diluted blood and tissue samples were subjected for taking the absorbance (OD) at 440 nm. Similarly, diluted samples from normal group of mice were used as blank, while measuring the OD in the experimental samples.

Separation of LDH isozymes—Polyacrylamide gel electrophoresis (PAGE) was performed according to Davis²¹ with certain modifications. Loading samples were prepared in 50 mM of Tris-Cl (pH 6.8) containing glycerol (50%) and bromophenol blue (0.0025%; marker dye). On 10% of polyacrylamide gel, samples containing proteins (25 μg) were loaded in each lane. Electrophoresis was done for 1.5 hr at 30 mA/gel in the cold room. After electrophoresis, gels were subjected to activity stain following the method of Dietze and Lubrano²². Specific staining mixture was composed of 0.125 M, Tris-Cl (pH 7.4); 0.5 mM, MgCl₂; 0.1 mM, li-lactate; 1 mg/ml, NAD; 0.01 M, NaCl; 0.25 mg/ml, nitro blue tetrazolium (NBT); and 0.025 mg/ml, phenyl methyl sulphonate (PMS). After development of LDH bands, gels were washed several times in TDW and photographed.

Results and Discussion

Complexes prepared were found thermally stable in DMSO. During evaluation of cytotoxicity, all the complexes except reported in Table 1 got precipitated. Nevertheless, results indicated that all Ru(II) containing flavones showed potent cytotoxicity against all the cell lines examined. When compared with the cytotoxicity of diazo Ru(II) containing polypyridyl complexes as reported earlier,³ it is evident that the effect is enhanced by introduction of 3-hydroxy flavones (LH) in the complex. Such a potent cytotoxicity of the compound is suggestive of its anti-tumor properties in vitro. Results in Table 2 indicated that all complexes containing flavones showed no effect on viral replication within the cytotoxic range of the complexes tested. However, other Ru (II) complexes have been seen to show strong anti-HIV activities.³⁶ In order to understand the mechanism of cytotoxic and anti-viral actions of these complexes, we have recently demonstrated that Ru(II) polypyridyl complexes containing flavones do not bind to PBR322 DNA.⁷ With a view to extend the work at biochemical level, structurally similar complex bearing two DMSO labile group viz. – [Ru(L)₂(DMSO)₃]SH₂O (M⁵⁺) has been evaluated using LDH profile as a marker for tumor growth.

Table 1—Cytotoxicity (IC₅₀ μg/ml)³ of Ru(II) complexes against different tumor cells

<table>
<thead>
<tr>
<th>Complexes</th>
<th>MCF</th>
<th>CAKI</th>
<th>HOS</th>
<th>KB-VIN</th>
<th>KB</th>
<th>SK-MEL</th>
<th>U87-MG</th>
<th>HCT-8</th>
<th>IA9</th>
<th>A549</th>
<th>FC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ru(L(bpy)₃)]PF₆, M⁵⁺</td>
<td>0.5</td>
<td>2.3</td>
<td>5.5±1.4</td>
<td>&gt;10(8)</td>
<td>0.85</td>
<td>0.81</td>
<td>0.49</td>
<td>4.3±0.1</td>
<td>0.45</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>[Ru(L(phen)₃)PF₆₂H₂O, M⁵⁺</td>
<td>4.2</td>
<td>8.7</td>
<td>&gt;10(5)</td>
<td>&gt;10(9)</td>
<td>3.5</td>
<td>3.8</td>
<td>4.2</td>
<td>&gt;10(32-37)</td>
<td>2.1</td>
<td>4.0</td>
<td>7.7</td>
</tr>
</tbody>
</table>

*Cytotoxicity IC₅₀ is the concentration that causes a 50% reduction in cell population as measured at 562 nm relative to untreated cells.

If inhibition < 50% at 10 μg/ml per cent inhibition observed is the value given in brackets.

MCF = Breast tumour cell; CAKI = Renal tumour cell; HOS = Bone tumour cell; KB = Nasopharynx; SK-MEL-2 = Melanoma; U87-MG = Glioblastoma; HCT-8 = Ileocecal; IA9 = Ovarian; A549 = Lung; FC3 = Prostate

Table 2—Anti-HIV activity of Ru(II) and Fe(III) complexes on H9 cells

<table>
<thead>
<tr>
<th>Complexes</th>
<th>IC₅₀ (μg/ml)</th>
<th>EC₅₀ (μg/ml)</th>
<th>Therapeutic Index (TI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ru (L) (bpy)₃</td>
<td>PF₆, M⁵⁺</td>
<td>0.553</td>
<td>No suppression</td>
</tr>
<tr>
<td>[Ru (L) (phen)₃]PF₆₂H₂O, M⁵⁺</td>
<td>5.29</td>
<td>No suppression</td>
<td>No suppression</td>
</tr>
<tr>
<td>[Ru (LH) (bpy)₂]PF₆₂H₂O, M⁵⁺</td>
<td>5.89</td>
<td>No suppression</td>
<td>No suppression</td>
</tr>
<tr>
<td>[Ru (LH) (phen)₃]PF₆₂EtOH, M⁵⁺</td>
<td>60.4</td>
<td>No suppression</td>
<td>No suppression</td>
</tr>
<tr>
<td>[Ru (L)₆(DMSO)₃]SH₂O, M⁵⁺</td>
<td>41.9</td>
<td>No suppression</td>
<td>No suppression</td>
</tr>
<tr>
<td>AZT</td>
<td>500.0</td>
<td>0.0175</td>
<td>28600</td>
</tr>
</tbody>
</table>

AZT-Azido-thymidine; IC₅₀-Inhibitory concentration for 50% inhibition of viral replication; EC₅₀-Effective concentration for 50% toxicity; TI-the ratio of IC₅₀ to EC₅₀.

No suppression-no effect on viral replication within the survival range (IC₅₀) of H9 cells.
In higher vertebrates, physiologically acceptable compounds have a fixed route of transport from absorption to excretion via blood circulation. Having seen the reproducibility of concentration dependent linearity at 440 nm, absorption profile of the compound (Fig. 2A) was studied in blood and other tissues coming in direct route of the circulation. A linear increase in OD at 440 nm in intestine, blood, heart and kidney of mice up to 3 hr after feeding the compound suggested that M₅ got absorbed across the intestine and kept on increasing in blood up to 3 hr after feeding. It was difficult to collect urine of mice as per our experimental design. However, significant increase of M₅ in kidney after 3-4 hr of feeding the compound (Fig. 2A) might be correlated with excretion timing of the compound. Therefore, it was apparent that after oral administration, the compound had followed a normal physiological route from absorption to excretion in the mice.

A normal pattern of protein synthesis at cellular level is a critical parameter to assess integrity of biosynthetic machinery of the cells. Compared to the normal and DMSO control groups, M₅ treated mice showed only a little variation in the total protein of liver and heart up to 3 hr of treatment (Fig. 2B). A significant but transitory decline in the protein contents in brain, kidney and intestine was observed after 1-2 hr of treatment. Nevertheless, marked recovery (even more than the normal value) in the protein level was observed in all the tissues after 4 hr of treatment. This unusual increase coincided with the decreasing trend of the compound in blood (Fig. 2A). Such an increase in protein content after remaining under heat and other stresses for several hours has been correlated with the synthesis of stress proteins in mammalian cells as a strategy against the stressor.²³ In the present context, being non-native compounds to the mammalian system, DMSO itself and M₅ both might have acted as stressors. Therefore, marked increase in the level of cellular proteins after 4 hr of feeding the compound raised the possibility of synthesis of additional proteins in all the tissues studied under the influence of DMSO and M₅ in vivo.

Few glycolytic enzymes have been found to be fairly interactive with a variety of organic and inorganic compounds.²⁴ Recently, a variety of chemical compounds have been designed as anti-cancer agents targeted to inhibit critical enzymes of DNA synthesis and signal transduction pathway.²⁶ LDH holds a special biochemical importance so far tumor growth and proliferations are concerned.²⁸ Thus, designing a compound to inhibit this enzyme for tumor regression is of special interest.

As compared to the normal and DMSO control sets, the tissues (intestine, kidney and heart), that come under direct route of blood flow, showed 80-90% of decrease in LDH activity within 1 hr of M₅ treatment (Figs 3, 4). Furthermore, continued inhibition of LDH up to 3 hr of treatment in these tissues was indicative of a relationship between contact period and inactivation of LDH in the respective tissues. The explanation got further support from a significant recovery (~60-80%) in the enzyme activity between 3 and 4 hr after the treatment (clearance phase of the compound from blood) in intestine, liver and heart. Similarly, slow rate of recovery in kidney might be correlated with the existence of still high level of the compound in this organ between 3 and 4 hr of treatment (Fig. 2A).

In contrast to liver that receives most of the drugs and metabolites without much hindrance, brain is highly selective in receiving foreign compounds due to blood-brain barrier. Nevertheless, brain is highly sensitive to even a minor stressor present in the blood. Significantly low (< 50%) LDH activity even in DMSO control sets followed by further decrease up to 3 hr in M₅ treated animals also seemed to reflect more
of a stress than the contact effect of the compound in mice brain. This was further indicated by start of recovery in LDH activity after 4 hr of treatment when M5 level started declining in the blood (Fig. 2A).

Though, a further study on tissue concentration and interaction of M5 with the purified LDH is desirable to understand the basic mechanism of action of this compound, the results presented provide preliminary evidence in favour of strong but reversible inactivation of LDH in almost all the tissues studied during the presence of M5 in blood.

With a view to investigate the effect of this compound on biosynthetic activities, tissue specific expression pattern of LDH isozymes were studied after the treatment of M5. The results of this study have been shown in Fig. 5. Liver (L) and kidney (K) of normal mice showed predominant expression of M4-LDH, whereas, heart (H), brain (B) and skeletal muscle (M) showed differential expression of all five molecular forms of the enzyme. DMSO treated group revealed similar pattern in case of H, B and L. However, increased expression of H-LDH was quite evident in case of kidney. Overall, tissue specific isoenzymic patterns of LDH after feeding the compound indicated that biosynthetic patterns of the enzyme were not affected much by M5. However, in comparison to the control animals, DMSO and M5 fed mice could show apparent repression of M4 and increased intensities of H-LDH bands in kidney, liver, intestine and brain up to 2-3 hr of treatment. This might signify a transient increase in aerobic respiration in these tissues as a part of one of the biochemical adaptations against the imposed chemical stress. Such metabolic adaptations via alterations in the isozymic patterns and kinetic properties of LDH have also been reported earlier under varieties of physiological stresses.

Moreover, in comparison to the extent of inhibition of LDH observed in the tissue extracts of treated mice (Figs 3, 4), electrophoretically separated, LDH from the same samples (Fig. 5) showed uninhibited activity stain in the gel. Thus, it seemed that the enzyme got inactivated in tissue extracts either by M5 directly or

![Fig. 3—Activity profiles of LDH in intestine (I), liver (L) and kidney (K) of mice at different time after feeding of M5 complex. C-normal control, CI-DMSO fed mice. Data has been presented as mean ± SEM of 3 observations at each time interval.

* P < 0.001 (control vs other sets).](image)

![Fig. 4—Activity profiles of LDH in brain (B) and heart (H) of mice at different time interval after feeding of M5 complex. C-normal control, CI-DMSO fed mice. Data has been presented as mean ± SEM of 3 observations at each time point.

* P < 0.001 (control vs other sets).](image)
Fig. 5—Expression pattern of LDH isozymes in different tissues of control and in liver (L), brain (B), kidney (K), heart (H) and intestine (I) of DMSO control and M5 fed mice. Samples containing 25 μg proteins were loaded in each lane on 10% non-denaturing PAGE.

by certain transiently produced cellular factors. These were likely to get separated from the enzyme during electrophoresis, making the enzyme free to take sufficient activity stain in the gel. Though, studies on purified LDH may throw some light in this direction, but as per the results presented in the Figs 3, 4 and 5, involvement of some specific cellular factors in bringing out reversible inactivation of LDH during [Ru(L)2(DMSO)2]·5H2O treatment also seemed to be a logical possibility.

A variety of chemically synthesized compounds have recently been reported to show some anti-tumor activities26,31,32 including irreversible inhibition of LDH33. Cisplatin has also been reported to inhibit LDH during regression of lymphomas in mice34. In this context, [Ru(L)2(DMSO)2]·5H2O is evident to be the another strong, but reversible inhibitor of LDH, an enzyme that gets activated during proliferation of tumor in mammalian models13,14. Taking into account of its cytotoxic effects in vitro and physiological acceptability of organism, testing of this compound on tumor bearing animals holds a high promise in this direction. Results also provided biochemical basis for further studies on the mechanism of action of [Ru(L)2(DMSO)2]·5H2O complexes at cellular and macromolecular levels under a variety of pathophysiological conditions in animal models.

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