The purpose of the present study was to investigate and compare the effects of two ruthenium complexes with trifluoperazine on acetylcholinesterase enzyme activity and lactate dehydrogenase levels \textit{in vivo} under physiological conditions in rats blood. Complexes 1 and 2 showed positive effects on acetylcholinesterase at all doses and did not disturb its normal activity. Total LDH activity was inhibited in the presence of both complexes, but Ru(II) complexes showed different effects on the activity of LDH isoenzymes. The activities of LDH₁ and LDH₂ isoenzymes were decreased in all applied doses of the complex 2, while the activity of LDH₂ reduced using complex 1 in the same doses. Results of the present study suggest the neuro- and cardio protective potential of oral administration of complexes 1 and 2, as non-toxic compounds under physiological conditions. These protective effects are the result of their potent antioxidant activity.

\textbf{Key words:} Ruthenium complexes; N-alkylphenothiazines; Acetylcholinesterase; Lactate dehydrogenase

\section*{INTRODUCTION}

Metal complexes of ruthenium have been for more than twenty years in the focus of many researches in chemistry [1-4] as well as in medicine [5,6]. Their properties can be altered by the choice of ligands, which means that complexes potentially have multiple applications. Ruthenium complexes show an antimicrobial activity [5,7] but their most interesting activity is on tumor cells [8-10] with developed resistance to the usual medicinal treatment, including cisplatin. The advantage of ruthenium complexes is their relatively low toxicity [6,11], lower than cisplatin, which can partly be explained by its reduced reactivity to DNA in intact cells. Many biological properties have been attributed to ruthenium complexes including antioxidant activity [12,13] and cytotoxicity [3,9,10]. Anticancer activities of Ru(II)-DMSO complexes are known for

*Corresponding author: e-mail: milena@vet.bg.ac.rs
many years and some of them are in intensively clinical trials [14,15], while Ru(II)-
arene complexes have been also widely studied as anticancerogenic compounds [3,16].
Previous investigations of enzyme activities of ruthenium complexes demonstrated
a slight induction of superoxide dismutase (SOD) and catalase (CAT) in the rat liver
tissue [17], but also an inhibition of succinate dehydrogenase (SDH) and cytochrome
oxidase (COX) activities in the brain, heart, skeletal muscle, liver and kidney of rats
[18]. Ruthenium(II) and ruthenium(III) complexes exhibit antioxidative enzyme
activities (SOD, CAT) and might be responsible for complex–induced cytotoxicity
[19]. Expression of lactate dehydrogenase (LDH) isoenzymes and tumors was
investigated [20] and it was found that inhibition of LDH can be considered one of
the ways to control tumor growth [21]. Ruthenium complexes have a good cytotoxic
effect and their possible applications as non-competitive inhibitors of LDH were
reported [22,23]. Ru(II) complexes can decrease total levels of serum LDH and LDH4
in tumor cells, as well as increase serum glutamate oxaloacetate transaminase (SGOT)
activity, without toxic effects on the liver [23]. A relationship between the increase
of acetylcholinesterase and apoptosis of tumor cells was also reported [24,25].
Acetylcholinesterase (AChE) is a hydrolase in the nervous system that plays a key role
in cholinergic transmission by catalyzing the rapid hydrolysis of the neurotransmitter
acetylcholine (ACh) [26]. This enzyme was identified in the amyloid plaques found in
Alzheimer’s disease and the isoforms of AChE have different effects on the extent of
plaque development [27].

Among the numerous drugs reported, phenothiazine and its N–alkyl derivatives
are specific. Phenothiazines are sulfur- and nitrogen-containing tricyclic aromatic
compounds, famous as dyes in the middle of the nineteen century, after what they
found their application in pharmacy and medicine as antipsychotic, antihistaminic,
antiemetic and analgetic drugs [5, 28]. In the literature are described few mononuclear
complexes with phenothiazines as ligands coordinated in a monodentate fashion
through heterocyclic sulfur [29-31] or through heterocyclic nitrogen [32]. Despite
the description of synthetic derivatives with transition metals [33,34], only partial
biological activities of these compounds were tested.

New Ru(II)-DMSO and Ru(II)-p-cymene complexes with N-alkylphenothiazines were
previously synthesized and characterized by elemental analysis and spectroscopic
methods (FT-IR, UV-Vis, 1H and 13C NMR) [35,36]. These complexes showed effects
on antioxidative enzymes, as well as malondyaldehyde and nitrite levels in rats blood.
Therefore, in vitro cytotoxic activity of complexes was assayed in four human carcinoma
cell lines MCF-7, MDA-MB-453 (breast carcinoma), SW-480 (colon carcinoma) and
IM9 (myeloma multiple cells).

In continuation of our previous research [35,36], the aim of the present study was
to investigate the effect of two ruthenium complexes with trifluoperazine, previously
prepared and characterized in our laboratory (Scheme 1), on acetylcholinesterase
enzyme activity in rats blood as well as their ability to influence LDH levels in vivo
under physiological conditions.
MATERIALS AND METHODS

Chemicals

All reagents and chemicals were purchased from commercial sources. Enzyme activities were measured on CECIL CE 2021 UV/VIS spectrophotometer, while electrophoresis was performed on a vertical device Mini Ve Hoffer, LKB 2117, Bromma, Uppsala, Sweden. The band intensities of the isoenzymes of LDH were estimated using Scion Image Beta 4.02 software for Windows.

The complex (TF.2H)[Ru(DMSO)₃Cl₃]Cl·0.5C₂H₅OH (1) was prepared by the reaction of complex [RuCl₂(DMSO)₄] with trifluoperazine dihydrochloride (TF.2HCl) in absolute ethanol [35]. In the reaction the starting complex [RuCl₂(η⁶-p-cymene)] with TF.2HCl in 2-propanol as solvent, complex 2 (TF.2H)[RuCl₃(η⁶-p-cymene)]Cl·0.5C₃H₇OH·0.5H₂O was synthesized [36]. Both complexes were characterized by elemental analysis, spectroscopic methods and X-ray diffraction as previously described [35,36].

Biological assays

The experiments were carried out on adult male Wistar rats (200–300 g) housed in stainless steel cages with wired floors and with free access to food and water, in a room under controlled conditions (12 h light–dark cycle, temperature 22 ± 2 °C). Rats were randomly assigned to an experimental or to the control group. All experiments were done according to our institutional guidelines for animal research and principles of the European Convention for the Protection of Vertebrate Animals Used for
Rats were divided into seven groups of eight animals each: the control (group I), and three separate groups for each compound treated i.p. with complexes dissolved in water, at doses of 0.4 μmol kg\(^{-1}\) bw (group II), 4.5 μmol kg\(^{-1}\) bw (group III) and 90.4 μmol kg\(^{-1}\) bw (group IV). Blood samples (6-8 ml) were obtained via aorta abdominalis puncture (from all rats, including the control group, 24 h after treatment) and collected in tubes containing sodium-citrate (3.8% w/v) as anticoagulant. Erythrocytes and plasma were separated by centrifugation (3000 rpm), then the erythrocytes were washed in saline solution three times, and the enzymes activities were determined immediately.

**Determination of AChE activity in erythrocytes**

AChE activity was determined as described at an earlier date [37]. The principle of the method is the measurement of the production rate of thiocholine as acetylthiocholine is hydrolyzed. This is accomplished by the continuous reaction of the thiol with 5,5′-dithiobis-2-nitrobenzoate ion to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The rate of color production is measured at 412 nm. Activity was expressed as U/g Hb.

**Determination of LDH activity in plasma**

The total LDH activity in plasma was determined spectrophotometrically according to the method of McQueen [38]. In the reaction between sodium piruvate and NADH at pH 7.2 lactate is formed, which reacts with the enzyme from the plasma. The activity of LDH is determined by the decrease in absorbance at 340 nm and expressed as U/mg proteins.

LDH isoenzymes (LDH\(_{1-5}\)) were detected by vertical electrophoresis on non-denaturing 7% PAGE using a Tris-glycine buffer, following the methods described [39]. The isoenzymes bands were detected using lithium lactate as a substrate in the presence of cofactors NADH and tetrazolium blue. The color intensity of each isoenzyme strip was measured by densitometry. The density of each band was estimated with respect in percent to the total area. The band intensities of the LDH isoenzymes were estimated using Scion Image Beta 4.02 software [40].

**Statistical analysis**

Data are expressed as the means ± SD. Statistical significance was tested by one-way Anova followed by Dunnett’s t-test. The minimum level of statistical significance was set to p < 0.05.
RESULTS

The AChE activity was significantly increased (p < 0.01) by using 4.5 and 90.4 μmol kg\(^{-1}\) bw of complex 1 as present in Fig. 1a. The effect of complex 2 on AChE activity in erythrocytes was different and significantly higher in all applied doses compared to the control group (Fig. 1b). Therefore, almost the same effect was noticed using lower doses of complex 2 (0.4 and 4.5 μmol kg\(^{-1}\) bw). A significant increase of AChE activity with 90.4 μmol kg\(^{-1}\) bw complex 2 was also observed.

![Figure 1](image1.png)

**Figure 1.** AChE activities in rats erythrocytes: I– control group; II – rats treated i.p. with complexes at a dose of 0.4 μmol kg\(^{-1}\) bw; III – rats treated i.p. with complexes at a dose of 4.5 μmol kg\(^{-1}\) bw; IV – rats treated i.p. with complexes at a dose of 90.4 μmol kg\(^{-1}\) bw, a) for complex 1 and b) complex 2; Hb: Haemoglobin

The present study demonstrated that the ruthenium(II) complexes inhibited total LDH activity in all applied doses compared to the control group, however the difference was not statistically significant (p < 0.05) (Fig. 2).

![Figure 2](image2.png)

**Figure 2.** Total LDH activity: I–control group; II – rats treated i.p. with complexes at a dose of 0.4 μmol kg\(^{-1}\) bw; III – rats treated i.p. with complexes at a dose of 4.5 μmol kg\(^{-1}\) bw; IV – rats treated i.p. with complexes at a dose of 90.4 μmol kg\(^{-1}\) bw, a) for complex 1 and b) complex 2

The effects of ruthenium(II) complexes on the activity of LDH isoenzymes are here presented (Table 1 and 2). Complex 1 reduced the activity of LDH\(_{2}\) isoenzyme at all applied doses, while it significantly increased (p < 0.01) LDH\(_{1}\) isoenzyme activity in
a dose of 4.5 μmol kg\(^{-1}\) bw (group III) (Table 1). The activity of LDH\(_3\) isoenzyme increased linearly with increasing doses of complex 1, but the difference was not statistically significant. A dose of 0.4 μmol kg\(^{-1}\) (group II) induced a stronger positive effect on LDH\(_1\), while doses of 4.5 and 90.4 μmol kg\(^{-1}\) of complex 1 had a negative influence and significantly increased (p < 0.05) the activity of LDH\(_4\) and LDH\(_5\), respectively.

**Table 1.** The effects of complex 1 on the activity of LDH isoenzymes

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Isoenzyme</th>
<th>LDH(_1)</th>
<th>LDH(_2)</th>
<th>LDH(_3)</th>
<th>LDH(_4)</th>
<th>LDH(_5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>40.11 ± 3.41</td>
<td>37.28 ± 5.88</td>
<td>9.34 ± 2.49</td>
<td>7.07 ± 2.36</td>
<td>3.23 ± 2.05</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>33.59 ± 7.09**</td>
<td>27.34 ± 14.75</td>
<td>9.94 ± 4.12</td>
<td>17.29 ± 13.49</td>
<td>4.85 ± 2.77</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>47.20 ± 4.87***</td>
<td>20.38 ± 12.62***</td>
<td>11.67 ± 3.77</td>
<td>12.92 ± 4.96**</td>
<td>4.61 ± 2.71</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>42.69 ± 2.54</td>
<td>17.93 ± 6.64***</td>
<td>15.94 ± 7.15</td>
<td>12.26 ± 2.53</td>
<td>8.35 ± 4.09**</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. Significantly different from I group (control) for the same isoenzyme ***p < 0.01, ** p < 0.05

The influence of complex 2 on LDH activity is given in Table 2. Complex 2 caused a significant decline in the activity of LDH\(_2\) in all treated groups with significant inhibition (p < 0.05) at doses of 4.5 μmol kg\(^{-1}\) (group III) and 90.4 μmol kg\(^{-1}\) (group IV) (Table 2). A significant increase (p < 0.05) was also observed in the activity of LDH\(_4\) (group treated with 0.4 μmol kg\(^{-1}\) bw), LDH\(_3\) (group treated with 4.5 μmol kg\(^{-1}\) bw) and LDH\(_5\) (group treated with 90.4 μmol kg\(^{-1}\) bw). A dose of 4.5 μmol kg\(^{-1}\) (group III) induced a very positive effect on LDH\(_2\), while a dose of 90.4 μmol kg\(^{-1}\) had a negative influence and increased the activity of LDH\(_5\).

**Table 2.** The effects of complex 2 on the activity of LDH isoenzymes

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Isoenzyme</th>
<th>LDH(_1)</th>
<th>LDH(_2)</th>
<th>LDH(_3)</th>
<th>LDH(_4)</th>
<th>LDH(_5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>38.78 ± 3.15</td>
<td>35.86 ± 6.29</td>
<td>10.64 ± 2.62</td>
<td>8.08 ± 4.54</td>
<td>5.30 ± 3.81</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>37.98 ± 3.21</td>
<td>29.90 ± 8.50</td>
<td>10.88 ± 3.29</td>
<td>16.44 ± 5.48**</td>
<td>7.15 ± 1.40</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>38.04 ± 7.60</td>
<td>20.98 ± 9.84**</td>
<td>16.03 ± 3.59**</td>
<td>15.18 ± 8.45</td>
<td>9.93 ± 5.05</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>35.30 ± 3.53</td>
<td>23.54 ± 9.95**</td>
<td>10.27 ± 2.91</td>
<td>11.87 ± 4.85</td>
<td>16.29 ± 5.98**</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. Significantly different from I group (control) for the same isoenzyme ** p < 0.05
Reactive oxygen species (ROS) are involved in many biochemical processes in living organisms. Small amounts of ROS, including hydroxyl radicals (\(\cdot\)OH), superoxide anions (\(\cdot\)O\(_2\)) and hydrogen peroxide (H\(_2\)O\(_2\)) are constantly generated in aerobic organisms. Their low levels are vital for a number of cell signaling events and they are essential for proper cell function. Otherwise, high concentrations of ROS result in oxidative stress and as a result damage the biological macromolecules. To protect against oxidative damage, organisms developed a variety of antioxidant defenses that include specialized antioxidant enzymes as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). Ruthenium(II) complexes showed effects on the activity of antioxidative blood enzymes (SOD and CAT) in rats treated i.p. with complexes at three different doses: 0.4, 4.5 and 90.4 μmol kg\(^{-1}\) bw [35,36].

The biological effect of these Ru(II) complexes were investigated. AChE activity as a parameter of their influence on brain function was tested. Acetylcholinesterase is an enzyme present in the nervous tissue, muscle and erythrocytes that catalyzes the hydrolysis of acetylcholine to choline and acetic acid. This enzyme is present throughout the body, but the activity of AChE is higher in motor than sensor neurons. Inhibition of AChE leads to accumulation of acetylcholine in the synapses, signaling is interrupted, which results in dysfunctioning nerve impulses. Many diseases are connected to the inhibition of AChE, as myasthenia gravis, Parkinson’s and Alzheimer’s diseases [41].

Both complexes showed a strong positive effect at the highest dose (4.5 and 90.4 μmol kg\(^{-1}\) bw), while applied in 4.5 μmol kg\(^{-1}\) bw complex 1 increased AChE activity more than complex 2. Conversely, a better effect on AChE complex 2 was shown at lower doses compared to complex 1. As it is well known, the activity of AChE is in correlation with apoptosis of cancer cells and these results agree with cytotoxic effects of complexes 1 and 2 [35,36].

The disturbance of the cell membrane integrity causes an increase in the extracellular concentration of big macromolecules, such as LDH. Lactate dehydrogenase is a tetrameric cytoplasmatic enzyme present in almost all major organs. Its role in the metabolism is to catalyse the oxidation of L-lactate to pyruvate as the final step in the metabolic chain of anaerobic glycolysis. Four peptide chains form two types of lactate dehydrogenase: heart (H) subunit or muscle (M) subunit, which are so named because of their predominance in the respective tissues. LDH has five different isoenzyme forms which catalyze the same biochemical reaction but differ in their molecular structure, and organ specificity. The isoenzymes are separable by electrophoresis. The subunit compositions of five isoenzymes, in order of decreasing anodal mobility in an alkaline medium are: LDH\(_1\) and LDH\(_2\) (found primarily in the heart and red blood cells), LDH\(_3\) (kidneys and brain), LDH\(_4\) (lung and spleen) and LDH\(_5\) (dominantly in the liver and muscle) [42]. Determination of the isoenzyme forms of this enzyme enables us to define the type and the level of cell damage in some tissues [43].
Total LDH activities were reduced by both ruthenium complexes, compared to the activity of this enzyme in the control group. Complex 1 slightly decreased total LDH activity with increasing applied doses. In the case of complex 2 the biological effect was the opposite. Complex 2 caused an increase of total enzyme activity from 0.4 to 90.4 μmol kg⁻¹ bw, but without a significant difference (p < 0.05).

Complex 1 decreases the activity of LDH₃ isoenzyme in all applied doses, while the activities of the LDH₁ and LDH₄ are increased if complex 1 is applied at a dose of 4.5 μmol kg⁻¹ bw. These results are in agreement with previous studies [22] and show a strong inhibition of LDH isoenzymes induced by Ru(II)-DMSO complex. Both complexes show a significant increase in the activity of LDH₅ in the highest applied dose, which may suggest potential hepatotoxic effects, but at the same time these doses of complexes significantly reduce activity of LDH₂ primarily from red blood cells. The activity of LDH₃ isoenzyme is decreased at all applied doses of complex 2, while the effect of this complex on LDH₄ and LDH₅ shows an increase of their activities (Fig. 3). This study clearly demonstrates that LDH isoenzymes are sensitive to all applied doses of the investigated Ru(II)complex.

![Image](Figure 3. Electrophoresis of LDH isoenzymes on non-denaturing 7% PAGE: 1-5– control group; 6-10 – rats treated i.p. with complex 2 at a dose of 0.4 μmol kg⁻¹ bw; 11-15 – rats treated i.p. with complex 2 at a dose of 4.5 μmol kg⁻¹ bw; 16-20 – rats treated i.p. with complex 2 at a dose of 90.4 μmol kg⁻¹ bw)

An important effect of LDH activity inhibition is the decrease anaerobic glycolysis in cells which is crucial for the growth of tumor cells. Earlier researches [23,44] have shown that metal complexes can bind to LDH and thus reduce its activity or change the conformation of the enzyme. Ru(II) complexes 1 and 2 reduce total LDH₃ probably in a similar fashion, which can be connected with previously proved cytotoxicity [35,36].

In summary, biological assays of Ru(II) complexes with N–alkylphenothiazine ligands provide clear evidence that complex 1, under physiological conditions can increase the activity of AChE at higher doses, while complex 2 has a positive influence at all applied doses. These results suggest that the complexes do not lead to enhanced production of acetylcholine and thus do not cause neuromuscular diseases.

Results of LDH isoenzymes activities measuring exhibit different sensitivity, depending on the applied concentration of the complexes 1 and 2, but also a positive effect on the heart muscle due to the decreased activity of isoenzymes LDH₂ with increasing
concentration of these complexes, compared to the control group. Also, complex 1 does not damage the brain tissue (LDH₃ is not significant increased, while AChE activity significantly increased), while both complexes show positive effects on liver in lower doses (LDH).

Results of the present study suggest the neuro- and cardioprotective potential of orally administered complexes 1 and 2, as non-toxic compounds under physiological conditions, induced through its potent antioxidant activity. This investigation confirms a great potential of Ru(II)-cymene complexes as pharmacological drugs and potential biological compounds with promising applications. The whole mechanism of influence of Ru(II) complexes on cancer cells via anaerobic glycolysis will be the subject of our future research.

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Authors’ contributions
KM synthesized and characterized compounds and participated in the design of the experiment and drafted the manuscript. BS participated in the design of the study, biochemical analysis and interpretation of results, performed the statistical analysis and helped to drafted the manuscript. SS and GŠS participated in the design of compounds and their characterization. PJ helped to the interpretation of characterization compounds. All authors read and approved the final manuscript.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**IN VIVO ISPITIVANJE ENZIMSKE AKTIVNOSTI NEKIH Ru(II) JEDINJENJA SA N-ALKILFENOTIAZINIMA**

KRSTIĆ P. Milena, BOROZAN Z. Sunčica, SOVILJ P. Sofija, GRGURIĆ-ŠIPKA R. Sanja, POLJAREVIĆ M. Jelena

Gaji ovog rada je da se ispitaju i uporede efekti dva kompleksa rutenijuma sa trifluoperazinom na aktivnost enzima acetilholinesteraze i laktat-dehidrogenase in vivo pod fiziološkim uslovima u krvi pacova. Kompleksi 1 i 2 pokazali su pozitivan efekat na aktivnost acetilholinesteraze u svim primenjenim dozama. Ukupna aktivnost LDH je inhibirana u prisustvu oba kompleksa, ali kompleksi Ru(II) pokazuju različite rezultate na izoenzimske oblike ovog enzima. Aktivnosti izoenzima LDH1 i LDH2 su smanjene u svim primenjenim dozama kompleksa 2, dok kompleks 1 smanjuje aktivnost samo izoenzima LDH2 u tim istim koncentracijama. Rezultati prikazanog istraživanja ukazuju na neuro - i kardio zaštitni potencijal oralne primene kompleksa 1 i 2, kao netoksičnih jedinjenja pod fiziološkim uslovima, indukovano preko njihovog snažnog antioksidativnog efekta.