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Structural and functional characterization of an organometallic ruthenium complex as a potential myorelaxant drug



Tomaž Trobec^a, Monika C. Žužek^a, Kristina Sepčič^b, Jerneja Kladnik^c, Jakob Kljun^c, Iztok Turel^c, Evelyne Benoit^d, Robert Frangež^{a,*}

^a Institute of Preclinical Sciences, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia

^b Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia

^c Department of Chemistry and Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Večna pot 113, 1000 Ljubljana, Slovenia

^d Université Paris-Saclay, CEA, Institut des sciences du vivant Frédéric Joliot, Département Médicaments et Technologies pour la Santé (DMTS), Service d'Ingénierie Moléculaire pour la Santé (SIMoS), ERL CNRS/CEA 9004, 91191 Gif sur Yvette, France

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ABSTRACT

In addition to antibacterial and antitumor effects, synthetic ruthenium complexes have been reported to inhibit several medicinally important enzymes, including acetylcholinesterase (AChE). They may also interact with muscle-type nicotinic acetylcholine receptors (nAChRs) and thus affect the neuromuscular transmission and muscle function. In the present study, the effects of the organometallic ruthenium complex of 5-nitro-1,10-phenanthroline (nitrophen) were evaluated on these systems. The organoruthenium-nitrophen complex $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{nitrophen})\text{Cl}]\text{Cl}$; $\text{C}_{22}\text{H}_{21}\text{Cl}_2\text{N}_3\text{O}_2\text{Ru}$ (C1-Cl) was synthesized, structurally characterized and evaluated *in vitro* for its inhibitory activity against electric eel acetylcholinesterase (eeAChE), human recombinant acetylcholinesterase (hrAChE), horse serum butyrylcholinesterase (hsBChE) and horse liver glutathione-S-transferase. The physiological effects of C1-Cl were then studied on isolated mouse phrenic nerve-hemi-diaphragm muscle preparations, by means of single twitch measurements and electrophysiological recordings. The compound C1-Cl acted as a competitive inhibitor of eeAChE, hrAChE and hsBChE with concentrations producing 50 % inhibition (IC_{50}) of enzyme activity ranging from 16 to 26 μM . Moreover, C1-Cl inhibited the nerve-evoked isometric muscle contraction ($\text{IC}_{50} = 19.44 \mu\text{M}$), without affecting the directly-evoked muscle single twitch up to 40 μM . The blocking effect of C1-Cl was rapid and almost completely reversed by neostigmine, a reversible cholinesterase inhibitor. The endplate potentials were also inhibited by C1-Cl in a concentration-dependent manner ($\text{IC}_{50} = 7.6 \mu\text{M}$) without any significant change in the resting membrane potential of muscle fibers up to 40 μM . Finally, C1-Cl (5–40 μM) decreased (i) the amplitude of miniature endplate potentials until a complete block by concentrations higher than 25 μM and (ii) their frequency at 10 μM or higher concentrations. The compound C1-Cl reversibly blocked the neuromuscular transmission *in vitro* by a non-depolarizing mechanism and mainly through an action on postsynaptic nAChRs. The compound C1-Cl may be therefore interesting for further preclinical testing as a new competitive neuromuscular blocking, and thus myorelaxant, drug.

1. Introduction

1,10-Phenanthroline (phen) is a very attractive ligand in

coordination chemistry, due to its structural and chemical properties (e.g. rigidity, planarity, aromaticity, basicity). Therefore, a countless library of compounds containing the phen building block has been

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; eeAChE, electric eel acetylcholinesterase; EtOH, ethanol; C1-Cl, organoruthenium-nitrophen complex $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{nitrophen})\text{Cl}]\text{Cl}$, $\text{C}_{22}\text{H}_{21}\text{Cl}_2\text{N}_3\text{O}_2\text{Ru}$; ChEs, cholinesterases; EPPs, endplate potentials; hrAChE, human recombinant acetylcholinesterase; hsBChE, horse serum butyrylcholinesterase; IC_{50} , concentrations producing 50 % inhibition; IQR, interquartile range; MEPPs, miniature endplate potentials; n_{H} , Hill number; nAChRs, muscle-type nicotinic acetylcholine receptors; nitrophen, 5-nitro-1,10-phenanthroline; PF_6^- , hexafluorophosphate; rVm, resting membrane potential

* Corresponding author.

E-mail addresses: Tomaz.Trobec@vf.uni-lj.si (T. Trobec), Monika.Zuzek@vf.uni-lj.si (M.C. Žužek), Kristina.Sepcic@bf.uni-lj.si (K. Sepčič), Jerneja.Kladnik@fkkt.uni-lj.si (J. Kladnik), Jakob.Kljun@fkkt.uni-lj.si (J. Kljun), Iztok.Turel@fkkt.uni-lj.si (I. Turel), Evelyne.Benoit@cea.fr (E. Benoit), Robert.frangez@vf.uni-lj.si (R. Frangež).

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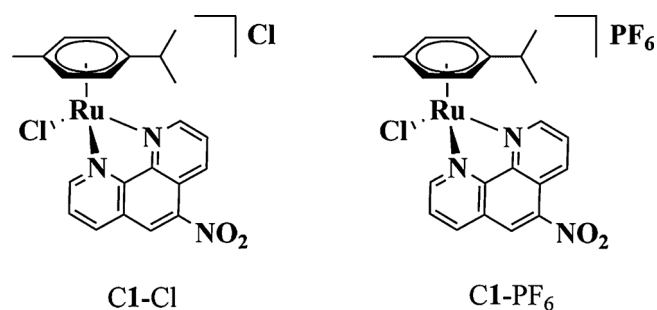


Fig. 1. Chemical structures of organometallic ruthenium complexes C1-Cl and C1-PF₆ of 5-nitro-1,10-phenanthroline (nitrophen).

isolated [1]. Phen can be substituted with various functional groups that tune physicochemical and biological properties. Two nitrogen donor atoms make phen a bidentate chelating ligand that binds to many metal ions. Consequently, free ligands, as well as various complexes with metals of the d and f block, have been used in many applications, e.g. analytical probes, optoelectronic devices, herbicides, and pharmaceuticals [1–3]. The pharmacological effect caused by the DNA-binding and recognition properties of metal complexes with phen ligands have been extensively reported for copper, platinum, rhodium and ruthenium complexes which act as metallo-intercalators. Some of them also trigger DNA cleavage first by photoactivation. The capability of interfering with DNA was further related to their anticancer [1,4] and antibacterial effects, and some are also very active against drug-resistant strains [5]. Organometallic ruthenium complex C1-Cl of 5-nitro-1,10-phenanthroline (nitrophen; C1-Cl, Fig. 1) was first isolated by Süss-Fink and collaborators [6].

The nitro group is well-known to exhibit an electron-withdrawing inductive and resonance effects. Aromatic ring systems, to which such group is bonded, are very electron-poor, and electrophilic aromatic substitution reactions on such arenes proceed much slower [7]. It was also reported that the presence of a nitro group affects photochemical properties [8]. Within the same research group, catalytic properties of C1-Cl (and other related compounds) were tested in transfer hydrogenation reactions [6].

During the last years, our group has focused on the synthesis of ruthenium complexes, including ruthenium(III) complexes with phen [9] and on their biological activities. Several isolated and characterized complexes have expressed versatile biological properties, ranging from antibacterial and antitumor effects to inhibition of several medicinally important enzymes, such as cholinesterases (ChEs) and glutathione-S-transferases (GSTs) [9–18]. Acetylcholinesterase (AChE) is the key enzyme acting in nerve synapses and neuromuscular junctions, where it hydrolyses the neurotransmitter acetylcholine (ACh). AChE inhibitors are therefore commonly used in some medical conditions to treat myasthenia gravis, glaucoma, postural orthostatic tachycardia syndrome, and as non-depolarizing muscle relaxant reversals [19,20].

Cholinesterase inhibitors which pass the blood-brain barrier have been used for symptomatic treatment in some brain neurodegenerative diseases. However, some AChE inhibitors, such as physostigmine [21,22], bis(7)-tacrine [23], BW284c51 [24], and natural alkyipyridinium salts and their synthetic analogs [25–27] can have dual effects on the cholinergic system. Besides inhibiting AChE, they can interact with muscle-type nicotinic acetylcholine receptors (nAChRs) in a concentration-dependent manner, and disrupt the neuromuscular transmission.

Recently, the organoruthenium compound (η^6 -p-cymene)Ru(pyridithionato)Cl (Fig. S1a) was reported to act as a potent inhibitor of the human recombinant acetylcholinesterase (hrAChE). In addition, this compound, characterized physiologically on the peripheral neuromuscular system, showed no undesirable peripheral cholinergic effects and therefore, represents an interesting potential candidate for

symptomatic treatment of Alzheimer's disease [15].

The present study is focused on C1-Cl, an organoruthenium complex, based on nitrophen, that also shows very potent inhibitory effect of the electric eel acetylcholinesterase (eeAChE) and human recombinant acetylcholinesterase (hrAChE). To our knowledge, no biological experiment with C1-Cl has been conducted so far. Therefore, we have decided to test the eventual anticholinergic effects of this compound on the neuromuscular junction properties. Based on its observed *in vitro* activity against hrAChE, and assuming that some AChE inhibitors can interact with muscle-type nAChRs, we studied the effects of C1-Cl on the mouse peripheral neuromuscular system where the muscle-type nAChRs play a critical role in neuromuscular transmission and muscle function. We found that, at the tested concentrations, C1-Cl did not affect the neuromuscular junction AChE but reversibly blocked the neuromuscular transmission. Therefore, this compound may be an interesting candidate for further preclinical testing as a potential myorelaxant drug.

2. Materials and methods

2.1. Compound synthesis

C1-Cl was prepared according to previously reported procedure [6]. As crystallization attempts of C1-Cl were unsuccessful we substituted the chlorido counterion with hexafluorophosphate (PF₆⁻) to obtain crystals of C1-PF₆ containing the same ruthenium complex species which were suitable for X-ray structural analysis (Fig. S2).

Ruthenium chloride cymene precursor [RuCl₂(p-cymene)]₂ and ligand nitrophen were purchased from commercial suppliers (Strem Chemicals and Acros Organics, USA), and used as received. Pre-coated TLC sheets ALUGRAM® SIL G/UV254 (Macherey-Nagel, Germany) were used to follow the progress of the reactions and were visualized under UV light. The orange solution of [RuCl₂(p-cymene)]₂ (48.0 mg, 0.078 mmol, 1 mol eq.) and nitrophen (35.3 mg, 0.156 mmol, 2 mol eq.) in dichloromethane (20 mL) was refluxed for 2 h. Then, the solvent was evaporated, the residue was redissolved in acetone (20 mL), an excess of KPF₆ (8 mol eq.) was added and left to stir at room temperature for 1.5 h. The solvent was concentrated, the solution filtered over Celite and clear yellow mother liquid was again evaporated under reduced pressure to approximately 2 mL. Diethyl ether was added and the precipitated yellow solid was filtered under reduced pressure, washed with diethyl ether and left to dry overnight at 45 °C. The product was isolated in quantitative yield. The NMR spectra of C1-Cl and C1-PF₆ are presented in the Supplementary Information (Figs. S4a and S4b, respectively).

C1-Cl: ¹H NMR (500 MHz, (CD₃OD)): δ = 10.00 (dd, 1H, *J* = 5.3, 1.1 Hz, Ar-*H*_{nitrophen}), 9.98 (dd, 1H, *J* = 5.3, 1.1 Hz, Ar-*H*_{nitrophen}), 9.39 (dd, 1H, *J* = 8.6, 1.1 Hz, Ar-*H*_{nitrophen}), 9.23 (s, 1H, Ar-*H*_{nitrophen}), 9.05 (dd, 1H, *J* = 8.3, 1.1 Hz, Ar-*H*_{nitrophen}), 8.29–8.21 (m, 2H, Ar-*H*_{nitrophen}), 6.27 (d, 2H, *J* = 6.7 Hz, Ar-*H*_{cym}), 6.06–6.02 (m, 2H, Ar-*H*_{cym}), 2.72 (hept, 1H, *J* = 6.9 Hz, Ar-CH(CH₃)_{2cym}), 2.26 (s, 3H, Ar-CH_{3cym}), 1.05 (d, 6H, *J* = 6.9 Hz, Ar-CH(CH₃)_{2cym}).

C1-PF₆: ¹H NMR (500 MHz, (CD₃)₂CO): δ = 10.14 (dd, 1H, *J* = 5.3, 1.1 Hz, Ar-*H*_{nitrophen}), 10.11 (dd, 1H, *J* = 5.3, 1.1 Hz, Ar-*H*_{nitrophen}), 9.39 (dd, 1H, *J* = 8.6, 1.1 Hz, Ar-*H*_{nitrophen}), 9.31 (s, 1H, Ar-*H*_{nitrophen}), 9.20 (dd, 1H, *J* = 8.3, 1.2 Hz, Ar-*H*_{nitrophen}), 8.36–8.30 (m, 2H, Ar-*H*_{nitrophen}), 6.38 (d, 2H, *J* = 6.7 Hz, Ar-*H*_{cym}), 6.16 (d, 2H, *J* = 6.7 Hz, Ar-*H*_{cym}), 2.85 (hept, 1H, *J* = 7.0 Hz, Ar-CH(CH₃)_{2cym}), 2.28 (s, 3H, Ar-CH_{3cym}), 1.10 (d, 6H, *J* = 7.0 Hz, Ar-CH(CH₃)_{2cym}). ³¹P NMR (202 MHz, (CD₃)₂CO): δ = -144.31 (hept, *J*_{PF} = 708 Hz, PF₆).

2.2. Physicochemical characterization

NMR spectroscopy was performed using Bruker Avance III 500 spectrometer at room temperature. ¹H NMR spectra were recorded at 500 MHz. Chemical shifts are referenced to the deuterated solvent

residual peaks of CD₃OD or (CD₃)₂CO at 3.31 or 2.05 ppm (both referenced against the central line of quintet), respectively. ³¹P spectrum was recorded at 202 MHz and chemical shift is reported relative to external standard. The splitting of proton resonances is defined as s = singlet, d = doublet, t = triplet, hept = heptet or m = multiplet. Chemical shift (δ) and coupling constants (J) are given in ppm and Hz, respectively. All NMR data processing was carried out using MestReNova version 11.0.4.

X-ray diffraction data were collected on an Oxford Diffraction SuperNova diffractometer with Mo/Cu microfocus X-ray source ($K\alpha$ radiation, $\lambda_{\text{Mo}} = 0.71073 \text{ \AA}$, $\lambda_{\text{Cu}} = 1.54184 \text{ \AA}$) with mirror optics and an Atlas detector at 150(2) K. The structure was solved in Olex² graphical user interface [28] by direct methods implemented in SHELXT and refined by a full-matrix least-squares procedure based on F^2 using SHELXL [29]. All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were placed at calculated positions and treated using appropriate riding models. The crystal structure has been submitted to the CCDC and has been allocated the deposition number 1937228. Additional details on structural properties and refinement details are given in the Supplementary Information (Table S1, Fig. S3).

2.3. Cholinesterase inhibition assay

Cholinesterase activity was measured by Ellman's method [30] adapted for microtiter plates, as described in [31]. Stock solutions of C1-Cl (1 mg/mL), or neostigmine bromide (Sigma-Aldrich, USA) as a positive control, were prepared in 100 % ethanol (EtOH) and progressively diluted in 100 mM potassium phosphate buffer (pH 7.4) in the final volume of 50 μL . Then, 100 μL of acetylthiocholine chloride and 5,5'-dithiobis-2-nitrobenzoic acid, at concentrations of 1 mM and 0.5 mM, respectively, dissolved in the same buffer, were added to the wells of the microtiter plate. The three ChEs used as the source of enzyme were dissolved in the same buffer at the final concentration of 0.0075 U/mL: eeAChE, hrAChE and hsBChE (Sigma, USA). 50 μL of each ChE solution were added to start the reaction which was followed spectrophotometrically at 405 nm and 25 °C for 5 min using a kinetic microplate reader (Dynex Technologies, USA). Blank reactions, without the inhibitors, were run in the presence of the appropriate dilution of EtOH. Each measurement was repeated at least three times. For the determination of inhibitory constants (K_i), the kinetics was monitored using three different final substrate concentrations (0.125, 0.25 and 0.5 mM, respectively).

2.4. Glutathione S-transferase inhibition assay

GST activity was measured according to the method described by Habig et al. (1974) [32], using a VIS microplate reader (Anthos, UK). The stock solution of C1-Cl (1 mg/mL) was prepared in 100 % EtOH and progressively diluted in 100 mM sodium phosphate buffer (pH 6.5). 1-Chloro-2,4-dinitrobenzene (Sigma, USA) was dissolved in EtOH to obtain a 50 mM solution, which was afterward diluted with 100 mM sodium phosphate buffer (pH 6.5) to a final concentration of 4 mM. This solution (50 μL) was mixed with 100 μL of 2 mM reduced glutathione (Sigma, USA) in 100 mM sodium phosphate buffer (pH 6.5), and with 50 μL of diluted C1-Cl. 50 μL of glutathione S-transferase from horse liver (Sigma, USA), diluted in 100 mM sodium phosphate buffer (pH 6.5), were added to start the reaction. The final enzyme concentration was 0.044 U/mL. A blank reaction was performed by replacing C1-Cl with the appropriate dilution of EtOH. The reaction was followed spectrophotometrically at 340 nm and 25 °C for 4 min using a VIS microplate reader (Anthos, UK).

2.5. Experimental animals and neuromuscular preparations

Conventional 16–20-week adult male Balb/C mice (22–26 g) were obtained from the animal breeding facility at the Veterinary Faculty

(University of Ljubljana, Slovenia). Five mice were housed in each cage (1284 L EUROSTANDARD TYPE II L, 365 × 207 × 140 mm, floor area 530 cm²; Tecniplast, Italy) in temperature and relative humidity controlled room (T = 20–24 °C; RH = 40–60 %), on a 12/12 light/dark cycle (lights on at 02:00 a.m.). For bedding were used autoclaved wood fibers (LIGNOCEL 3/4-S; J. Rettenmaier & Söhne GmbH + Co KG, Germany). Food (Teklad global 16 % protein extruded, irradiated, 2916; Envigo, Italy) and tap water acidified with the addition of HCl (pH 3–4) were provided *ad libitum*. The experiments on isolated neuromuscular preparations followed ethical standards, were carried out in strict accordance with the Slovenian legislation, as harmonized with the European Communities Council Guidelines (Directive 86/609/EGS on 24 November 1986; Directive 2010/63/EU on 22 September 2010), and were approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection (permit no. 34401–12/2012/2).

Mice were sacrificed by cervical dislocation followed by immediate exsanguination. After dissection of diaphragm muscle with both phrenic nerves, the neuromuscular preparations were prepared by splitting the diaphragm muscle into two hemidiaphragms. Each hemidiaphragm was maintained in oxygenated standard Krebs-Ringer solution composed of 154 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, and 11 mM D-glucose (pH 7.4). Just prior to experiments, C1-Cl was dissolved in a Krebs-Ringer solution containing 10 % (v/v) of EtOH at a stock concentration of 2 mg/mL. The final concentration of EtOH in the organ bath was always 0.11 % (v/v). Neostigmine methyl sulfate (Tokyo Chemical Industry CO./LTD, Japan), 3,4-diaminopyridine (3,4-DAP; Sigma-Aldrich, USA) and μ -conotoxin GIIIB (Bachem, Switzerland) were of the highest grade of purity available. All experiments were performed at room temperature (22–24 °C).

2.6. Muscle contraction recordings

The hemidiaphragm was pinned to the bottom of the organ bath on its lateral side. It was connected to an isometric mechano-electrical transducer (Grass Instruments, West Warwick, RI, USA) by silky thread *via* a stainless-steel hook attached to its tendinous end. The corresponding motor nerve was stimulated using an S-48 square pulse stimulator (Grass Instruments, West Warwick, RI, USA) *via* a suction electrode with pulses of 0.1 ms duration and supramaximal voltage (typically 5–8 V) applied at a 0.1-Hz frequency. Direct muscle stimulation was evoked by electric field stimulation *via* two parallel platinum electrodes in the organ bath connected to the S-48 Grass stimulator and producing pulses of 0.1 ms duration and 50–80 V applied at a 0.1-Hz frequency. Directly- (muscle stimulation) or indirectly- (nerve stimulation) evoked tetanic contraction was obtained with a pulse train duration of 1000 ms and a stimulation frequency of 80 Hz. To achieve the maximal contractile response upon nerve-evoked stimulation, the stable resting tension for each neuromuscular preparation (typically 1.5–2.5 g) was adjusted approximately 20–30 min before starting the experiment. Muscle single twitch or tetanic contraction was recorded using a Grass FT03 force transducer (Grass Instruments, West Warwick, RI, USA) connected to P122 strain gauge amplifier (Grass Instruments, West Warwick, RI, USA). The amplified signal was digitized using a data acquisition system (Digidata 1440A; Molecular Devices, Sunnyvale, CA, USA) at a sampling rate of 1 kHz. The inhibitory response of C1-Cl was continuously measured during 60 min after compound application. The muscle twitch or tetanic contraction blockade produced by C1-Cl was expressed as the percentage of the initial maximal response. The concentrations of C1-Cl studied were 5, 10, 20, 25 and 40 μM .

2.7. Membrane potential recordings

Electrophysiological recordings on hemidiaphragm muscles fibers were performed using conventional microelectrode techniques.

Hemidiaphragm preparations were equilibrated for 30 min in standard Krebs-Ringer physiological solution supplemented with 1.6 μM $\mu\text{-conotoxin GIIIB}$, which selectively blocks the $\text{Na}_v1.4$ sodium channel subtype of muscle fibers [33,34]. Under these conditions, endplate potentials (EPPs), miniature endplate potentials (MEPPs) and resting membrane potential ($r\text{Vm}$) were recorded from superficial hemidiaphragm muscle fibers using intracellular borosilicate glass microelectrodes and Axon Axoclamp 900A microelectrode amplifier (Molecular Devices, Sunnyvale, CA, USA). Microelectrodes were pulled using a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA, USA) and were filled with 3 M KCl solution. Only microelectrodes having a 10–20 M Ω resistance were used. EPPs were recorded by stimulating the phrenic nerve *via* the bipolar suction electrode with supramaximal square pulses of 0.1 ms at 0.1 Hz using the S-48 Grass stimulator. MEPPs and EPPs were digitized at 25 kHz using Digidata 1440A and pCLAMP 10.7 (Molecular Devices, Sunnyvale, CA, USA), and were stored for later analysis using pCLAMP-Clampfit 10.7 software (Molecular Devices, Sunnyvale, CA, USA). The recordings were performed (i) before applying C1-Cl, (ii) 1, 30 and 60 min after the compound application, and (iii) 15 min after the compound wash-out. MEPP and EPP amplitudes were normalized to a $r\text{Vm}$ of -70 mV using the formula $V_c = V_o (-70)/r\text{Vm}$, where V_c is the normalized amplitude of MEPPs and EPPs, and V_o is their recorded amplitude [35]. The concentrations of C1-Cl studied were 5, 10, 20, 25 and 40 μM .

2.8. Data analysis and statistics

The results are presented as means \pm S.D. The C1-Cl concentrations producing 50 % inhibition of initial responses (IC_{50}) and the Hill number (n_H) were determined from the theoretical concentration-response relationships calculated from typical sigmoid nonlinear regressions through data points (correlation coefficient = r^2), using the four-parameter nonlinear regression model (GraphPad Prism version 6.00). The data were statistically analyzed using Sigma Plot for Windows version 12.5 (Systat Software Inc., USA). Data were first tested for normality (Shapiro–Wilk test) and equal variance for assignment to parametric or nonparametric analysis. For paired comparison between two groups, when equal variance were not met, nonparametric analysis was performed using Mann-Whitney Rank Sum Test and are presented as box-and-whisker plots using median and the interquartile range (IQR). A P -value ≤ 0.05 was considered to be statistically significant. Student's two-tailed t -test was used for statistical analysis of the normally distributed data. Statistical significance was set at a P -value ≤ 0.05 .

3. Results

3.1. Synthesis and crystal structure of C1-Cl

C1-Cl was first synthesized and characterized by Süss-Fink and collaborators, using standard physicochemical methods [6]. However, no crystal structure has been reported so far. We have managed to obtain suitable crystals of C1-Cl with a hexafluorophosphate (PF_6^-) as a counterion, *i.e.* C1- PF_6 (Fig. 1). The exchange of counterion is one of the common methods to obtain crystals for ionic compounds that otherwise do not crystallize. The crystal structure of C1- PF_6 revealed the expected pseudo-octahedral geometry (Fig. 2) with the cymene ligand π -bonded to the central ruthenium ion, while the remaining three coordination sites are occupied by the chloride and the N,N -bound nitrophen ligands. All bonds and angles are within the expected range of values for previously reported structures of organoruthenium-phen complexes [36–38].

Refinement of this structure presented a unique challenge as the determined asymmetric unit consists of one half of the molecule of C1- PF_6 with a mirror plane intersecting the cymene ring and the central ruthenium ion. Details on crystallization, structure solution and

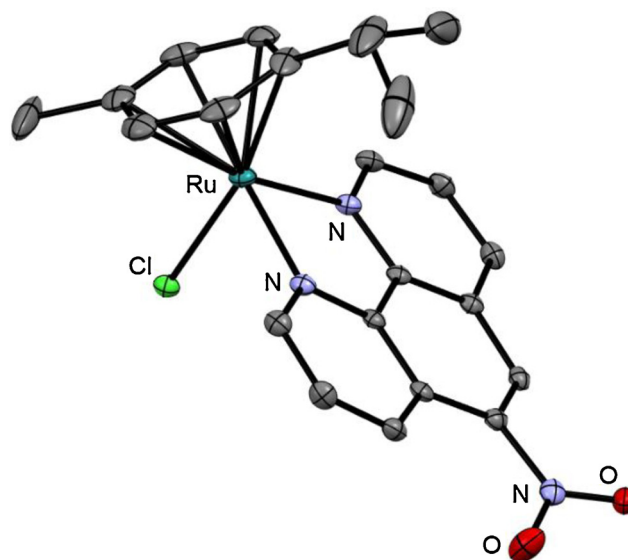


Fig. 2. Crystal structure of C1- PF_6 . Thermal ellipsoids are shown at 25 % probability level. Hydrogen atoms and hexafluorophosphate anion are omitted for better clarity of presentation (this part of the structure is the same in C1-Cl and C1- PF_6).

refinement as well as structure analysis are given in the SI (Table S1, Fig. S3).

3.2. Inhibition of cholinesterase and GST enzymes by C1-Cl, nitrophen and ruthenium precursor

The inhibitory potential of C1-Cl towards ChE enzymes of animal or human origin was within a pharmaceutically interesting range, with IC_{50} values of 26.2, 24.2 and 16.1 μM against eeAChE, hrAChE and hsBChE, respectively. Our results also illustrate that nitrophen ligand is a potent inhibitor of eeAChE and hsBChE, with respective IC_{50} values of 5.3 and 4.0 μM . However, its activity was not so pronounced against hrAChE, with an IC_{50} value of 40 μM . In our previous study we have shown that the ruthenium precursor complex does not inhibit eeAChE or hrAChE, while moderate inhibition of hsBChE (IC_{50} value 32.7 μM) was observed (Table 1) [15].

The C1-Cl inhibition of all three ChEs was apparently of a reversible competitive type, suggesting that the tested inhibitor interacts with the enzyme active site inside the gorge (Fig. 3).

The inhibitory constant against both tested AChEs was the same (17 μM) and it was considerably lower against BChE (5 μM). Interestingly, and despite the fact that ruthenium-based compounds are often associated with GST inhibitory properties, C1-Cl failed to inhibit the GST of animal origin (data not shown).

3.3. Effect of C1-Cl on skeletal muscle contraction *in vitro*

The effects of C1-Cl (5–40 μM) were first determined on nerve-evoked and directly muscle-elicited isometric single twitch and tetanic contraction in mouse hemidiaphragm preparations. Fig. 4 shows representative recordings of the C1-Cl effects on indirectly and directly evoked single twitch and tetanic contraction, as well as graphs illustrating concentration-response relationships for C1-Cl induced inhibition of muscle contraction. The results reveal that C1-Cl (20 μM) reduced the amplitude of nerve-evoked muscle single twitch by approximately 50 %. This effect was reversible since washing the preparation with a standard physiological solution free of C1-Cl, for about 15 min, completely restored the nerve-evoked muscle twitch (Fig. 4a). In contrast, directly muscle-elicited single twitch and tetanic contraction were not affected by the same (20 μM) and even a highest

Table 1Inhibition of eeAChE or hrAChE, and hsBChE by C1-Cl, its ligand nitrophen (5-nitro-1,10-phenanthroline) and ruthenium precursor [RuCl₂(*p*-cymene)]₂.

Compound	eeAChE		hrAChE		hsBChE	
	IC ₅₀ (μM) ^a	K _i (μM)	IC ₅₀ (μM) ^a	K _i (μM)	IC ₅₀ (μM) ^a	K _i (μM)
C1-Cl	26.2	17	24.2	17	16.1	5
nitrophen	5.3	1.8	40.0	18.7	4.0	0.7
[RuCl ₂ (<i>p</i> -cymene)] ₂	/	/	/	/	32.7	23.3
Neostigmine bromide	6.16	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	75.09	<i>n.d.</i>

^a IC₅₀ was determined as the concentration of the compound inducing 50 % inhibition of the enzyme activity. *n.d.*: non determined. (eeAChE) electric eel acetylcholinesterase; (hrAChE) human recombinant acetylcholinesterase; (hsBChE) horse serum butyrylcholinesterase.

(40 μM) C1-Cl concentration (Fig. 4a, d and e). The C1-Cl -induced inhibition of muscle contraction evoked by nerve stimulation occurred in a concentration-dependent manner (Fig. 4d and e). The IC₅₀ values, determined from the concentration-response curves, were 19.44 μM for nerve-evoked muscle contraction (Fig. 4d) and 9.68 μM for nerve-evoked tetanic muscle contraction (Fig. 4e). It is worth noting that small drop in muscle twitch amplitude (1–3 %) were observed during 60 min of exposure of neuromuscular preparations to 0.11 % (v/v) of EtOH, and that no decrease of muscle twitch amplitude occurred during 60 min control experiments during which neuromuscular preparations were kept in the Krebs-Ringer solution, as shown in representative recordings (Fig. 5a).

During the development of the muscle twitch block, obtained within 30 min after the mouse hemidiaphragm exposure to 20 μM C1-Cl, 3 μM neostigmine, known to almost completely inhibit AChE in mouse diaphragm neuromuscular junctions [39], rapidly restored muscle single twitch (Fig. 5c). Additionally, 300 μM 3,4-diaminopyridine, a well-known potassium channel blocker in nerve terminals [40], significantly increased single twitch and tetanic contraction amplitudes to approximately 114 ± 1 % (n = 4) of their initial value (data not shown).

3.4. Effects of C1-Cl on membrane potentials

To gain further mechanistic insight of the C1-Cl blocking action on nerve evoked skeletal muscle single twitch and tetanic contraction, the compound effects were investigated on rVm, MEPPs and EPPs.

3.4.1. C1-Cl did not affect rVm of muscle fibers

C1-Cl, at concentrations (5–40 μM) that partially or completely inhibited nerve-evoked single twitch and tetanic contraction of mouse hemidiaphragms (see Fig. 4), did not affect the rVm recorded in the endplate regions of the surface muscle fibers after 1, 30 and 60 min of compound exposure (Fig. 6). In particular, the mean rVm was -67.5 ± 0.5 mV under control conditions (*i.e.* at time zero, just before C1-Cl application), and -67.1 ± 0.5 mV and -66.1 ± 0.6 mV, 30 and 60 min after exposure to 40 μM C1-Cl, respectively.

3.4.2. C1-Cl produced decreased EPP and MEPP amplitudes

Electrophysiological recordings of EPPs from the endplates of the mouse hemidiaphragm exposed to C1-Cl (5–40 μM) for 60 min revealed that the compound caused a concentration-dependent decrease of EPP amplitude (Fig. 7). The IC₅₀ value for the C1-Cl -induced reduction of EPP amplitude was 7.61 μM (Fig. 7b). No significant change in the EPP half-decay time was observed among the various C1-Cl concentrations studied (Fig. 7a).

The effects of C1-Cl on the MEPP frequency and amplitude are shown in Fig. 8. The compound (5–40 μM), in a concentration-dependent manner, significantly reduced the amplitude of MEPPs recorded in superficial muscle fibers of the mouse hemidiaphragm (Fig. 8a and b), without significant decrease of their frequency at low C1-Cl concentration (Fig. 8c). At the highest concentration (40 μM), C1-Cl completely abolished MEPPs (Fig. 8b). No significant change in the MEPP half-decay time was observed among the concentrations studied (Fig. 8a). Estimations of the frequency of MEPPs when these potentials were blocked by about 45 % (by 5 μM C1-Cl), but easily recognized from basal noise, revealed that the MEPP frequency was not significantly modified by C1-Cl.

4. Discussion

Due to the possible applications of organoruthenium compounds in medicine the synthesis of new series of organometallic compounds [15], including C1-Cl, has been vastly extended. This study newly reports: (i) crystal structure, (ii) the mode of AChE inhibition, and (iii) the effects on neuromuscular transmission and contractility, the most fundamental skeletal muscle function, of 5-nitro-1,10-phenanthroline-based organoruthenium compound C1-Cl.

Almost seven decades ago, it was shown that tris(1,10-phenanthroline)ruthenium(II) complex (Fig. S1b) can act as inhibitor of AChE [41]. Since then, several ruthenium-based complexes have been described as ChE inhibitors [15,42–47]. Anti-ChE compounds represent the main class of drugs used in the therapeutic strategy in some neurodegenerative, eye and autoimmune diseases [48]. In this study, C1-Cl was evaluated for its ChE inhibitory activity as a potential anti-ChE compound. Inhibition type of all three ChEs was apparently reversible

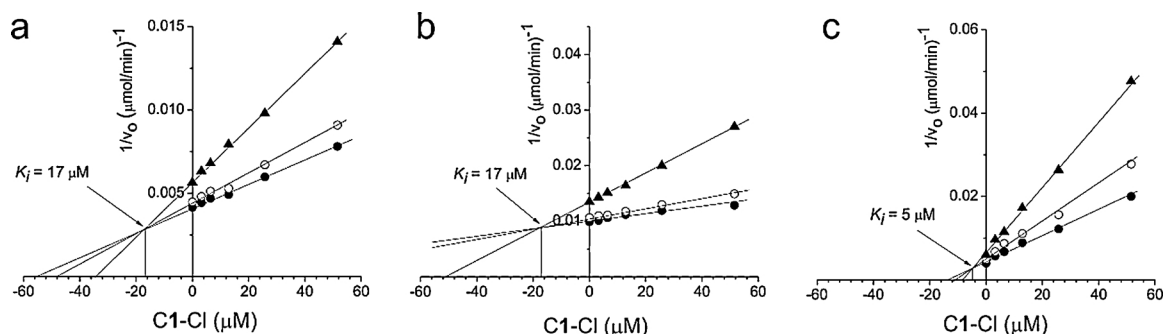
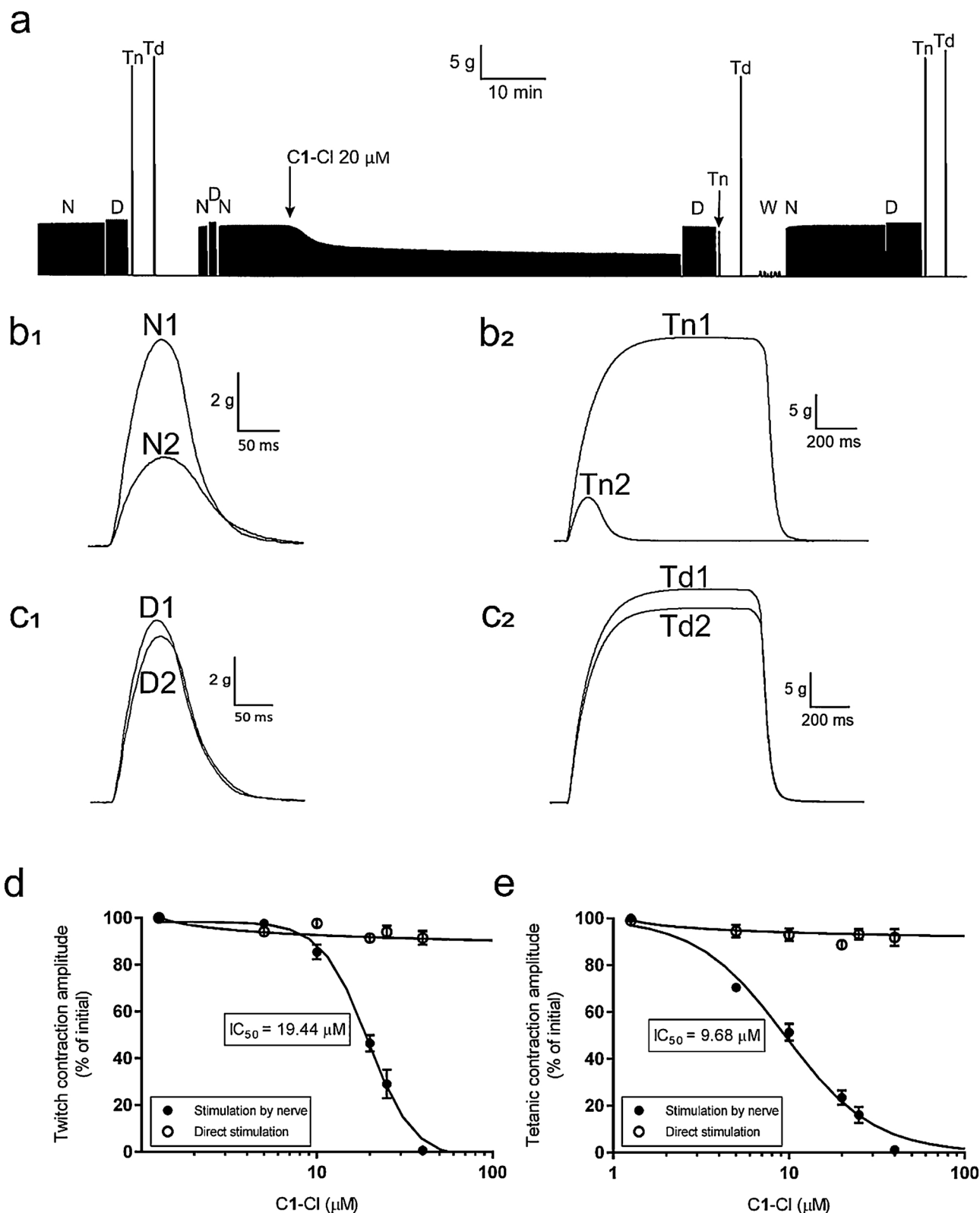


Fig. 3. Determination of the type of inhibition and inhibitory constant (K_i) for C1-Cl towards eeAChE (a), hrAChE (b) and hsBChE (c). The concentrations of the substrate acetylthiocholine were 0.125 mM (full triangles), 0.25 mM (open circles) and 0.5 mM (full circles).



(caption on next page)

and competitive, suggesting that the inhibitor interacts with the enzyme active site inside the gorge. The inhibitory potential of C1-Cl towards ChE enzymes from animal origin was in a pharmaceutically interesting range, comparable to that of other ruthenium-based ChE inhibitors, whose IC₅₀ values span in the range from 0.2–50 μ M [15,42–45,47]. The inhibitory constant against both tested AChEs was

the same (17 μ M) and was considerably lower (5 μ M) against BChE. Interestingly, despite the fact that ruthenium-based compounds are often associated with GST-inhibitory properties, C1-Cl failed to inhibit GSTs from horse liver. Furthermore, ruthenium precursor [RuCl₂(*p*-cymene)]₂ showed no activity against eeAChE or hrAChE, and only moderately inhibited hsBChE, whereas the free ligand nitrophen was

Fig. 4. Effects of C1-Cl on nerve-evoked and directly muscle-elicited single twitch and tetanic contraction of isolated mouse nerve-hemidiaphragm preparation. (a) Representative time-course of C1-Cl (20 μ M, arrow) effects on muscle contraction showing the partial and reversible block of nerve-evoked and no change in contractile response of directly muscle-elicited twitch and tetanic contraction. N denotes nerve-evoked muscle single twitch; D denotes directly muscle-elicited single twitch; Tn denotes nerve-evoked muscle tetanic contraction; Td denotes directly muscle-elicited tetanic contraction; W denotes wash out. (b1) Traces of nerve-evoked twitch recorded before (N1) and 60 min after (N2) the application of 20 μ M C1-Cl. (b2) Traces of tetanic contraction recorded before (Tn1) and 60 min after (Tn2) the application of 20 μ M C1-Cl. (c1) Representative recordings of twitch elicited by direct muscle stimulation, before (D1) and 60 min after (D2) the application of 20 μ M C1-Cl. (c2) Representative recordings of tetanic contraction elicited by direct muscle stimulation, before (Td1) and 60 min after (Td2) the application of 20 μ M C1-Cl. (d) Concentration-response curves for the inhibition of directly muscle-elicited and nerve-evoked twitch by C1-Cl. Muscle twitch was expressed as a percentage of its maximal initial value. Each value represents the mean \pm S.D. obtained from four–six different muscles. The IC_{50} and n_H values, determined from typical sigmoid nonlinear regressions through data points ($r^2 = 0.996$), were $19.44 \pm 1.05 \mu$ M and 3.3 ± 0.5 , respectively, for nerve-evoked single twitches. (e) Concentration-response curves for the inhibition of directly muscle-elicited and nerve-evoked tetanic contraction by C1-Cl. The tetanic contraction was expressed as the percentage of its maximal initial value. Each value represents the mean \pm S.D. obtained from four–six different muscles. The IC_{50} and n_H values, determined from typical sigmoid nonlinear regressions through data points ($r^2 = 0.992$), were $9.68 \pm 1.14 \mu$ M and 1.6 ± 0.3 , respectively, for nerve-evoked tetanic contractions.

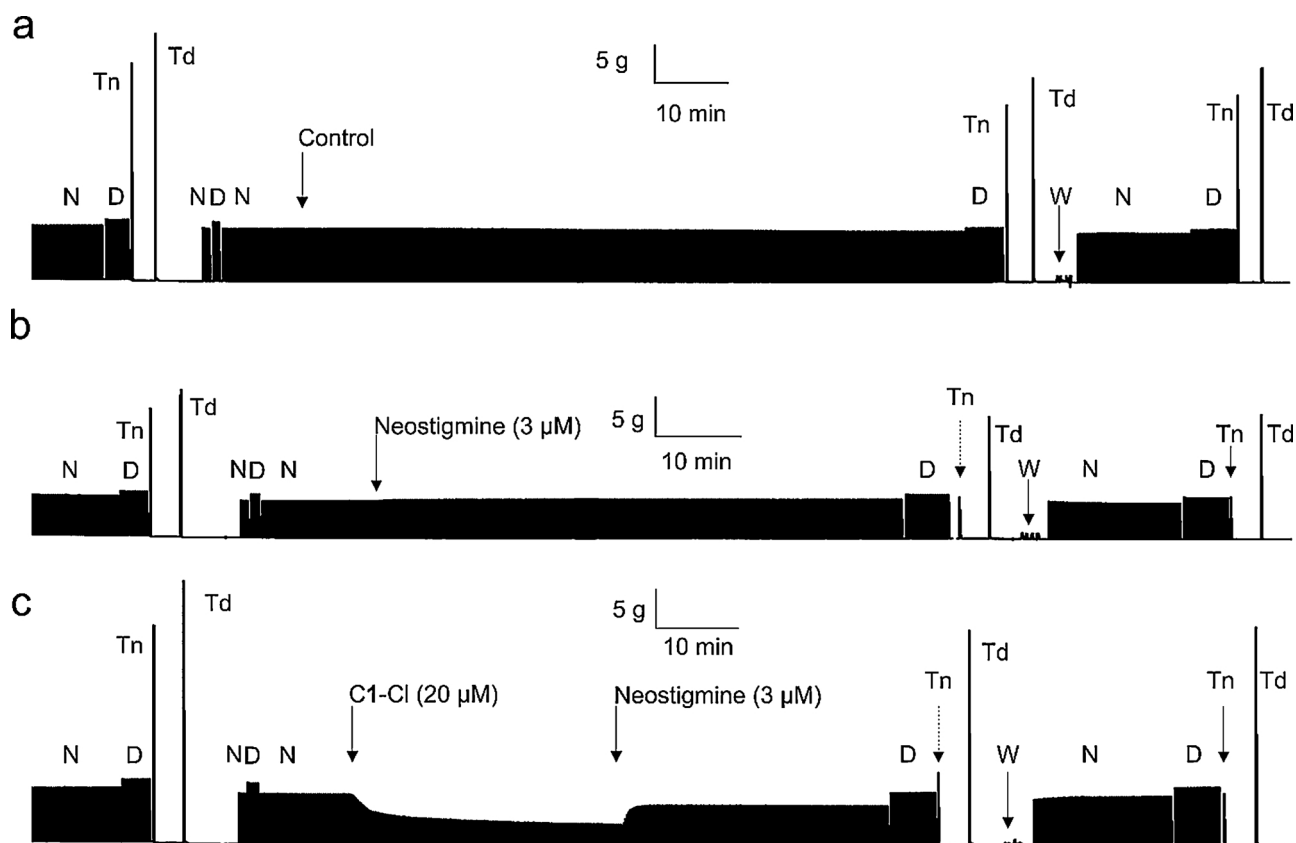


Fig. 5. Kinetics of C1-Cl effects on muscle single twitch and tetanic contraction of isolated mouse nerve-hemidiaphragm preparation. (a) Representative control tracing. (b) Representative ‘positive’ control tracing using neostigmine (3 μ M, arrow). (c) Reversal effects of neostigmine (3 μ M, arrow) on the blockade of nerve-evoked single twitches produced by C1-Cl (20 μ M, arrow). N denotes nerve-evoked muscle single twitch; D denotes directly muscle-elicited single twitch; Tn denotes nerve-evoked muscle tetanic contraction; Td denotes directly muscle-elicited tetanic contraction; W denotes wash out.

active against all three ChEs. However, we need to emphasize that the free ligand is only poorly soluble in the aqueous solution used for electrophysiological experiments and precipitates upon the addition of Krebs-Ringer solution. In contrast, its organoruthenium(II) complex C1-Cl was readily dissolved in the same solution. In the view of drug development, the solubility is an important parameter that must be considered when planning new potential drugs. Therefore, we see the organoruthenium of nitrophen as a big advantage also due to increased solubility of the ligand, which otherwise could not be examined by itself in our electrophysiological experiments with required conditions. The latter experiments are of utmost importance as some of the AChE inhibitors can produce unwanted cholinergic side effects in the neuromuscular system, like single twitch potentiation in skeletal muscle, and the inability of skeletal muscle to sustain tetanic contraction [49–51]. Therefore, it was of interest to examine the effects of C1-Cl on the skeletal neuromuscular transmission to reveal its eventual unwanted side-effects on neuromuscular transmission and underlying molecular

mechanism of action. According to the literature, bidentate *N,N*-donor ligands (like nitrophen) bind strongly to ruthenium(II) metal core and are not easily substituted [52]. Although there might be possibility of some release of nitrophen, the use of metal complex represents many advantages over the free organic ligand as either complex itself triggers the biological effect, or it acts as an efficient shuttle to transfer the ligand to the site of the action where the ligand could be released [53].

The results from muscle twitch experiments revealed that C1-Cl at concentrations ranging from 5 to 40 μ M, progressively decreased the amplitude of nerve-evoked isometric muscle twitch and tetanic contraction without a noticeable change in their decline phase. In contrast, the well-known competitive AChE antagonist neostigmine induced characteristic facilitation of neuromuscular transmission associated with anti-ChE treatment. Neostigmine produced the complete block of tetanic contractions evoked by repetitive nerve stimulation and had no effect on the amplitude of directly evoked muscle twitch and tetanic muscle contraction. Additionally, a relatively small decrease of

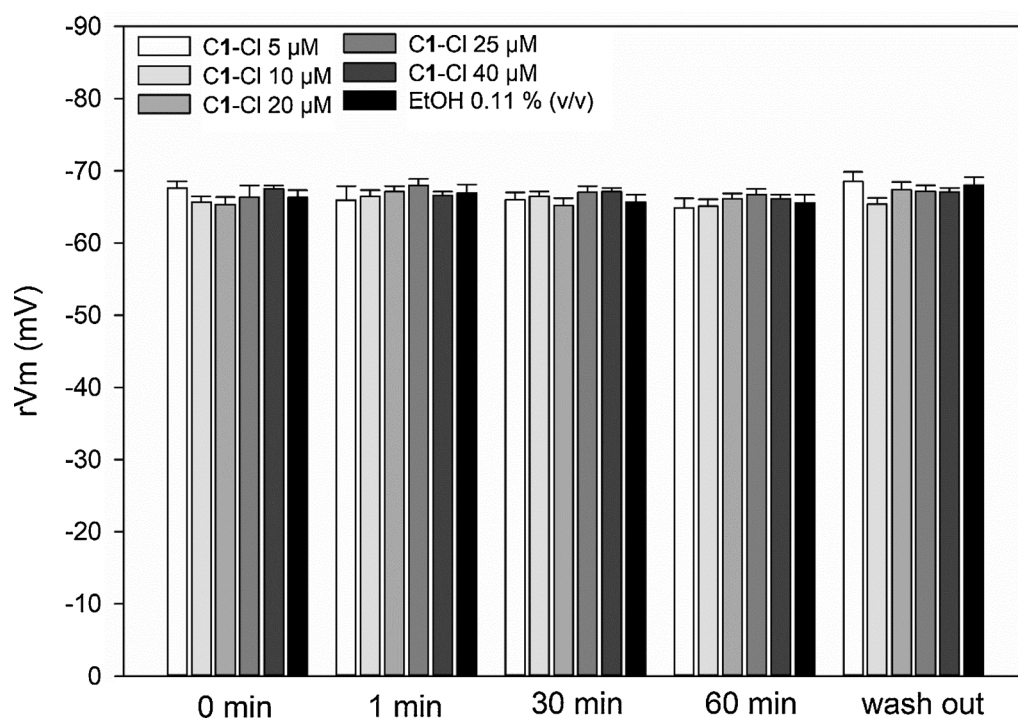


Fig. 6. Concentration- and time-dependent effect of C1-Cl on the resting membrane potential (rVm) of isolated mouse hemidiaphragm fibers. The neuromuscular preparations were exposed to 5–40 μM C1-Cl. Measurements were performed before (time 0), 1, 30 and 60 min after C1-Cl application, and after washing the preparation with a C1-Cl-free standard Krebs-Ringer physiological solution for about 15 min. Mean values \pm S.D. recorded from 4–8 different hemidiaphragms ($n = 7$ –10 fibers from each).

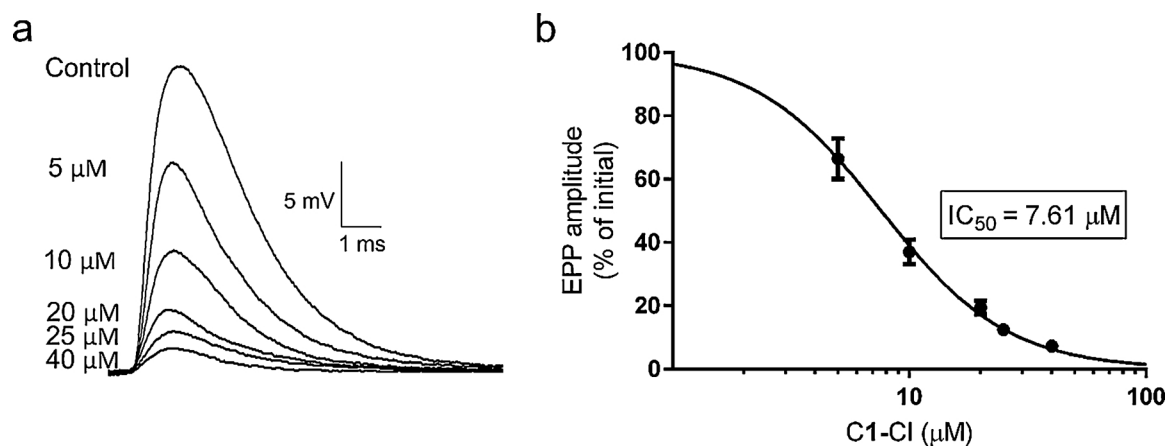


Fig. 7. Effects of C1-Cl on EPP amplitude in mouse hemidiaphragm skeletal muscle fibers. C1-Cl blocked EPPs in a concentration-dependent manner. (a) Representative EPP traces recorded under control conditions and 60 min after exposure of muscle to various concentrations of C1-Cl (5–40 μM). (b) Concentration-response curve for the inhibition of EPP amplitude by C1-Cl, expressed as a percentage of its maximal initial value. Each value represents the mean values of absolute values EPP \pm S.D. recorded from 4–8 different hemidiaphragms ($n = 7$ –10 muscle fibers from each). The IC_{50} and n_{H} values, determined from typical sigmoid nonlinear regressions through data points ($r^2 = 0.997$), were $7.61 \pm 1.04 \mu\text{M}$ and 1.6 ± 0.1 , respectively.

maximal amplitude was observed on directly muscle-elicited twitch and tetanic contraction probably due to the effects of EtOH since a similar small drop of twitch amplitude were observed in experiments with the EtOH only. These results altogether indicate that C1-Cl affects neuromuscular transmission, but does not affect excitation-contraction coupling of the skeletal muscle fibers. Exposure of neuromuscular preparation, treated with 20 μM C1-Cl which produced approximately 50 % muscle twitch amplitude block, was exposed to the 3 μM AChE inhibitor neostigmine which inhibits AChE in mouse diaphragm muscle by 96 % [39,54]. At his concentration neostigmine produced rapid and almost complete recovery of single twitch response in the presence of C1-Cl. These results strongly suggest that C1-Cl does not inhibit AChE in the neuromuscular junction at the concentration used since concentration-dependent blockade of muscle twitch produced by C1-Cl and no potentiation of single twitch were noticed in the presence of C1-Cl. Furthermore, no functional evidence for anti-ChE activity in the

neuromuscular junction of C1-Cl was obtained from measurements of the decay phase of synaptic potentials (MEPPs and full-size EPPs) in the present study. Their eventual prolongation would have resulted in potentiation of twitch amplitude and a prolongation of decay phase of EPPs and MEPPs due to persistence of ACh in the synaptic cleft and repetitive nAChRs activation [55]. However, if such an action was masked by C1-Cl-induced nAChR antagonism, the number of functional receptors available for repetitive ACh binding is expected to be reduced. AChE inhibition characteristics such as an increase in the EPPs and MEPPs amplitude, as well as increased MEPPs frequency [56,57], were also not observed. The postsynaptic extra junctional effect of C1-Cl related to muscle twitch amplitude was excluded by the observation that C1-Cl even at the highest concentration (i) did not affect the amplitude of direct muscle stimulation, (ii) did not change the passive membrane properties, and (iii) did not produce an increase in basal muscle tension (contracture), which is a sign of myotoxic action [54].

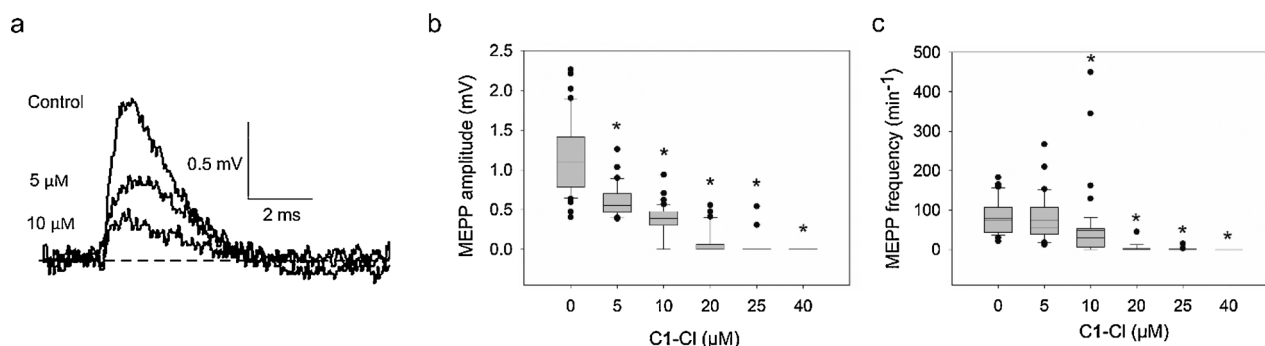


Fig. 8. Effects of C1-Cl on the MEPP amplitude and frequency in the muscle fibers of isolated mouse hemidiaphragm. (a) MEPP traces recorded under control conditions and after the indicated concentrations of C1-Cl applied for 60 min. (b) MEPP amplitude recorded from mouse hemidiaphragms exposed for 60 min to different C1-Cl concentrations. At 40 μM, C1-Cl completely blocked MEPPs. (c) MEPP frequency in the same preparations exposed for 60 min to different C1-Cl concentrations. Box plots indicate the median values of the data obtained from 4–8 different hemidiaphragms ($n = 7–10$ fibers from each), as well as its IQR, with whiskers showing the full range of data, and all outliers. * $P < 0.05$ versus control (Shapiro–Wilk with Mann–Whitney Rank Sum test).

Muscle twitch and tetanic contraction evoked by nerve stimulation were rapidly and almost completely reversed by neostigmine and washing of the nerve-muscle preparations with a compound-free medium, suggesting competitive blocking action of C1-Cl. Independently on the concentration and time of exposure to C1-Cl, the resting membrane muscle fibers potential at the end-plate region remain unchanged. The intracellular electrophysiological technique has been used to record the effect of C1-Cl on membrane potentials to find out if reduced muscle contraction by C1-Cl was associated with changes of evoked EPPs amplitude. The concentration and time-dependent block of EPPs amplitude to the subthreshold for the generation of a muscle fiber action potential can explain observed concentration dependent evoked nerve block of muscle twitch and tetanic muscle contraction produced by C1-Cl. Postsynaptic action of C1-Cl in the neuromuscular junction by reducing the activity of muscle-type nAChR receptors is strongly suggested altogether by (i) the reduction of the amplitude of spontaneous MEPPs without significant change in the ACh quantal release as indicated with no effects on the MEPPs frequency up to 5 μM, (ii) stable resting membrane potential of muscle fibers, and (iii) the block of EPPs without altering muscle fiber passive membrane properties. Results obtained from MEPPs frequency measurements (without change), where the amplitude of MEPPs has decreased approximately to 45 % indicate that C1-Cl does not have a pre-synaptic action that would modify the rate of quantal ACh release. Additional evidence that C1-Cl exerts postsynaptic action on muscle-type nAChR comes from muscle contraction experiments in which 3,4-diaminopyridine antagonized neuromuscular block produced by C1-Cl. 3,4-diaminopyridine is a well-known potassium channel blocker [40,58–60], which increases ACh release from motor nerve terminals and antagonizes the competitive blocking action of the muscle type nAChR competitive antagonists. Taken together, the physiological data obtained in the present study indicate that C1-Cl blocks mouse neuromuscular transmission, which can explain the skeletal muscle paralysis.

5. Conclusion

This study is the first report which reveals that organoruthenium compound C1-Cl inhibits nerve-evoked skeletal muscle twitch and tetanic contraction with no effect on directly muscle-elicited twitch and tetanic contraction. The inhibition is concentration-dependent, with IC_{50} being observed in the low micromolar range. The most likely mechanism underlying for this inhibitory activity of C1-Cl includes reversible inhibition of muscle-type nAChRs since C1-Cl reduced the number of open nAChRs by altering neither skeletal muscle fiber passive membrane properties nor neurotransmitter ACh release at low concentrations of C1-Cl at which it affects the amplitude of MEPPs. This blocking nAChR activity remains, however, to be confirmed by studying

the natural compound effects of C1-Cl on muscle-type nAChRs incorporated into the membrane of *Xenopus* oocytes. Despite the evidence of considerable inhibitory activity against mammalian AChE *in vitro*, no evidence from the electrophysiological measurements was found in the present study for an eventual anti-AChE effect of C1-Cl in the neuromuscular junction. The blocking effect of C1-Cl *in vitro* is rapid and almost completely reversal by the reversible AChE inhibitor neostigmine and maybe therefore interesting for further preclinical testing as a new competitive neuromuscular blocking drug.

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Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2020.110161>.

References

- [1] A. Bencini, V. Lippolis, 1,10-Phenanthroline: a versatile building block for the construction of ligands for various purposes, *Coord. Chem. Rev.* 254 (17) (2010) 2096–2180, <https://doi.org/10.1016/j.ccr.2010.04.008>.
- [2] G. Accorsi, A. Listorti, K. Yoosaf, N. Armadori, 1,10-Phenanthrolines: versatile building blocks for luminescent molecules, materials and metal complexes, *Chem. Soc. Rev.* 38 (6) (2009) 1690–1700, <https://doi.org/10.1039/B806408N>.
- [3] P.G. Sammes, G. Yahioğlu, 1,10-Phenanthroline: a versatile ligand, *Chem. Soc. Rev.* 23 (5) (1994) 327–334, <https://doi.org/10.1039/CS9942300327>.
- [4] B.M. Zeglis, V.C. Pierre, J.K. Barton, Metallo-intercalators and metallo-insertors, *Chem. Commun.* 28 (44) (2007) 4565–4579, <https://doi.org/10.1039/B710949K>.
- [5] L. Viganor, O. Howe, P. McCarron, M. McCann, M. Devereux, The antibacterial activity of metal complexes containing 1,10-phenanthroline: potential as alternative therapeutics in the era of antibiotic resistance, *Curr. Top. Med. Chem.* 17

- (11) (2017) 1280–1302, <https://doi.org/10.2174/1568026616666161003143333>.
- [6] J. Canivet, L. Karmazin-Brelot, G. Süß-Fink, Cationic arene ruthenium complexes containing chelating 1,10-phenanthroline ligands, *J. Organomet. Chem.* 690 (13) (2005) 3202–3211, <https://doi.org/10.1016/j.jorganchem.2005.02.050>.
- [7] K.S. Ju, R.E. Parales, Nitroaromatic compounds, from synthesis to biodegradation, *Microbiol. Mol. Biol. Rev.* 74 (2) (2010) 250–272, <https://doi.org/10.1128/MMBR.00006-10>.
- [8] A. Gabriellsson, P. Matousek, M. Towrie, F. Hartl, S. Zališ, A. Vlček, Excited states of nitro-polypyridine metal complexes and their ultrafast decay. Time-resolved IR absorption, spectroelectrochemistry, and TD-DFT calculations of fac-[Re(Cl)(CO)₃(5-nitro-1,10-phenanthroline)], *J. Phys. Chem. A* 109 (28) (2005) 6147–6153, <https://doi.org/10.1021/jp051677h>.
- [9] I. Turel, A. Golobič, J. Kljun, P. Samastur, U. Batista, K. Sepčić, New synthetic routes for ruthenium-1,10-phenanthroline complexes. Tests of cytotoxic and antibacterial activity of selected ruthenium complexes, *Acta Chim. Slov.* 62 (2) (2015) 337–345, <https://doi.org/10.107344/acs.2014.1130>.
- [10] J. Kladničnik, J. Kljun, H. Burmeister, I. Ott, I. Romero-Canelon, I. Turel, Towards identification of essential structural elements of organoruthenium(II)-pyrithionato complexes for anticancer activity, *Chem. Eur. J.* 25 (62) (2019) 14169–14182, <https://doi.org/10.1002/chem.201903109>.
- [11] J. Kljun, M. Anko, K. Traven, M. Sinreih, R. Pavlič, Š. Peršič, Ž. Ude, E.E. Codina, J. Stojan, T. Lanišnik Rižner, I. Turel, Pyrithione-based ruthenium complexes as inhibitors of aldo-keto reductase 1C enzymes and anticancer agents, *Dalton Trans.* 45 (29) (2016) 11791–11800, <https://doi.org/10.1039/C6DT00668J>.
- [12] J. Kljun, I. Bratsos, E. Alessio, G. Psomas, A. Repnik, M. Butinar, B. Turk, I. Turel, New uses for old drugs: attempts to convert quinolone antibacterials into potential anticancer agents containing ruthenium, *Inorg. Chem.* 52 (15) (2013) 9039–9052, <https://doi.org/10.1021/ic401220x>.
- [13] A. Mitrović, J. Kljun, I. Sosić, S. Gobec, I. Turel, J. Kos, Clitoxinol-ruthenium complex impairs tumour cell invasion by inhibiting cathepsin B activity, *Dalton Trans.* 45 (42) (2016) 16913–16921, <https://doi.org/10.1039/C6DT02369J>.
- [14] A. Mitrović, J. Kljun, I. Sosić, M. Uršič, A. Meden, S. Gobec, J. Kos, I. Turel, Organoruthenated nitroxoline derivatives impair tumor cell invasion through inhibition of cathepsin B activity, *Inorg. Chem.* 58 (18) (2019) 12334–12347, <https://doi.org/10.1021/acs.inorgchem.9b01882>.
- [15] S. Ristovski, M. Uzelac, J. Kljun, T. Lipec, M. Uršič, S.Z. Jokhadar, M.C. Žužek, T. Trobec, R. Frangež, K. Sepčić, I. Turel, Organoruthenium prodrugs as a new class of cholinesterase and glutathione-S-transferase inhibitors, *ChemMedChem* 13 (20) (2018) 2166–2176, <https://doi.org/10.1002/cmdc.201800432>.
- [16] M.C. Ruiz, J. Kljun, I. Turel, A.L. Di Virgilio, I.E. Leon, Comparative antitumor studies of organoruthenium complexes with 8-hydroxyquinolines on 2D and 3D cell models of bone, lung and breast cancer, *Metallomics* 11 (3) (2019) 666–675, <https://doi.org/10.1039/C8MT00369F>.
- [17] S. Seršen, K. Traven, J. Kljun, I. Turel, C.T. Supuran, Organoruthenium(II) complexes of acetazolamide potentially inhibit human carbonic anhydrase isoforms I, II, IX and XII, *J. Enzyme Inhib. Med. Chem.* 34 (1) (2019) 388–393, <https://doi.org/10.1080/14756366.2018.1547288>.
- [18] Y. Sheng, Z. Hou, S. Cui, K. Cao, S. Yuan, M. Sun, J. Kljun, G. Huang, I. Turel, Y. Liu, Covalent versus noncovalent binding of ruthenium η²-p-cymene complexes to zinc-finger protein NcP7, *Chem. Eur. J.* 25 (5) (2019) 12789–12794, <https://doi.org/10.1002/chem.201902434>.
- [19] M.B. Colović, D.Z. Krstić, T.D. Lazarević-Pašti, A.M. Bondžić, V.M. Vasić, Acetylcholinesterase inhibitors: pharmacology and toxicology, *Curr. Neuropharmacol.* 11 (3) (2013) 315–335, <https://doi.org/10.2174/1570159X11311030006>.
- [20] K.A. Petrov, A.D. Kharlamova, O.A. Lenina, A.R. Nurtdinov, M.E. Sitydkova, V.I. Ilyin, I.V. Zueva, E.E. Nikolsky, Specific inhibition of acetylcholinesterase as an approach to decrease muscarinic side effects during myasthenia gravis treatment, *Sci. Rep.* 8 (1) (2018) 304, <https://doi.org/10.1038/s41598-017-18307-9>.
- [21] K.O. Okonjo, J. Kuhlmann, A. Maelicke, A 2nd pathway of activation of the Torpedo acetylcholine-receptor channel, *Eur. J. Biochem.* 200 (3) (1991) 671–677, <https://doi.org/10.1111/j.1432-1033.1991.tb16231.x>.
- [22] S.M. Sherby, A.T. Eldefrawi, E.X. Albuquerque, M.E. Eldefrawi, Comparison of the actions of carbamate anticholinesterases on the nicotinic acetylcholine-receptor, *Mol. Pharmacol.* 27 (3) (1985) 343–348.
- [23] E. Ros, J. Aleu, I.G. De Aranda, C. Canti, Y.P. Pang, J. Marsal, C. Solsona, Effects of bis(7)-tacrine on spontaneous synaptic activity and on the nicotinic ACh receptor of Torpedo electric organ, *J. Neurophysiol.* 86 (1) (2001) 183–189, <https://doi.org/10.1152/jn.2001.86.1.183>.
- [24] S. Olivera-Bravo, I. Ivorra, A. Morales, The acetylcholinesterase inhibitor BW284c51 is a potent blocker of Torpedo nicotinic AChRs incorporated into the *Xenopus* oocyte membrane, *Br. J. Pharmacol.* 144 (1) (2005) 88–97, <https://doi.org/10.1038/sj.bjp.0705965>.
- [25] M. Grandič, R. Araoz, J. Molgo, T. Turk, K. Sepčić, E. Benoit, R. Frangež, The non-competitive acetylcholinesterase inhibitor APS12-2 is a potent antagonist of skeletal muscle nicotinic acetylcholine receptors, *Toxicol. Appl. Pharm.* 265 (2) (2012) 221–228, <https://doi.org/10.1016/j.taap.2012.09.024>.
- [26] M. Grandič, R. Araoz, J. Molgo, T. Turk, K. Sepčić, E. Benoit, R. Frangež, Toxicity of the synthetic polymeric 3-alkylpyridinium salt (APS3) is due to specific block of nicotinic acetylcholine receptors, *Toxicology* 303 (1) (2013) 25–33, <https://doi.org/10.1016/j.tox.2012.10.013>.
- [27] M.C. Žužek, M. Grandič, E. Benoit, R. Frangež, Natural polymeric 3-alkylpyridinium salt affects vertebrate skeletal muscle contractility by preferentially blocking neuromuscular transmission, *Toxicol. Lett.* 281 (2017) 95–101, <https://doi.org/10.1016/j.toxlet.2017.09.007>.
- [28] O.V. Dolomanov, L.J. Bourhis, R.J. Gildea, J.A.K. Howard, H. Puschmann, OLEX2: a complete structure solution, refinement and analysis program, *J. Appl. Crystallogr.* 42 (2009) 339–341, <https://doi.org/10.1107/S0021889808042726>.
- [29] G. Sheldrick, SHELXT - integrated space-group and crystal-structure determination, *Acta Crystallogr. A* 71 (1) (2015) 3–8, <https://doi.org/10.1107/S2053273314026370>.
- [30] G.L. Ellman, K.D. Courtney, V. Andres Jr., R.M. Feather-Stone, A new and rapid colorimetric determination of acetylcholinesterase activity, *Biochem. Pharmacol.* 7 (2) (1961) 88–95, [https://doi.org/10.1016/0006-2952\(61\)90145-9](https://doi.org/10.1016/0006-2952(61)90145-9).
- [31] E.K. Olsen, E. Hansen, L.W.K. Moodie, J. Isaksson, K. Sepčić, M. Cergolj, J. Svenson, J.H. Andersen, Marine AChE inhibitors isolated from *Geodia barretti*: natural compounds and their synthetic analogs, *Org. Biomol. Chem.* 14 (5) (2016) 1629–1640, <https://doi.org/10.1039/C5OB02416A>.
- [32] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases. The first enzymatic step in mercapturic acid formation, *J. Biol. Chem.* 249 (22) (1974) 7130–7139.
- [33] L.J. Cruz, W.R. Gray, B.M. Olivera, R.D. Zeikus, L. Kerr, D. Yoshikami, E. Moczydlowski, Conus-geographus toxins that discriminate between neuronal and muscle sodium-channels, *J. Biol. Chem.* 260 (16) (1985) 9280–9288.
- [34] S.J. Hong, C.C. Chang, Use of geographutoxin-ii (Mu-conotoxin) for the study of neuromuscular-transmission in mouse, *Br. J. Pharmacol.* 97 (3) (1989) 934–940, <https://doi.org/10.1111/j.1476-5381.1989.tb12034.x>.
- [35] N.E. Pardo, R.K. Hajela, W.D. Atchison, Acetylcholine release at neuromuscular junctions of adult tottering mice is controlled by N-(Ca(v)2.2) and R-type (Ca(v)2.3) but not L-type (Ca(v)1.2) Ca²⁺ channels, *J. Pharmacol. Exp. Ther.* 319 (3) (2006) 1009–1020, <https://doi.org/10.1124/jpet.106.108670>.
- [36] S. Betanzos-Lara, L. Salassa, A. Habtemariam, O. Novakova, A.M. Pizarro, G.J. Clarkson, B. Liskova, V. Brabec, P.J. Sadler, Photoactivatable organometallic pyridyl ruthenium(II) arene complexes, *Organometallics* 31 (9) (2012) 3466–3479, <https://doi.org/10.1021/om201177y>.
- [37] M. Ganeshpandian, M. Palaniandavar, A. Muruganatham, S.K. Ghosh, A. Riyasdeen, M.A. Akbarsha, Ruthenium(II)-arene complexes of diimines: effect of diimine intercalation and hydrophobicity on DNA and protein binding and cytotoxicity, *Appl. Organomet. Chem.* 32 (3) (2018) 1–17, <https://doi.org/10.1002/aoc.4154>.
- [38] F. Martinez-Pena, S. Infante-Tadeo, A. Habtemariam, A.M. Pizarro, Reversible pH-responsive behavior of ruthenium(II) arene complexes with tethered carboxylate, *Inorg. Chem.* 57 (9) (2018) 5657–5668, <https://doi.org/10.1021/acs.inorgchem.8b00625>.
- [39] J. Minic, A. Chatonnet, E. Krejci, J. Molgo, Butyrylcholinesterase and acetylcholinesterase activity and quantal transmitter release at normal and acetylcholinesterase knockout mouse neuromuscular junctions, *Br. J. Pharmacol.* 138 (1) (2003) 177–187, <https://doi.org/10.1038/sj.bjp.0705010>.
- [40] J. Molgo, H. Lundh, S. Thesleff, Potency of 3,4-diaminopyridine and 4-aminopyridine on mammalian neuromuscular-transmission and the effect of pH changes, *Eur. J. Pharm.* 61 (1) (1980) 25–34, [https://doi.org/10.1016/0014-2999\(80\)90378-7](https://doi.org/10.1016/0014-2999(80)90378-7).
- [41] F.P. Dwyer, E.C. Gyarfas, W.P. Rogers, J.H. Koch, Biological activity of complex ions, *Nature* 170 (4318) (1952) 190–191, <https://doi.org/10.1038/170190a0>.
- [42] N. Alatrash, E.S. Narh, A. Yadav, M.J. Kim, T. Janaratne, J. Gabriel, F.M. MacDonnell, Synthesis, DNA cleavage activity, cytotoxicity, acetylcholinesterase inhibition, and acute murine toxicity of redox-active ruthenium(II) polypyridyl complexes, *ChemMedChem* 12 (13) (2017) 1055–1069, <https://doi.org/10.1002/cmdc.201700240>.
- [43] C.R. Cardoso, I. de Aguiar, M.R. Camilo, M.V. Lima, A.S. Ito, M.S. Baptista, C. Pavani, T. Venancio, R.M. Carlos, Synthesis, spectroscopic characterization, photochemical and photophysical properties and biological activities of ruthenium complexes with mono- and bi-dentate histamine ligand, *Dalton Trans.* 41 (22) (2012) 6726–6734, <https://doi.org/10.1039/C2DT12136K>.
- [44] S.P. Mulcahy, S. Li, R. Korn, X. Xie, E. Meggers, Solid-phase synthesis of tris-heteroleptic ruthenium(II) complexes and application to acetylcholinesterase inhibition, *Inorg. Chem.* 47 (12) (2008) 5030–5032, <https://doi.org/10.1021/ic800808b>.
- [45] D.E. Silva, M.P. Cali, W.M. Pazin, E. Carlos-Lima, M.T. Salles Trevisan, T. Venancio, M. Arcisio-Miranda, A.S. Ito, R.M. Carlos, Luminescent Ru(II) phenanthroline complexes as a probe for real-time imaging of beta self-aggregation and therapeutic applications in alzheimer's disease, *J. Med. Chem.* 59 (19) (2016) 9215–9227, <https://doi.org/10.1021/acs.jmedchem.6b01130>.
- [46] N.A. Vyas, S.S. Bhat, A.S. Kumbhar, U.B. Sonawane, V. Jani, R.R. Joshi, S.N. Ramteke, P.P. Kulkarni, B. Joshi, Ruthenium(II) polypyridyl complex as inhibitor of acetylcholinesterase and Abeta aggregation, *Eur. J. Med. Chem.* 75 (2014) 375–381, <https://doi.org/10.1016/j.ejmech.2014.01.052>.
- [47] N.A. Vyas, S.N. Ramteke, A.S. Kumbhar, P.P. Kulkarni, V. Jani, U.B. Sonawane, R.R. Joshi, B. Joshi, A. Erleben, Ruthenium(II) polypyridyl complexes with hydrophobic ancillary ligand as Abeta aggregation inhibitors, *Eur. J. Med. Chem.* 121 (2016) 793–802, <https://doi.org/10.1016/j.ejmech.2016.06.038>.
- [48] L.W.K. Moodie, K. Sepčić, T. Turk, R. Frangež, J. Svenson, Natural cholinesterase inhibitors from marine organisms, *Nat. Prod. Rep.* 36 (8) (2019) 1053–1092, <https://doi.org/10.1039/C9NP00010K>.
- [49] F. Bosch, M. Morales, A. Badia, J.E. Banos, Effects of velnacrine, tacrine and physostigmine on tetanic twitch responses at the rat neuromuscular junction, *Eur. J. Pharmacol.* 222 (1) (1992) 163–166, [https://doi.org/10.1016/0014-2999\(92\)90832-O](https://doi.org/10.1016/0014-2999(92)90832-O).
- [50] A.L. Clark, F. Hobbiger, Twitch potentiation by organophosphate anticholinesterases in rat phrenic nerve diaphragm preparations, *Br. J. Pharmacol.* 78 (1) (1983) 239–246, <https://doi.org/10.1111/j.1476-5381.1983.tb09385.x>.
- [51] J.D. Morrison, The generation of nerve and muscle repetitive activity in the rat phrenic nerve-diaphragm preparation following inhibition of cholinesterase by ecotiopate, *Br. J. Pharmacol.* 60 (1) (1977) 45–53, <https://doi.org/10.1111/j>.

- 1476-5381.1977.tb16745.x.
- [52] B. Sarkar, A. Mondal, Y. Madaan, N. Roy, A. Moorthy, Y.C. Kuo, P. Paira, Luminescent anticancer ruthenium(II)-*p*-cymene complexes of extended imidazo-phenanthroline ligands: synthesis, structure, reactivity, biomolecular interactions and live cell imaging, *Dalton Trans.* 48 (32) (2019) 12257–12271, <https://doi.org/10.1039/c9dt00921c>.
- [53] E. Alessio, Thirty years of the drug candidate NAMI-A and the myths in the field of ruthenium anticancer compounds: a personal perspective, *Eur. J. Inorg. Chem.* (12) (2017) 1549–1560, <https://doi.org/10.1002/ejic.201600986>.
- [54] A.L. Harvey, A. Barfaraz, E. Thomson, A. Faiz, S. Preston, J.B. Harris, Screening of snake-venoms for neurotoxic and myotoxic effects using simple in-vitro preparations from rodents and chicks, *Toxicol.* 32 (3) (1994) 257–265, [https://doi.org/10.1016/0041-0101\(94\)90078-7](https://doi.org/10.1016/0041-0101(94)90078-7).
- [55] W. Van der Kloot, O.P. Balezina, J. Molgo, L.A. Naves, The timing of channel opening during miniature endplate currents at the frog and mouse neuromuscular junctions: effects of fasciculin-2, other anti-cholinesterases and vesamicol, *Pflugers Arch.* 428 (2) (1994) 114–126, <https://doi.org/10.1007/BF00374848>.
- [56] C.C. Chang, S.J. Hong, J.L. Ko, Mechanisms of the inhibition by neostigmine of tetanic contraction in the mouse diaphragm, *Br. J. Pharmacol.* 87 (4) (1986) 757–762, <https://doi.org/10.1111/j.1476-5381.1986.tb14594.x>.
- [57] B. Katz, R. Miledi, The binding of acetylcholine to receptors and its removal from the synaptic cleft, *J. Physiol.* 231 (3) (1973) 549–574, <https://doi.org/10.1113/jphysiol.1973.sp010248>.
- [58] S.J. Hong, C.C. Chang, Facilitation by 3,4-diaminopyridine of regenerative acetylcholine-release from mouse motor-nerve, *Br. J. Pharmacol.* 101 (4) (1990) 793–798, <https://doi.org/10.1111/j.1476-5381.1990.tb14159.x>.
- [59] J. Molgo, M. Lemeignan, F. Peradejordi, P. Lechat, Presynaptic effects of aminopyridines in the vertebral neuromuscular-junction, *J. Pharmacol.* 16 (2) (1985) 109–144.
- [60] W. Vanderkloot, J. Molgo, Quantal acetylcholine-release at the vertebrate neuromuscular-junction, *Physiol. Rev.* 74 (4) (1994) 899–991, <https://doi.org/10.1152/physrev.1994.74.4.899>.