



**HAL**  
open science

## New insights into the effects of organometallic ruthenium complexes on nicotinic acetylcholine receptors

Tomaž Trobec, Nicolas Lamassiaude, Evelyne Benoit, Monika Cecilija Žužek, Kristina Sepčić, Jerneja Kladnik, Iztok Turel, Rómulo Aráoz, Robert Frangež

### ► To cite this version:

Tomaž Trobec, Nicolas Lamassiaude, Evelyne Benoit, Monika Cecilija Žužek, Kristina Sepčić, et al.. New insights into the effects of organometallic ruthenium complexes on nicotinic acetylcholine receptors. *Chemico-Biological Interactions*, 2024, 402, pp.111213. 10.1016/j.cbi.2024.111213 . hal-04681985

**HAL Id: hal-04681985**

**<https://hal.science/hal-04681985v1>**

Submitted on 30 Aug 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## New insights into the effects of organometallic ruthenium complexes on nicotinic acetylcholine receptors

Tomaž Trobec<sup>1,2</sup>, Nicolas Lamassiaude<sup>1</sup>, Evelyne Benoit<sup>1</sup>, Monika Cecilija Žužek<sup>2</sup>, Kristina Sepčič<sup>3</sup>, Jerneja Kladnik<sup>4</sup>, Iztok Turel<sup>4</sup>, Rómulo Aráoz<sup>1</sup>, Robert Frangež<sup>2,\*</sup>

<sup>1</sup> 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), EMR CNRS/CEA 9004, 91191 Gif-sur-Yvette, France.

<sup>2</sup> Institute of Preclinical Sciences, Veterinary Faculty, University of Ljubljana, 1000 Ljubljana, Slovenia.

<sup>3</sup> Department of Biology, Biotechnical Faculty, University of Ljubljana, 1000 Ljubljana, Slovenia.

<sup>4</sup> Faculty of Chemistry and Chemical Technology, University of Ljubljana, 1000 Ljubljana, Slovenia.

\* Corresponding author: Robert Frangež; robert.frangez@vf.uni-lj.si; Institute of Preclinical Sciences, Veterinary Faculty, University of Ljubljana, 1000 Ljubljana, Slovenia.

**Short title:** Effects of organometallic ruthenium complexes on nAChRs.

**Abstract.** Nicotinic acetylcholine receptors (nAChRs) are expressed in excitable and non-excitable cells of the organism. Extensive studies suggest that nAChR ligands have therapeutic potential, notably for neurological and psychiatric disorders. Organometallic ruthenium complexes are known to inhibit several medically important enzymes such as cholinesterases. In addition, they can also interact with muscle- and neuronal-subtype nAChRs. The present study aimed to investigate the direct effects of three organometallic ruthenium complexes,  $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{II})(5\text{-nitro-1,10-phenanthroline})\text{Cl}]\text{Cl}$  (**C1-Cl**),  $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{II})(1\text{-hydroxypyridine-2(1H)-thionato})\text{Cl}]$  (**C1a**) and  $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{II})(1\text{-hydroxy-3-methoxypyridine-2(1H)-thionato})\text{pta}]\text{PF}_6$  (**C1**), on muscle-subtype (*Torpedo*) nAChRs and on the two most abundant human neuronal-subtype nAChRs in the CNS ( $\alpha 4\beta 2$  and  $\alpha 7$ ) expressed in *Xenopus laevis* oocytes, using the two-electrode voltage-clamp. The results show that none of the three compounds had agonistic activity on any of the nAChR subtypes studied. In contrast, **C1-Cl** reversibly blocked *Torpedo* nAChR (half-reduction of ACh-evoked peak current amplitude by 332 nM of compound). When tested at 10  $\mu\text{M}$ , **C1-Cl** was statistically more potent to inhibit *Torpedo* nAChR than  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs. Similar results of **C1** effects were obtained on *Torpedo* and  $\alpha 4\beta 2$  nAChRs, while no action of the compound was detected on  $\alpha 7$  nAChRs. Finally, the effects of **C1a** were statistically similar on the three nAChR subtypes but, in contrast to **C1-Cl** and **C1**, the inhibition was hardly reversible. These results, together with our previous studies on isolated mouse neuromuscular preparations, strongly suggest that **C1-Cl** is, among the three compounds studied, the only molecule that could be used as a potential myorelaxant drug.

**Keywords:** organometallic ruthenium complexes; nicotinic acetylcholine receptors; nicotinic antagonists; electrophysiology; two-electrode voltage-clamp technique.

### Abbreviations:

Acetylcholine (ACh); nicotinic acetylcholine receptors (nAChRs); central nervous system (CNS); dimethyl sulfoxide (DMSO); mean  $\pm$  standard error of the mean (S.E.M.); peripheral nervous system (PNS); potassium acetate (KAc); two-electrode voltage-clamp (TEVC);  $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{II})(1\text{-hydroxy-3-methoxypyridine-2(1H)-thionato})\text{pta}]\text{PF}_6$  (**C1**);  $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{II})(1\text{-hydroxypyridine-2(1H)-thionato})\text{Cl}]$  (**C1a**);  $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{II})(5\text{-nitro-1,10-phenanthroline})\text{Cl}]\text{Cl}$  (**C1-Cl**).

### Introduction

Acetylcholine receptors are ligand-gated receptors that are activated by the binding of acetylcholine

(ACh) [1, 2]. The main acetylcholine receptor types are metabotropic muscarinic and ionotropic nicotinic receptors. These types of receptors are expressed in various cells of the organism, including neuronal and non-neuronal cells [1, 3].

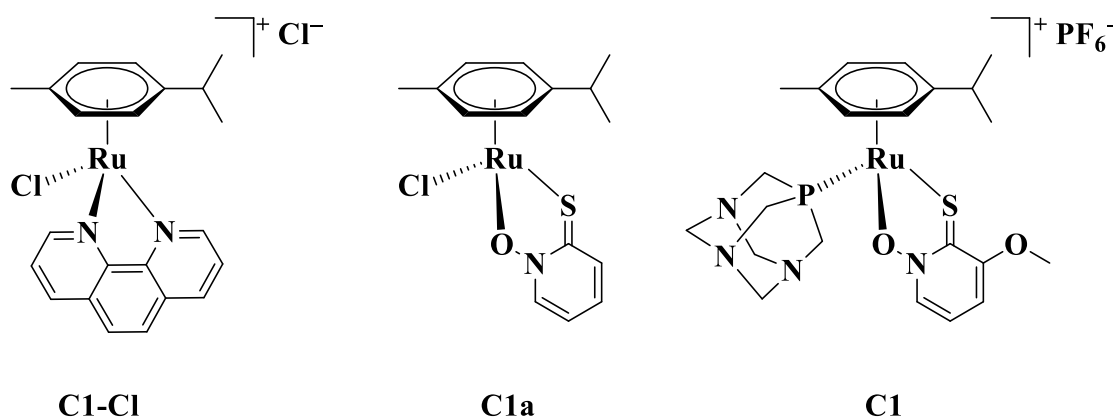
Nicotinic acetylcholine receptors (nAChRs) belong to the “Cys-loop” superfamily of ligand-gated ion channels which also includes GABA<sub>A</sub>, glycine and serotonin (5-HT<sub>3</sub>) receptors [4, 5]. They are ligand-gated cationic channels that are permeable to Na<sup>+</sup>, K<sup>+</sup> and, to some extent, to Ca<sup>2+</sup> ions, depending on the nAChR subtype [3, 5]. In mammals, there are several subtypes of nAChRs that differ in their subunit composition and, for some of them, in their localisation in the organism [1, 3, 4, 6]. Muscle-subtype nAChRs are heteropentamers and consist of five subunits:  $\alpha 1$ ,  $\beta 1$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$ . From these subunits, two muscle-subtypes are formed, the embryonic ( $\alpha 1\beta 1\delta\gamma$ ) and the adult ( $\alpha 1\beta 1\delta\epsilon$ ) ones in the stoichiometry of 2:1:1:1 [1, 3, 4, 6-8]. Neuronal subtypes of nAChRs (central and peripheral nervous systems), as well as those expressed in non-excitatory cells are composed as homopentamers or heteropentamers. In mammals, eight  $\alpha$  ( $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\alpha 9$  and  $\alpha 10$ ) and three  $\beta$  ( $\beta 2$ ,  $\beta 3$  and  $\beta 4$ ) neuronal and non-excitatory cell subunits are known. The structural and functional diversity of these nAChR subtypes arises notably from the many possible combinations of subunits. The most common subtypes in the mammalian CNS are  $\alpha 4\beta 2$  heteromeric and  $\alpha 7$  homomeric receptors [2-4, 8].

A large number of nAChRs are located in the central (CNS) and peripheral (PNS) nervous systems. This indicates that nAChRs perform a wide range of functions in the nervous systems, from mediating various cognitive processes in the CNS to neuromuscular transmission in the PNS [6]. Therefore, to treat the various neurological diseases, there is a great interest to modulate, in particular, homomeric  $\alpha 7$  and heteromeric  $\alpha 4\beta 2$  subtypes because of their involvement in the pathogenesis of several neurological and non-neurological diseases [2-4, 8, 9]. In addition, dysfunction of the muscle-subtype of nAChRs results in congenital myasthenic syndromes [10].

For more than three decades, efforts were focused on the development of nAChR ligands as new therapies, and progress has been made in the design and development of selective molecules targeting the different nAChR subtypes involved in CNS and PNS disorders. To date, however, only few synthetic nAChR ligands have been approved for clinical use to aid smoking cessation (varenicline, bupropione, nicotine, cytisine), hypertension (mecamylamine) and for the treatment of dry eye disease (varenicline) [11, 12]. Some non-depolarizing and short-acting skeletal muscle relaxants (atracurium, pancuronium, pipecuronium, vecuronium), that bind to and block the muscle-subtype of nAChRs, are clinically used as adjuncts to general anesthesia for muscle relaxation, neuromuscular blockade in mechanically ventilated patients in the intensive care unit, and endotracheal intubation [13].

Among emergent nAChR ligands, the organometallic ruthenium complex  $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{II})(5\text{-nitro-1,10-phenanthroline})\text{Cl}]\text{Cl}$  (**C1-Cl**) was recently reported to inhibit (i) the nerve-evoked single twitch and tetanic contraction of mouse isolated skeletal muscles, without affecting the directly-evoked muscle single twitch and tetanic contraction, and (ii) the end-plate potentials, without significant change in the resting membrane potential [14]. The other organometallic ruthenium complex studied,  $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{II})(1\text{-hydroxypyridine-2(1H)-thionato})\text{Cl}]$  (**C1a**), had no significant effect on the mouse muscle contraction and membrane potential parameters of isolated muscles at pharmacologically relevant concentrations [15], as did the third organometallic ruthenium complex studied  $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{II})(1\text{-hydroxy-3-methoxypyridine-2(1H)-thionato})\text{pta}]\text{PF}_6$  (**C1**) [16]. Therefore, it is likely that **C1-Cl**, but not **C1a** and **C1**, interacts with muscle-subtype nAChRs, no information being available for interactions between these compounds and neuronal-subtype nAChRs. The aim of the present study was thus to investigate the effects of the three organometallic ruthenium complexes **C1-Cl**, **C1a** and **C1** (**Figure 1**) on muscle-subtype (*i.e.* *Torpedo*) nAChRs and the two most abundant human neuronal nAChR subtypes in the CNS (*i.e.*  $\alpha 4\beta 2$  and  $\alpha 7$ ) [4, 7], expressed in *Xenopus*

*laevis* oocytes and using the two-electrode voltage-clamp (TEVC) technique. It is worth noting that the incorporation of *Torpedo marmorata* membranes, rich in native nAChR muscle-subtype, into the oocyte membrane allows microtransplantation of fully processed complexes while maintaining their native environment (*i.e.* clusters of rapsyn protein and lipids). In addition, these membranes have often been used to characterize a given molecule action on the nAChR muscle-subtype, in particular at the molecular level (see [17] and, for a recent review, [18]).



**Figure 1. Chemical structures of the organometallic ruthenium complexes C1-Cl, C1a and C1.**

## Material and Methods

### Organometallic ruthenium complexes and drugs

**C1-Cl** (molecular weight of 486.40, purity rate > 95%), **C1a** (molecular weight of 396.90, purity rate > 95%) and **C1** (molecular weight of 693.59, purity rate > 95%) were chemically synthesized and prepared as previously reported [14-16, 19]. Kanamycin and ACh chloride were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). All chemicals were of the highest grade available.

### Expression of *Torpedo*, $\alpha 4\beta 2$ and $\alpha 7$ nAChRs in *Xenopus laevis* oocytes

Standard procedures were used to express human  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs, as well as to prepare electrocyte membrane aliquots rich of muscle-subtype ( $\alpha 1$ )<sub>2</sub> $\beta 1\delta\gamma$  nAChRs from *Torpedo marmorata* electrical organ that is an extremely abundant source of nAChRs [20, 21]. Briefly, oocytes of stages V-VI were harvested from anesthetized *Xenopus laevis* female and maintained in OR2 medium of the following composition: 88 mM NaCl, 1 mM MgCl<sub>2</sub>, 5 mM hydroxyethylpiperazine ethane sulfonic acid (HEPES), pH 7.4 adjusted with NaOH. Oocytes were manually defolliculated and maintained at 19°C in Barth's medium containing 88 mM NaCl, 1 mM KCl, 0.41 mM CaCl<sub>2</sub>, 0.82 mM MgSO<sub>4</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 2.4 mM NaHCO<sub>3</sub>, 7.5 mM HEPES (pH 7.4), and supplemented with kanamycin (500 µg/mL). Purified *Torpedo* membranes enriched in ( $\alpha 1$ )<sub>2</sub> $\beta 1\delta\gamma$  nAChRs (150 ng in 5 mM glycine), human  $\alpha 4\beta 2$  cDNA (2.5 ng of each subunit) or human  $\alpha 7$  mRNA (5 ng) were microinjected into the cytoplasm (*Torpedo* membranes and mRNA) or the nucleus (cDNA) of oocytes with a Nanoliter2000 Micro4 Controller (World Precision Instruments Inc., Stevenage, Herts, UK) mounted on a microscope. Recordings were performed 2-5 days after microinjections. It is worth noting that the injection of *Torpedo* electrocyte membranes into oocytes also results in the incorporation of ClC-0 to the oocyte membrane which is much more efficient than that of nAChRs [22, 23]. Therefore, the presence of ClC-0 channels would be expected in the plasma membrane of oocytes injected with *Torpedo* electrocyte membranes. However, the current responses to ACh were shown to be similar for oocytes injected with *Torpedo* electrocyte membranes and *Torpedo* mRNA [24]. In addition, the density of ClC-0 has

been reported to be 2-4% of the total membrane proteins [25] whereas that of nAChRs is up to 40-50% [26]. Therefore, it is unlikely that the presence of CIC-0 affects markedly the present results.

#### Electrophysiological recordings from *Xenopus* oocytes having incorporated *Torpedo* nAChRs

TEVC recordings from *Xenopus* oocytes transplanted with *Torpedo* nAChRs were manually performed at 20°C with an OC-725B amplifier (Warner Instruments, LLC, Hamden, MA, USA) [27]. Glass microelectrodes were filled with 1 M KCl and 1.5 M potassium acetate (KAc) and had resistances of 2-5 MΩ. The studied oocyte was placed in the recording chamber (300 μL capacity) and continuously perfused (8-12 mL/min) with a barium Ringer's solution containing 100 mM NaCl, 2.8 mM KCl, 1 mM MgCl<sub>2</sub>, 0.3 mM BaCl<sub>2</sub> and 5 mM HEPES (pH 7.4). The membrane potential was maintained at -60 mV. Experimental data were acquired using the pCLAMP-9 software (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA), and digitized using a Digidata-1322A A/D converter (Molecular Devices, Union City, CA, USA). The stock solution of ACh was prepared in deionized water at 1 M, those of **C1-CI** and **C1a** were prepared in barium Ringer's solution, and that of **C1** was prepared in deionized water with 5% dimethyl sulfoxide (DMSO). All stock solutions were diluted in the barium Ringer's solution to give the final concentrations indicated in the text. At the beginning of experiments, 50 μM ACh (two times the half-maximal effective ACh concentration) were applied three times for 15 seconds to each oocyte to record the ACh-induced current under control conditions. After each ACh application, the oocyte was perfused with barium Ringer's solution for 150 seconds. Then, the oocyte was perfused for 45 seconds with a specific concentration of a given ruthenium compound (**C1-CI**, **C1a** or **C1**) to test whether the compound alone had an agonistic effect on nAChRs and, immediately after, with a mixture of ACh and **C1a**, **C1-CI** or **C1** for 15 seconds to test the effects of a given compound on ACh-induced current. After 150 seconds of perfusion with barium Ringer's solution, each oocyte was re-exposed (for 15 seconds) to ACh alone to assess the current recovery.

The voltage dependence of ACh-induced current activation was assessed by applying a series of 90-ms voltage pulses (from -120 to 60 mV, in 20 mV steps), from a holding potential of -60 mV, to a given oocyte during the maximal current elicited by 50 μM ACh alone or together with **C1-CI**.

#### Electrophysiological recordings from *Xenopus* oocytes expressing human α4β2 or α7 nAChRs

An automated TEVC system, the HiClamp system (MCS GmbH., Reutlingen, Germany) was used for current recordings from *Xenopus* oocytes expressing human α4β2 or α7 nAChRs [28]. The studied oocyte was taken from a 96-well microtiter plate and placed in a silver wire basket which also serves as a reference bath electrode. The oocyte was automatically clamped at a holding potential of -60 mV using glass microelectrodes (2-5 MΩ resistance) filled with a mixture of 1 M KCl and 1.5 M KAc. The calcium Ringer's external solution contained 100 mM NaCl, 2.8 mM KCl, 1 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub> and 5 mM HEPES (pH 7.4). The clamped oocyte was then transferred into successive wells of the sample-microplate filled with 200 μL of (i) calcium Ringer's solution or (ii) 10 μM **C1-CI**, **C1a** or **C1** for 45 s, (iii) 100 μM ACh alone for 10 s (α4β2) and 5 s (α7) or (iib) 100 μM ACh together with 10 μM **C1-CI**, **C1a** or **C1** for 10 s (α4β2) and 5 s (α7), and (iii) calcium Ringer's solution. Periods of 150 s of perfusion with calcium Ringer's solution were programmed prior compounds and ACh application. The amplifier allowed continuous recordings, even during the transfer of oocytes between the different bathes.

#### Data analyses

Electrophysiological recordings were analysed using the Clampfit-11.1 (Axon Instruments, Molecular Devices) and DataMining for automated TEVC (HiClamp, Multichannel Systems) softwares. Then, subsequent data analyses and graph generation were performed using the SigmaPlot-14.5 (Systat Software Inc, San Jose, CA, USA), GraphPad Prism-9.5.1 (GraphPad Software, Boston, MA, USA) and Origin Pro 2021 (OriginLab, Northampton, MA, USA) softwares.

To evaluate the compound-induced current inhibition of a given oocyte, peak current amplitude ( $I$ ) values were measured in the presence of various compound concentrations  $[C]$  and normalized to the maximal value ( $I_{max}$ ) recorded under control conditions, to yield fractional ( $F$ ) response data. Theoretical concentration-response curves were obtained by typical sigmoid non-linear regressions through data points (coefficient of determination,  $r^2$ ), according to the following equation (Equation 1):  $F = 1/[1 + ([C]/IC_{50})^{n_H}]$ , where  $IC_{50}$  is the compound concentration that reduces the ACh-evoked peak current amplitude by 50%, and  $n_H$  is the Hill number.

### Statistical analyses

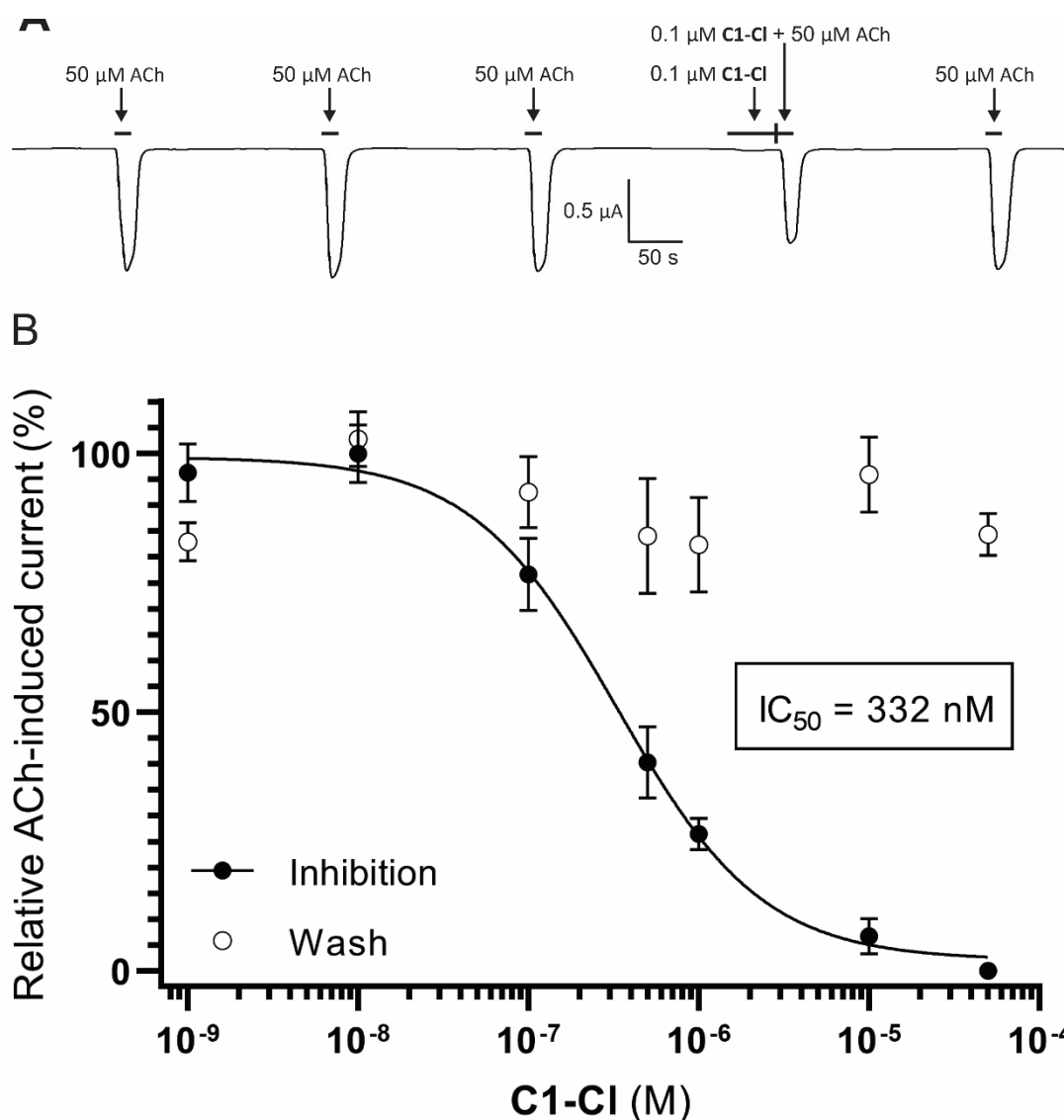
Data, presented as mean  $\pm$  standard error of the mean (S.E.M.) of  $n$  different oocytes, were statistically analysed using the SigmaPlot-14.5 software. Data were firstly tested for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe) to assign them to parametric or non-parametric analyses. Then, if the values of variables were normally distributed, the parametric two-tailed Student's  $t$ -test and the one-way analysis of variance (one-way ANOVA) followed by a Holm-Sidak test were used for statistical comparisons between two and at least three groups, respectively. These statistical analysis were used for all the present data except those concerning the current kinetic data that were non-normally distributed. In this case, a non-parametric Mann-Whitney test was used. Differences were considered to be statistically significant at  $P \leq 0.05$ .

## **Results and Discussion**

### Effects of **C1-CI** on *Torpedo* nAChRs

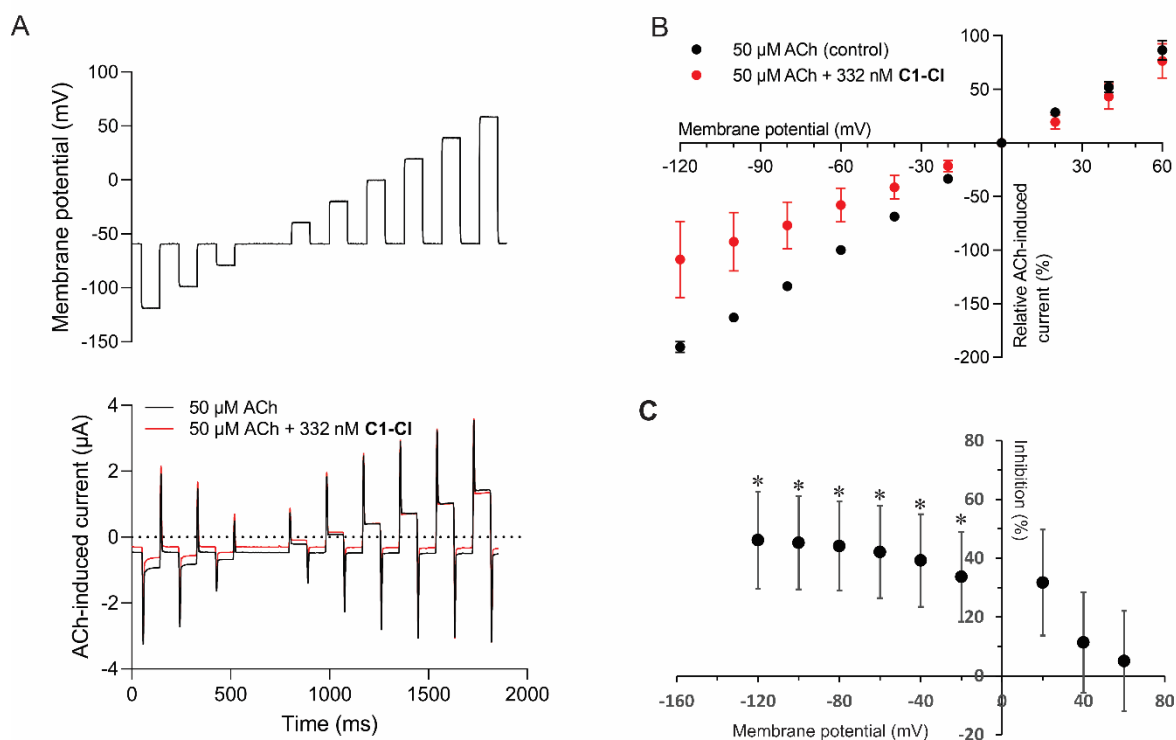
Superfusion of oocytes microtransplanted with *Torpedo* nAChRs, and clamped at  $-60$  mV, with  $50 \mu\text{M}$  ACh produced an inward and transient current activation. In contrast, superfusion of oocytes with  $0.5$ - $50 \mu\text{M}$  **C1-CI** did not evoke any current activation. This indicates that the compound, in the absence of the orthosteric agonist ACh, had no agonistic activity on *Torpedo* nAChRs, *i.e.* did not mimic the action of ACh on these receptors. However,  $0.1 \mu\text{M}$  **C1-CI** applied together with  $50 \mu\text{M}$  ACh induced an inward and transient current whose peak amplitude was about 75% that produced by  $50 \mu\text{M}$  ACh alone. The compound-induced inhibition of ACh-evoked current was fully reversible (**Figure 2A**). Moreover, at concentrations ranging from  $0.1$  to  $50 \mu\text{M}$ , **C1-CI** reversibly inhibited the ACh-induced current in a concentration-dependent manner. From the concentration-response curve of the effects of **C1-CI** on the peak ACh-induced current amplitude,  $IC_{50}$  and  $n_H$  values of  $332$  nM and  $1.02$  ( $n = 4-8$ ) were determined, respectively (**Figure 2B**). These results are in accordance with those previously obtained from **C1-CI** effects on nerve-evoked contraction of isolated mouse skeletal muscles, *i.e.* adult  $\alpha 1\beta 1\delta \epsilon$  (mouse) nAChR muscle-subtype [14], although the compound was between 30- and 60-fold more efficient to block *Torpedo* nAChRs.

The effects of **C1-CI** were then investigated on the voltage-dependence of activation of ACh-evoked current through *Torpedo* nAChRs. To this purpose, voltage pulses were applied to oocytes during the maximal current elicited by  $50 \mu\text{M}$  ACh alone or together with  $332$  nM **C1-CI**, *i.e.* at the  $IC_{50}$  (**Figure 3A**). As shown in **Figure 3B**, the voltage-dependence curves of current activation by ACh alone or together with **C1-CI** were apparently linear. A ratio of ACh and **C1-CI** conductance to ACh conductance of 61% could be determined from the curve slopes, a value close to the one expected with  $332$  nM of compound, *i.e.* 50% (see **Figure 2B**). In addition, the two curves intercepted the voltage axis at  $0$  mV, signifying that the current reversal potential was not modified by the compound. However, calculating the percentage of current inhibition at each test potential revealed that the **C1-CI**-induced inhibition was only significant at negative voltages (**Figure 3C**). These results indicate that the blocking effects of ACh-induced current produced by **C1-CI** were voltage-dependent.



**Figure 2. Effects of C1-Cl on ACh-induced current through *Torpedo* nAChRs.** (A) Representative traces of ACh-induced inward current recorded from an oocyte microtransplanted with *Torpedo* nAChRs, in response to applications of (i) 50  $\mu$ M ACh for 15 s (three times), (ii) 0.1  $\mu$ M C1-Cl for 45 s, (iii) 0.1  $\mu$ M C1-Cl together with 50  $\mu$ M ACh for 15 s, and (iv) 50  $\mu$ M ACh for 15 s (wash) (bars above recordings). The holding potential was -60 mV. (B) Concentration-response curves of the blocking effects of C1-Cl (Inhibition, filled circles) and their reversibility (Wash, open circles) on ACh-evoked current recorded from oocytes microtransplanted with *Torpedo* nAChRs. Currents were elicited by 15 s application of (i) 50  $\mu$ M ACh (three times, control), (ii) C1-Cl (0.001-50  $\mu$ M) together with 50  $\mu$ M ACh (one time), and (iii) again 50  $\mu$ M ACh (one time, wash). The peak current amplitude recorded in response to either a given C1-Cl concentration together with ACh or ACh alone (wash) was normalized to the mean one triggered by the first three-time applications of ACh alone (control), and plotted against the logarithm of C1-Cl concentration. The theoretical curve was calculated from typical sigmoid non-linear regression through data points ( $r^2 = 0.933$ ), according to Equation 1 (see Material and Methods) with IC<sub>50</sub> and n<sub>H</sub> values of 332 nM and 1.08, respectively. Mean  $\pm$  S.E.M. of 4-8 oocytes.

In conclusion, C1-Cl is able to reversibly block *Torpedo* nAChRs, the complex effects occurring only at negative voltages. At the molecular level, this implies that the C1-Cl-binding site on this subtype of nAChRs is located within the channel pore and that the compound binding is removed by positive voltages.



**Figure 3. Effects of C1-Cl on voltage-dependence of activation of ACh-induced current through *Torpedo* nAChRs.** (A) Upper: Protocol used to access to the voltage-dependence of activation of ACh-induced current consisting in a series of 90-ms voltage pulses (from  $-120$  to  $60$  mV, in  $20$  mV steps) applied from a holding potential of  $-60$  mV to oocytes microtransplanted with *Torpedo* nAChRs, during the maximal current. Lower: Representative traces of current recorded during the above protocol and elicited by either  $50$   $\mu$ M ACh alone (control, black trace) or together with  $332$  nM C1-Cl (red trace). (B) Voltage-dependence of activation. The values of peak current amplitude were normalized to those obtained at  $-60$  mV under control conditions. Mean  $\pm$  S.E.M. of 4 oocytes. (C) Percentage of current inhibition calculated at each test potential. \*:  $P < 0.034$  versus control.

#### Effects of C1-Cl on human neuronal-subtype ( $\alpha 4\beta 2$ and $\alpha 7$ ) nAChRs

To get information on the nAChR selectivity profile of C1-Cl, the effects of  $10$   $\mu$ M of compound were studied on human neuronal-subtype ( $\alpha 4\beta 2$  and  $\alpha 7$ ) nAChRs, the two most abundant neuronal-subtypes in the CNS [4, 7], and compared with those on *Torpedo* nAChRs. As observed on *Torpedo* nAChRs (see above), the compound had no agonistic activity on the two human neuronal-subtypes of nAChRs (Data not shown). In contrast, incubation of oocytes expressing human neuronal-subtype ( $\alpha 4\beta 2$  or  $\alpha 7$ ) nAChRs, clamped at  $-60$  mV, with  $100$   $\mu$ M ACh produced an inward and transient current activation that was reversibly decreased by incubating the same oocyte with  $10$   $\mu$ M C1-Cl together with  $100$   $\mu$ M ACh (Figure 4Aa). Under these conditions, the compound produced significant ( $P < 0.001$ ) mean decreases of  $58$  and  $75\%$  of the peak amplitude of ACh-evoked currents through human neuronal-subtype  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs, respectively (Figure 4B). It is worth noting that the effects of the compound on the ACh-evoked currents through the three nAChR subtypes were completely reversible since no significant ( $P > 0.742$ ) change in the mean peak amplitude of ACh-evoked currents was detected before and after compound application (Figure 4C).

Therefore, it appears that C1-Cl was statistically ( $P \leq 0.005$ ) more potent to reversibly inhibit *Torpedo* than  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs (Figures 2 and 4B). However, taking into account its previously reported *in vitro* action on cholinesterases [12], the compound was still more active to block the three nAChR subtypes than these enzymes. In addition, it is well known that some metal ions and their



organometallic complexes interact with cysteine-containing macromolecules. In particular, organometallic zinc complexes, in the low (sub)micromolar range, have been reported to exert potent *in vitro* inhibition of two cysteine proteases, cathepsin L and PL<sup>Pro</sup> involved in SARS-CoV-2 entry and replication, respectively, while **C1a** showed less effective inhibition of these enzymes in tens to hundreds of micromolar range [30-32].

#### Effects of **C1** or **C1a** on *Torpedo*, $\alpha 4\beta 2$ and $\alpha 7$ nAChRs

The effects of 10  $\mu\text{M}$  of two other organometallic ruthenium complexes, **C1** and **C1a**, were also evaluated on *Torpedo*,  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs, and compared with those of **C1-CI** on these three nAChR subtypes. As observed for **C1-CI** (see above), the two compounds had no agonistic activity on the three nAChR subtypes studied (Data not shown). The inward and transient ACh-evoked current through *Torpedo* or  $\alpha 4\beta 2$  nAChRs was reversibly decreased by applying 10  $\mu\text{M}$  **C1** together with 50-100  $\mu\text{M}$  ACh (**Figure 5Ab**). Indeed, the compound produced significant ( $P < 0.001$ ) mean decreases of 83 and 58% of the peak amplitude of ACh-evoked currents through *Torpedo* and  $\alpha 4\beta 2$  nAChRs, respectively (**Figure 5B**).

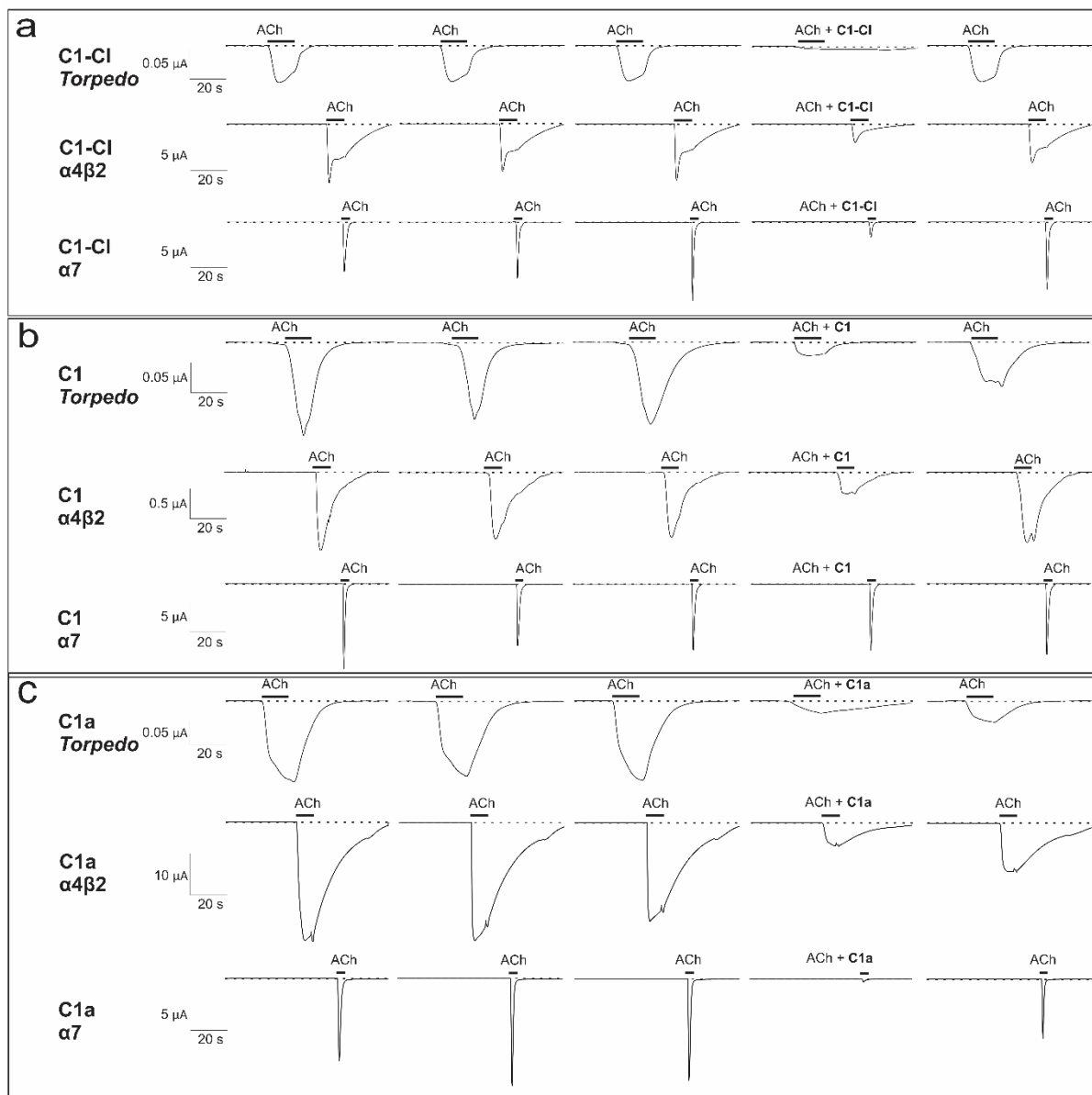
Therefore, the comparison of **C1** and **C1-CI** affinities points out that they were very close for *Torpedo* nAChRs ( $P = 0.948$ ) and even identical for  $\alpha 4\beta 2$  nAChRs ( $P = 0.986$ ). It is worth noting that the effects of **C1**, as those of **C1-CI**, on the ACh-evoked current through  $\alpha 4\beta 2$  nAChRs were completely reversible since no significant ( $P = 0.996$ ) change in the mean peak amplitude of ACh-evoked current was detected before and after compound application (**Figure 4C**). However, those on the ACh-evoked current through *Torpedo* nAChRs, in contrast to those of **C1-CI**, were only partly reversible since the mean peak amplitude of ACh-evoked current, after having being decreased to 17% by applying 10  $\mu\text{M}$  **C1** together with 50  $\mu\text{M}$  ACh (**Figure 4B**), returned to a mean value of only 61%, significantly ( $P = 0.007$ ) different from the control one, after the compound wash-out (**Figure 4C**). Finally, **C1** had no significant ( $P = 0.052$ ) effect on the ACh-evoked current through human neuronal-subtype  $\alpha 7$  nAChR (**Figure 4Ab, B, C**), indicating that this compound, at a concentration of 10  $\mu\text{M}$  and in contrast to **C1-CI**, did not bind to and block this neuronal-subtype of nAChRs.

The present results strongly suggest that **C1**, compared to **C1-CI**, might be the best suitable molecule for a long-lasting *in vivo* effect on muscle-subtype nAChRs. Moreover, the lack of effect of the compound on human neuronal-subtype ( $\alpha 7$ , although not  $\alpha 4\beta 2$ ) nAChRs is consistent with the potential clinical development of this molecule as a skeletal muscle relaxant, provided that its effects on the adult  $\alpha 1\beta 1\delta\epsilon$  muscle-subtype of nAChRs are similar to those observed on *Torpedo* nAChRs. However, it was recently reported that **C1**, at concentrations up to 120  $\mu\text{M}$ , had no marked effect on mouse muscle contraction and membrane potential parameters [16]. These unexpected results strongly suggest that the compound does not exert any action on the adult  $\alpha 1\beta 1\delta\epsilon$  nAChR muscle-subtype of mouse. Although further experiments are needed on the human adult  $\alpha 1\beta 1\delta\epsilon$  nAChR muscle-subtype to exclude some possible species differences in compound effects, a likely explanation is that the subunit  $\gamma$  of the embryonic muscle-subtype but not the  $\epsilon$  of the adult one is involved in the binding of **C1** to the receptor, as previously reported for  $\alpha$ A-conotoxin O1VB [33, 34]. In addition, although **C1** did not affect the electric eel and human recombinant acetylcholinesterase activities, at concentrations up to 180  $\mu\text{M}$ , the compound selectively inhibited those of horse serum ( $\text{IC}_{50} = 2.9 \mu\text{M}$ ) and human recombinant ( $\text{IC}_{50} = 144.2 \mu\text{M}$ ) butyrylcholinesterases [16].

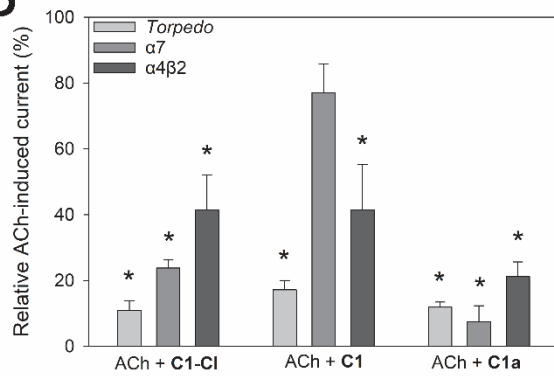
Finally, the inward and transient ACh-evoked current activation through *Torpedo* and human neuronal-subtype ( $\alpha 4\beta 2$  and  $\alpha 7$ ) nAChRs was decreased by applying 10  $\mu\text{M}$  **C1a** together with 50-100  $\mu\text{M}$  ACh, the decrease being hardly reversible (**Figure 4Ac**). The comparison between **C1a** (10  $\mu\text{M}$ ) and **C1-CI** (10  $\mu\text{M}$ ) effects pointed out that they were not significantly different for *Torpedo* ( $P = 0.984$ ),  $\alpha 4\beta 2$  ( $P = 0.205$ ) and  $\alpha 7$  ( $P = 0.369$ ) nAChRs (**Figure 4B**). However, the effects of **C1a** on the ACh-evoked current

through the three nAChR subtypes studied, in contrast to those of **C1-Cl**, were only slightly reversible (**Figure 4C**).

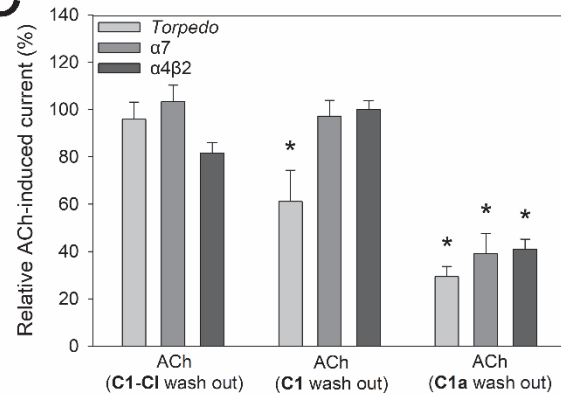
**A**



**B**



**C**



**Figure 4. Effects of C1-Cl, C1 and C1a on ACh-induced currents through muscle-subtype (*Torpedo*) and**

**human neuronal-subtype ( $\alpha 4\beta 2$  and  $\alpha 7$ ) nAChRs.** (A) Representative traces of ACh-induced inward currents recorded from oocytes expressing *Torpedo*,  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs, in response to applications of 50-100  $\mu\text{M}$  ACh for 15 s (*Torpedo* nAChRs), 10 s ( $\alpha 4\beta 2$  nAChRs) and 5 s ( $\alpha 7$  nAChRs) (three times), 50-100  $\mu\text{M}$  ACh together with 10  $\mu\text{M}$  **C1-Cl** (a), **C1** (b) and **C1a** (c) for 15 s (*Torpedo* nAChRs), 10 s ( $\alpha 4\beta 2$  nAChRs) and 5 s ( $\alpha 7$  nAChRs), and 50-100  $\mu\text{M}$  ACh for 15 s (*Torpedo* nAChRs), 10 s ( $\alpha 4\beta 2$  nAChRs) and 5 s ( $\alpha 7$  nAChRs) (bars above recordings). The holding potential was  $-60\text{mV}$ . (B,C) Histograms of the blocking effects (B) of 10  $\mu\text{M}$  **C1-Cl**, **C1** and **C1a** and their reversibility (C) on ACh-evoked currents through *Torpedo*,  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs, recorded as detailed above. The peak current amplitude recorded in response to applications of ACh and a given compound (B) or ACh alone (C) was normalized to that triggered by the first three-time applications of ACh alone (control). Mean  $\pm$  S.E.M. of 4-8 oocytes. \*:  $P \leq 0.048$  versus control.

The present results suggest that **C1a**, compared to **C1-Cl** and **C1**, might be the best suitable molecule for a long-lasting *in vivo* effect on muscle-subtype nAChRs, although the compound, at pharmacologically relevant concentrations (10  $\mu\text{M}$ ), also affected the activity of neuronal-subtype nAChRs (present study) and human recombinant acetylcholinesterase [15]. Moreover, as also observed with **C1**, the compound was shown to block the embryonic  $\alpha 1\beta 1\delta\gamma$  (*Torpedo*) muscle-subtype of nAChRs but was previously reported to have no marked effect on mouse muscle contraction and membrane potential parameters, *i.e.* on the adult  $\alpha 1\beta 1\delta\epsilon$  muscle-subtype (mouse) of nAChRs, at concentrations up to 38  $\mu\text{M}$  [15]. Here again, further experiments are needed to definitively conclude.

## Conclusion

The present results show that (i) **C1-Cl** preferentially and reversibly affected *Torpedo* nAChRs, (ii) **C1a** acted similarly on *Torpedo*,  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs but its effects were hardly reversible, and (iii) **C1** had similar effects on *Torpedo* and  $\alpha 4\beta 2$  nAChRs, while no effect of this compound was detected on  $\alpha 7$  nAChRs. These results, together with our previous studies on isolated mouse neuromuscular preparations [13-15], strongly suggest that **C1-Cl** is, among the three compounds studied, the only molecule that exerts *in vitro* effects on both muscle-subtype nAChRs and isolated mouse neuromuscular preparations, indicating thus that this compound blocks the nAChRs at the postsynaptic membrane of the neuromuscular junction. Therefore, **C1-Cl** may be of interest as the first member of a new class of potential peripheral myorelaxants if the next step, which will consist in appraising its pharmacokinetics and adverse effects *in vivo*, is successful.

## Funding sources

This work was supported by Slovenian research grants [P4-0053, P1-0207 and P1-0175], Proteus project (BI-FR/21-22-PROTEUS-002), Junior Investigator Grants for Tomaž Trobec and Jerneja Kladnik, and the French Alternative Energies and Atomic Energy Commission (CEA).

## Declaration of competing interest

The authors declare no competing interest.

## References

- [1] E.X. Albuquerque, E.F. Pereira, M. Alkondon, S.W. Rogers, Mammalian nicotinic acetylcholine receptors: from structure to function, *Physiol Rev*, 89 (2009) 73-120.
- [2] R.C. Hogg, M. Raggenbass, D. Bertrand, Nicotinic acetylcholine receptors: from structure to brain function, *Rev Physiol Biochem Pharmacol*, 147 (2003) 1-46.

- [3] A. Taly, P.J. Corringer, D. Guedin, P. Lestage, J.P. Changeux, Nicotinic receptors: allosteric transitions and therapeutic targets in the nervous system, *Nat Rev Drug Discov*, 8 (2009) 733-750.
- [4] D. Kalamida, K. Poulas, V. Avramopoulou, E. Fostieri, G. Lagoumintzis, K. Lazaridis, A. Sideri, M. Zouridakis, S.J. Tzartos, Muscle and neuronal nicotinic acetylcholine receptors. Structure, function and pathogenicity, *FEBS J*, 274 (2007) 3799-3845.
- [5] E. Sher, Y. Chen, T.J. Sharples, L.M. Broad, G. Benedetti, R. Zwart, G.I. McPhie, K.H. Pearson, T. Baldwinson, G. De Filippi, Physiological roles of neuronal nicotinic receptor subtypes: new insights on the nicotinic modulation of neurotransmitter release, synaptic transmission and plasticity, *Curr Top Med Chem*, 4 (2004) 283-297.
- [6] T.N.T. Ho, N. Abraham, R.J. Lewis, Structure-Function of Neuronal Nicotinic Acetylcholine Receptor Inhibitors Derived From Natural Toxins, *Front Neurosci*, 14 (2020) 609005.
- [7] J.A. Dani, Neuronal Nicotinic Acetylcholine Receptor Structure and Function and Response to Nicotine, *Int Rev Neurobiol*, 124 (2015) 3-19.
- [8] C.H. Wu, C.H. Lee, Y.S. Ho, Nicotinic acetylcholine receptor-based blockade: applications of molecular targets for cancer therapy, *Clin Cancer Res*, 17 (2011) 3533-3541.
- [9] S. Wang, Y. Hu, alpha7 nicotinic acetylcholine receptors in lung cancer, *Oncol Lett*, 16 (2018) 1375-1382.
- [10] L. Dresser, R. Wlodarski, K. Rezaia, B. Soliven, Myasthenia Gravis: Epidemiology, Pathophysiology and Clinical Manifestations, *J Clin Med*, 10 (2021).
- [11] H. Rollema, Bertrand, D., Hurst, R.S., Nicotinic Agonists and Antagonists. In: Stolerman, I.P. (eds) *Encyclopedia of Psychopharmacology*. , Springer, Berlin, Heidelberg, (2010) 1113-1131.
- [12] R. Hurst, H. Rollema, D. Bertrand, Nicotinic acetylcholine receptors: from basic science to therapeutics, *Pharmacol Ther*, 137 (2013) 22-54.
- [13] P.N. Patsalos, Neuromuscular Blocking Agents, in: P.N. Patsalos (Ed.) *Antiseizure Medication Interactions: A Clinical Guide*, Springer International Publishing, Cham, 2022, pp. 313-317.
- [14] T. Trobec, M.C. Zuzek, K. Sepcic, J. Kladnik, J. Kljun, I. Turel, E. Benoit, R. Frangez, Structural and functional characterization of an organometallic ruthenium complex as a potential myorelaxant drug, *Biomed Pharmacother*, 127 (2020) 110161.
- [15] S. Ristovski, M. Uzelac, J. Kljun, T. Lipec, M. Ursic, S. Zemljic Jokhadar, M.C. Zuzek, T. Trobec, R. Frangez, K. Sepcic, I. Turel, Organoruthenium Prodrugs as a New Class of Cholinesterase and Glutathione-S-Transferase Inhibitors, *ChemMedChem*, 13 (2018) 2166-2176.
- [16] T. Trobec, M.C. Zuzek, K. Sepcic, J. Kladnik, I. Turel, R. Frangez, Novel Organoruthenium(II) Complex C1 Selectively Inhibits Butyrylcholinesterase without Side Effects on Neuromuscular Transmission, *Int J Mol Sci*, 24 (2023).
- [17] M. Nys, E. Zarkadas, M. Brams, A. Mehregan, K. Kambara, J. Kool, N.R. Casewell, D. Bertrand, J.E. Baenziger, H. Nury, C. Ulens, The molecular mechanism of snake short-chain alpha-neurotoxin binding to muscle-type nicotinic acetylcholine receptors, *Nat Commun*, 13 (2022) 4543.
- [18] F.J. Barrantes, Structure and function meet at the nicotinic acetylcholine receptor-lipid interface, *Pharmacol Res*, 190 (2023).
- [19] J. Kljun, M. Anko, K. Traven, M. Sinreih, R. Pavlic, S. Persic, Z. Ude, E.E. Codina, J. Stojan, T.L. Rizner, I. Turel, Pyrithione-based ruthenium complexes as inhibitors of aldo-keto reductase 1C enzymes and anticancer agents, *Dalton T*, 45 (2016) 11791-11800.

- [20] R. Miledi, E. Palma, F. Eusebi, Microtransplantation of neurotransmitter receptors from cells to *Xenopus* oocyte membranes: new procedure for ion channel studies, *Methods Mol Biol*, 322 (2006) 347-355.
- [21] R. Araoz, P. Barnes, V. Sechet, M. Delepierre, S. Zinn-Justin, J. Molgo, A. Zakarian, P. Hess, D. Servent, Cyclic imine toxins survey in coastal european shellfish samples: Bioaccumulation and mode of action of 28-O-palmitoyl ester of pinnatoxin-G. first report of portimine-A bioaccumulation, *Harmful Algae*, 98 (2020) 101887.
- [22] I. Ivorra, A. Alberola-Die, R. Cobo, J.M. Gonzalez-Ros, A. Morales, *Xenopus* Oocytes as a Powerful Cellular Model to Study Foreign Fully-Processed Membrane Proteins, *Membranes (Basel)*, 12 (2022).
- [23] I. Ivorra, A. Fernandez, B. Gal, J. Aleu, J.M. Gonzalez-Ros, J.A. Ferragut, A. Morales, Protein orientation affects the efficiency of functional protein transplantation into the *xenopus* oocyte membrane, *J Membr Biol*, 185 (2002) 117-127.
- [24] J. Marsal, G. Tigyi, R. Miledi, Incorporation of acetylcholine receptors and Cl<sup>-</sup> channels in *Xenopus* oocytes injected with *Torpedo* electroplaque membranes, *Proc Natl Acad Sci U S A*, 92 (1995) 5224-5228.
- [25] A.F. Goldberg, C. Miller, Solubilization and functional reconstitution of a chloride channel from *Torpedo californica* electroplax, *J Membr Biol*, 124 (1991) 199-206.
- [26] J.L. Popot, J.P. Changeux, Nicotinic receptor of acetylcholine: structure of an oligomeric integral membrane protein, *Physiol Rev*, 64 (1984) 1162-1239.
- [27] R. Araoz, G. Ouanounou, B.I. Iorga, A. Goudet, D. Alili, M. Amar, E. Benoit, J. Molgo, D. Servent, The Neurotoxic Effect of 13,19-Didesmethyl and 13-Desmethyl Spirolide C Phycotoxins Is Mainly Mediated by Nicotinic Rather Than Muscarinic Acetylcholine Receptors, *Toxicol Sci*, 147 (2015) 156-167.
- [28] M. Cieslikiewicz-Bouet, M. Naldi, M. Bartolini, B. Perez, D. Servent, L. Jean, R. Araoz, P.Y. Renard, Functional characterization of multifunctional ligands targeting acetylcholinesterase and alpha 7 nicotinic acetylcholine receptor, *Biochem Pharmacol*, 177 (2020) 114010.
- [29] Z.Y. Li, C. Bon, Presence of a membrane-bound acetylcholinesterase form in a preparation of nerve endings from *Torpedo marmorata* electric organ, *J Neurochem*, 40 (1983) 338-349.
- [30] S.P. Fricker, Cysteine proteases as targets for metal-based drugs, *Metallomics*, 2 (2010) 366-377.
- [31] J. Kladnik, A. Dolinar, J. Kljun, D. Perea, J. Grau-Expósito, M. Genescà, M. Novinec, M.J. Buzon, I. Turel, Zinc Pyrithione is a Potent Inhibitor of PLPro and Cathepsin L Enzymes with Ex Vivo Inhibition of SARS-CoV-2 Entry and Replication (vol 37, pg 2158, 2022), *J Enzym Inhib Med Ch*, 37 (2022) 2574-2574.
- [32] K. Krzywoszynska, J. Swiatek-Kozłowska, S. Potocki, M. Ostrowska, H. Kozłowski, Triplet of cysteines - Coordinational riddle?, *J Inorg Biochem*, 204 (2020).
- [33] R.W. Teichert, J. Rivier, J. Torres, J. Dykert, C. Miller, B.M. Olivera, A uniquely selective inhibitor of the mammalian fetal neuromuscular nicotinic acetylcholine receptor, *J Neurosci*, 25 (2005) 732-736.
- [34] E.K. Lebbe, S. Peigneur, I. Wijesekara, J. Tytgat, Conotoxins targeting nicotinic acetylcholine receptors: an overview, *Mar Drugs*, 12 (2014) 2970-3004.
-