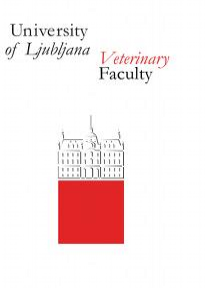


UDK
577.1:615.019:547.821:615.9:599.323.4(043.3)

Marjana Grandič, dr. vet. med.

PATOFIZIOLOŠKO DELOVANJE SINTETIČNIH POLIMERNIH ALKILPIRIDINIJEVIH SOLI *IN VITRO* IN *IN VIVO*

Doktorska disertacija

PATHOPHYSIOLOGIC ACTIONS OF SYNTHETIC POLYMERIC ALKYLPYRIDINIUM SALTS *IN VITRO* AND *IN VIVO*

Doctoral dissertation

Ljubljana, 2013

Marjana Grandič,

Patofiziološko delovanje sintetičnih polimernih alkilpiridinijevih soli *in vitro* in *in vivo*

Raziskovalno delo je bilo opravljeno na Inštitutu za fiziologijo, farmakologijo in toksikologijo Veterinarske fakultete v Ljubljani, na Katedri za biokemijo Oddelka za biologijo Biotehniške fakultete v Ljubljani in v Laboratoriju za nevrofiziologijo Inštituta CNRS v Gif-sur-Yvette v Franciji.

Javni zagovor je bil opravljen:

Mentor: prof. dr. Robert Frangež
Somentorica: prof. dr. Kristina Sepčić

Izjava o delu:

Izjavljam, da je doktorska disertacija rezultat lastnega raziskovalnega dela, da so rezultati korektno navedeni in da nisem kršila pravic intelektualne lastnine drugih.

Člani strokovne komisije za oceno in zagovor:

- predsednica: prof. dr. Silvestra Kobal
- član: prof. dr. Dušan Šuput
- član: prof. dr. Peter Maček

IZVLEČEK

Ključne besede: Spužva – kemija; acetilholinesteraza – kri; piridinijeve spojine – toksičnost; hemoliza; miokard; celična membrana; propustnost; podgane

Polimerne alkilpiridinijeve soli iz sredozemske morske spužve *Reniera sarai* so ene izmed najpogosteje proučevanih alkilpiridinijevih snovi. So amfifilne molekule, sestavljene iz hidrofilnih piridinijevih obročev in hidrofobnih alkilnih repov, ter spadajo v skupino kvartarnih amonijevih spojin. Imajo hemolitično, protimikrobno in protivegetativno aktivnost, uporabne so lahko kot orodje za transfekcijo celic, zavirajo encim acetilholinesterazo in rast celic pljučnega raka. Ker polimerne alkilpiridinijeve soli kažejo veliko uporabnost v medicini, farmaciji in industriji, so razvili številne sintetične analoge, pri katerih lahko s spreminjanjem kemijske strukture uravnavajo stopnjo bioloških aktivnosti. V doktorski disertaciji smo proučevali patofiziološko delovanje dveh sintetičnih analogov, to je APS3 in APS12-2. APS3 je kompetitivni zaviralec acetilholinesteraze in je nehemolitičen, APS12-2 pa je nekompetitivni zaviralec istega encima in je hemolitičen. Hemolitično aktivnost izbranih analogov smo dokazali na podganjih eritrocitih, membransko aktivnost pa smo proučevali na umetnih in naravnih lipidnih membranah. Ker bi izbrana sintetična analoga lahko bila uporabna v medicini, predvsem kot protitumorski snovi, smo želeli ugotoviti, ali imata morebitne neželene učinke na nivoju organov, tkiv, celic in na molekulskem nivoju ter ali vplivata na vitalne funkcije v sesalskem organizmu. Ugotovili smo, da APS12-2 (8 mg/kg) po intravenski aplikaciji povzroči pogin podgan zaradi srčnega zastoja, ki je verjetno posledica hiperkaliemije. Srednji letalni odmerek, ocenjen na miših, je za APS12-2 11,5 mg/kg, za APS3 pa 7,25 mg/kg. Oba sintetična analoga zmanjšata amplitudo mišične kontrakcije mišje hemidiafragme *in vitro* (IC_{50} (APS12-2) = 0,74 μM; IC_{50} (APS3) = 20,3 μM). Na neposredno izzvano mišično kontrakcijo nimata vpliva. Oba izbrana analoga zmanjšata amplitudo potencialov motorične ploščice (IC_{50} (APS12-2) = 0,36 μM; IC_{50} (APS3) = 7,28 μM) in miniaturnih potencialov motorične ploščice, medtem ko na mirovni membranski potencial nimata vpliva. Oba blokirata z acetilholinom izzvani ionski tok skozi nikotinske acetilholinske receptorje, izražene v membranah oocitov žabe *Xenopus laevis* (IC_{50} (APS12-2) = 0,0005 μM; IC_{50} (APS3) = 0,19 μM). Rezultati kažejo, da sta izbrana analoga učinkovita zaviralca nikotinskih acetilholinskih receptorjev, da je njuna antiacetilholinesterazna aktivnost zamaskirana s posledicami delovanja na nikotinske

acetilholinske receptorje ter da so toksični učinki, ugotovljeni *in vivo*, lahko posledica delovanja APS3 na nikotinske acetilholinske receptorje in hemolitične aktivnosti APS12-2.

ABSTRACT

Key words: Porifera – chemistry; acetylcholinesterase – blood; pyridinium compounds – toxicity; hemolysis; myocardium; cell membrane, permeability; rats

Polymeric alkylpyridinium salts (poly-APS) isolated from the Mediterranean marine sponge *Reniera sarai* are one of the most studied alkylpyridinium compounds. Poly-APS are amphipatic molecules bearing both hydrophilic cationic pyridinium headgroups and hydrophobic alkyl chains, and are structurally related to quaternary ammonium compounds. Poly-APS exert hemolytic, antimicrobial, antifouling and anti-acetylcholinesterase activity; they can be used as transfection tools, and are cytotoxic for lung cancer cells. Since poly-APS show a potential for use in medicine, pharmacology and industry, a number of their synthetic analogues with different chemical structures and rates of biological activities were synthesized. In the present work the pathophysiological effects of two synthetic analogues, APS12-2 and APS3, were studied. APS3 is a non-hemolytic competitive inhibitor of acetylcholinesterase, and APS12-2 is a hemolytic non-competitive inhibitor of the same enzyme. Hemolytic and pore-forming activity of these two analogues was studied on rat erythrocytes, on natural and artificial lipid membranes, respectively. As APS3 and APS12-2 are potential candidates for use in antitumor therapy, we investigated their possible adverse effects on organs, tissues, cells, and at the molecular level. We also aimed to assess their effects on vital functions in mammal organism. Intravenous application of APS12-2 (8 mg/kg) causes death of experimental rats due to cardiac arrest, probably induced by hyperkalemia. The estimated median lethal dose for APS12-2 and APS3 in mice is 11.5 and 7.25 mg/kg, respectively. Both synthetic analogues blocked nerve-evoked isometric muscle contraction of mouse hemidiaphragm *in vitro* (IC_{50} (APS12-2) = 0.74 μM; IC_{50} (APS3) = 20.3 μM), without affecting directly-elicited twitch tension. Both analogues also decreased the amplitude of endplate potentials (IC_{50} (APS12-2) = 0.36 μM; IC_{50} (APS3) = 7.28 μM) and miniature endplate potentials, without affecting the resting membrane potential. Both compounds also blocked acetylcholine-evoked inward currents in *Xenopus laevis* oocytes expressing muscle-type nicotinic acetylcholine receptors (IC_{50} (APS12-2) = 0.0005 μM; IC_{50} (APS3) = 0.19 μM). Results show that (i) APS12-2 and APS3 are potent inhibitors of nicotinic acetylcholine receptors, (ii) their anti-acetylcholinestrase activity is masked by effects on nicotinic acetylcholine receptors, and (iii) the toxic effects observed *in vivo* are due

to hemolytic activity of APS12-2, and inhibitory activity of APS3 on nicotinic acetylcholine receptors.

KAZALO VSEBINE

KAZALO PREGLEDNIC

KAZALO SLIK

SEZNAM OKRAJŠAV IN KRATIC

ACh	acetilholin
AChE	acetilholinesteraza
AP	akcijski potencial
A549	celična linija žleznega karcinoma
BW284c51	1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide
C	kapacitivnost
COX	ciklooksigenaza
CPC	cetilpiridinijev klorid
CTAB	cetiltrimetilamonijev bromid
DRG	dorsal root ganglia
DTC	D-tubokurarin
EC_{50}	srednja efektivna koncentracija
EKG	elektrokardiogram
EDCF	endotelijski kontraktilni faktor
EPP	endplate potential
HEK293	celična kultura iz človeških embrionalnih ledvičnih celic
IC_{50}	srednja inhibitorna koncentracija
ID_{50}	srednji inhibitorni odmerek
K*i*	konstanta inhibicije
LD_{50}	srednji letalni odmerek
MALDI-TOF	matrix assisted laser desorbtion/ionization time-of-flight
MEPP	miniature endplate potential
MIC	minimalna inhibitorna koncentracija
MP	membranski potencial
MPMP	miniaturni potencial motorične ploščice
nAChRs	nikotinski acetilholinski receptorji
NSCC	non selective cation channels (slov. neselektivni kationski kanalčki)
NSCLC	non small cell lung cancer
PMP	potencial motorične ploščice
poli-APS	polimerne alkilpiridinijeve soli
R	upor
RP	resting potential
SKMES-1	celična linija ploščatoceličnega karcinoma
τ	časovna konstanta
VDCC	voltage dependent calcium channels (slov. od napetosti odvisni kalcijevi kanalčki)

1 UVOD

V doktorski disertaciji smo opravili raziskave z dvema izbranima sintetičnima analogoma polimernih alkilpiridinijevih soli (poli-APS), to je z APS12-2 in APS3. Prvi deluje kot nekompetitivni zaviralec encima acetilholinesteraze (AChE) in je hemolitičen (Houssen in sod., 2010), drugi je kompetitivni zaviralec AChE in je nehemolitičen. Želeli smo dobiti odgovor na vprašanje, ali imata APS12-2 in APS3 v koncentracijski odvisnosti *in vivo* in *in vitro* morebitne neželene učinke na nivoju organov, tkiv, celic in na molekulskem nivoju ter ali vplivata na vitalne funkcije v sesalskem organizmu. Ugotavljali smo, ali izbrani snovi zavirata encim AChE tudi v živčno-mišičnem stiku in ali hkrati blokirata nikotinske acetilholinske receptorje (nAChRs), kar je znano za nekatere druge zaviralce AChE. Z ugotavljanjem molekulskih mehanizmov njihove vezave in interakcije z umetnimi lipidnimi in naravnimi membranami ter tarčnimi organi smo lahko dodatno razložili mehanizme učinkov izbranih sintetičnih analogov na nivoju celic, tkiv in organov ter na nivoju organizma kot integrirane celote.

Postavili smo naslednje hipoteze:

1. Oba izbrana sintetična analoga poli-APS po parenteralni aplikaciji v subletalnem odmerku statistično značilno znižata vrednosti srednjega arterijskega krvnega tlaka, v letalnem odmerku pa sta za pogin živali pri hemolitičnem APS12-2 odgovorna hiperkaliemija in srčni zastoj, pri nehemolitičnem APS3 pa respiratorni zastoj.

2. Izbrana sintetična analoga poli-APS vplivata na mišično kontrakcijo *in vitro* in *in vivo* tako, da v nizki koncentraciji povečata moč posredno izzvane mišične kontrakcije, v višjih koncentracijah pa nanjo delujeta zaviralno.

3. Izbrana sintetična analoga poli-APS v nizkih koncentracijah zavirata encim AChE v živčno-mišičnem stiku *in vitro* in *in vivo*.

4. Izbrana sintetična analoga poli-APS delujeta zaviralno na nikotinske receptorje.

5. Hemolitična aktivnost izbranih sintetičnih analogov poli-APS je odvisna od stopnje njune polimerizacije in od lastnosti okolja.

6. Sposobnost tvorbe por s sintetičnimi analogi poli-APS je odvisna od sestave lipidnih membran.

2 PREGLED LITERATURE

2.1 Naravne polimerne alkilpiridinijeve soli

Poli-APS, izolirane iz vodnega ekstrakta sredozemske morske spužve *Reniera sarai*, so biološko aktivne snovi, ki spužvi omogočajo nemoteno rast in razvoj ter jo varujejo pred zunanjim okoljem. Zaradi številnih zanimivih bioloških aktivnosti spadajo med najbolj proučevane snovi iz morskih organizmov (Sepčić in sod., 1997a; Sepčić in sod. 1997b). Poli-APS so amfifilne molekule, sestavljene iz hidrofilnih piridinijevih obročev in hidrofobnih alkilnih verig. Spadajo med kvartarne amonijeve spojine. So visoko polimerizirane snovi. Stopnja polimerizacije je povezana z biološko aktivnostjo (Mancini in sod., 2004; Turk in sod., 2008). Na podlagi analize MALDI-TOF (angl. matrix assisted laser desorbtion / ionization time-of-flight) je bilo ugotovljeno, da so poli-APS sestavljene iz dveh polimernih podenot z enako strukturo, vendar različno molekulsko maso, to je 18.900 Da in 5.520 Da, kar ustreza 99–100 oziroma 29 monomernim oktilpiridinijevim enotam (Sepčić in sod., 1997a). Novejše analize so pokazale, da so poli-APS sestavljene le iz ene populacije spojin, velikosti 5.520 Da (Mancini in Jaspars, neobjavljeni rezultati). Poli-APS so topne v vodi. V vodnih raztopinah tvorijo velike agregate, ki so micelom podobne strukture s sferično obliko. Velikost agregatov je približno 3×10^6 Da s hidrodinamskim radijem 23 ± 2 nm (Sepčić in sod. 1997b; Sepčić in sod., 1999).

Poli-APS delujejo hemolitično, protimikrobno in protivegetativno, zavirajo encim AChE in rast celic pljučnega raka (Turk in sod., 2008).

Hemolitična aktivnost poli-APS je posledica njihove detergentom podobne strukture. V vodnih raztopinah poli-APS tvorijo velike sferične strukture in kažejo lastnosti, podobne surfaktantom. Hemolitično aktivnost poli-APS so primerjali s hemolitično aktivnostjo kemično sorodnih kationskih detergentov, kot sta cetiltrimetilamonijev bromid (CTAB) in cetilpiridinijev klorid (CPC). Ugotovili so, da je hemolitična aktivnost teh spojin obratno sorazmerna kritični micelarni koncentraciji, to je koncentraciji, pri kateri se te spojine

združujejo v agregate (0,33 mg/ml). Za to je verjetno odgovorna slabša topnost monomerov v vodi in njihova večja afiniteta do nepolarnih eritrocitnih membran (Moni in sod., 1992). Ugotovili so, da prisotnost cinkovih in živosrebrovih ionov v raztopini zavre hemolitično aktivnost poli-APS. Ti naj bi zaprli pore v membrani, ki so posledica delovanja poli-APS. Poleg divalentnih kationov hemolizo, povzročeno s poli-APS, zavrejo tudi osmoprotektanti, kot je polietilenglikol, in nekateri lipidi, kot je fosfatidna kislina. Slednje nakazuje na to, da se poli-APS vežejo na negativno nabita področja na membrani. Hemoliza, ki jo v eritrocitih povzročijo poli-APS, je koloidno-osmoznega tipa (Malovrh in sod., 1999).

Poli-APS učinkujejo tudi protimikrobno: zavirajo rast po Gramu pozitivnih in negativnih bakterij, patogenih gliv, alg in virusov (Kawabata in Nishiguchi, 1988; Wainwright in Crosley, 2004; Madaan in Tyagi, 2008; De Muynck in sod., 2009). Vplivajo na celično steno mikrobov, ustavijo rast in povzročijo smrt celice (Thorsteinsson in sod., 2003). Protimikrobna učinkovitost je premo sorazmerna z dolžino alkilne verige in s stopnjo polimerizacije (Madaan in Tyagi, 2008; Shirai in sod., 2006), vendar mehanizmi delovanja in učinkov na celično steno bakterij še niso popolnoma pojasnjeni. Po Gramu negativne bakterije imajo kompleksnejšo sestavo celične stene. Zanje je značilen dodaten lipopolisaharidni ovoj, ki poveča negativni površinski naboj mikrobne celice in s tem občutljivost na piridinijeve soli, ki imajo visoko afiniteto do negativno nabitih površin (Madaan in Tyagi, 2008).

Ena izmed biološko pomembnih aktivnosti poli-APS je njihova protivegetativna (angl. antifouling) aktivnost. To pomeni, da imajo poli-APS sposobnost preprečevanja naseljevanja makro- in mikroorganizmov na površino spužve oziroma preprečujejo nastanek mikrobnega biofilma, kar je izrednega pomena za nemoteno rast in razvoj spužve (Baier, 1984; Wahl, 1989; Qian in sod., 2006). Ta lastnost je pomembna tudi za industrijo barv in lakov, ki preprečujejo naseljevanje morskih makro- in mikroorganizmov na površino potopljenih predmetov. Razvoj premazov, ki bi vsebovali naravne snovi iz morskih organizmov, se kaže kot obetavna alternativa sedanjim premazom, ki vsebujejo okolju škodljive težke kovine (Faimali in sod., 2003b; Sepčić in Turk, 2006; Turk in sod., 2008). Testiranja protivegetativne aktivnosti potekajo z različnimi produkti sesilnih morskih organizmov (Tsoukatou in sod., 2002; Faimali in sod., 2003a; Faimali in sod., 2003b; Almeida in sod., 2007; Eleršek in sod., 2008). Ugotovili so, da so poli-APS sicer približno desetkrat manj učinkovite od tako imenovanih »booster« biocidov, vendar so bistveno manj oziroma so netoksične (Faimali in

sod., 2003a). Možnih molekulskih mehanizmov, prek katerih poli-APS preprečijo naselitev organizmov, je več. Poli-APS močno zavirajo AChE. Acetilholin (ACh) namreč igra pomembno vlogo pri prepoznavanju substrata in posledični naselitvi organizmov nanj. Druga razlaga za preprečevanje naseljevanja je v surfaktantu podobnih lastnosti poli-APS (Sepčić in Turk, 2006).

Za poli-APS je med drugim značilno, da skozi membrane celic tvorijo začasne pore oziroma odprtine z radijem približno 2,9 nm. Na podganjih nevronih in celicah HEK293 se pri visokih koncentracijah poli-APS (50 μg/ml) zaradi nastanka por pojavi nepovraten padec membranskega potenciala (MP). Nižje koncentracije poli-APS (5 μg/ml) povzročijo nastanek začasnih por in le prehoden padec MP (McClelland in sod., 2003). Na nastanek začasnih por poleg amfifilnosti verjetno vpliva tudi sposobnost poli-APS, da tvorijo nekovalentne agregate s hidrodinamskim radijem 23 ± 2 nm (Sepčić in sod., 1997a; Malovrh in sod., 1999). Zaradi membranske depolarizacije, povzročene s poli-APS, pride do aktivacije od napetosti odvisnih kalcijevih kanalčkov in do vdora Ca^{2+} v celico skozi aktivirane kanalčke in novonastale pore (Tucker in sod., 2003; Koss in sod., 2007). Učinek poli-APS na MP lahko zavrejo cinkovi ioni, ki povečajo prevajalno upornost in zmanjšajo permeabilnost za ione Ca^{2+} ter tako preprečijo nastanek por (McClelland in sod., 2003). Tvorba začasnih por v celični membrani se lahko izkoristi za transfekcijo. To je vnos tuje DNK v sesalsko celico gostiteljico, v kateri pride pozneje do izražanja vnesene DNK. Pri tem je pomembno poudariti, da pri transfekciji z ustrezno količino poli-APS ne pride do poškodb, ki bi trajno ogrozile funkcionalnost plazemske membrane. V prihodnosti bi zato lahko poli-APS uporabili za zdravljenje genetskih bolezni, in sicer za vnos različnih genov v tarčne celice.

Zelo pomembna lastnost poli-APS je zaviranje AChE. Časovni potek encimskega zaviranja s poli-APS je nenavaden. Vzrok za to naj bi bila visoka stopnja polimerizacije poli-APS. V prvi stopnji pride do hitrega, nekompetitivnega, povratnega zaviranja, pri čemer se aktivnost encima zmanjšuje, vendar se ne izgubi popolnoma. V drugi stopnji vezave pride do počasnega vezanja na encim s konstanto inhibicije K_i = 3,23 μM. Tretja stopnja je faza nepovratne inaktivacije encima. Za začetno povratno in nato za nepovratno fazo zaviranja je odgovorno periferno anionsko mesto. Ugotovili so, da se poli-APS po prvi stopnji vežejo tudi na druga negativno nabita mesta na encimu. To naj bi vodilo do agregacije in precipitacije kompleksa encim-inhibitor ter posledičnega nepovratnega zaviranja encima (Sepčić in sod., 1998).

Poleg vseh naštetih bioloških aktivnosti imajo poli-APS tudi protitumorsko aktivnost. Ugotovljeno je, da zavirajo rast celic nedrobnoceličnega pljučnega raka (angl. non small cell lung cancer – NSCLC) v nanomolarnih koncentracijah (EC_{50} = 0,86 nM), pri tem pa ne vplivajo na normalne pljučne fibroblaste (Paleari in sod., 2006). Protitumorska aktivnost poli-APS naj bi bila posledica zaviralnega delovanja na AChE. Celice pljučnega raka na svoji površini izražajo številne acetilholinske receptorje, zaviralci AChE pa naj bi sprožili apoptozo tumorskih celic (Paleari in sod., 2006). Kljub temu nekatere raziskave kažejo, da je učinek inaktivacije AChE ravno nasproten (Jin in sod., 2004; Wang in sod., 2009; Ye in sod., 2010). Zaradi neaktivne AChE je namreč količina ACh višja, kar povzroča stalno stimulacijo nAChRs in posledično razmnoževanje tumorskih celic. V prisotnosti poli-APS do razmnoževanja tumorskih celic ne pride, kar kaže na to, da antiacetilholinesterazna aktivnost ni vzrok za protitumorsko aktivnost. Sklepajo, da je mehanizem sprožitve apoptoze tumorskih celic zaviranje delovanja nAChR (Zovko, 2012).

Ker naravne poli-APS kažejo veliko uporabnost v farmacevtski in kemijski industriji, so nedavno sintetizirali analoge poli-APS z različnimi stopnjami polimerizacije (Mancini in sod., 2004; Houssen in sod., 2010; Zovko in sod., 2012). Sinteza strukturno točno opredeljenih analogov poli-APS z različno stopnjo polimerizacije in z različnimi kemijskimi lastnostmi omogoča nadzor in uravnavanje stopnje njihovih bioloških aktivnosti.

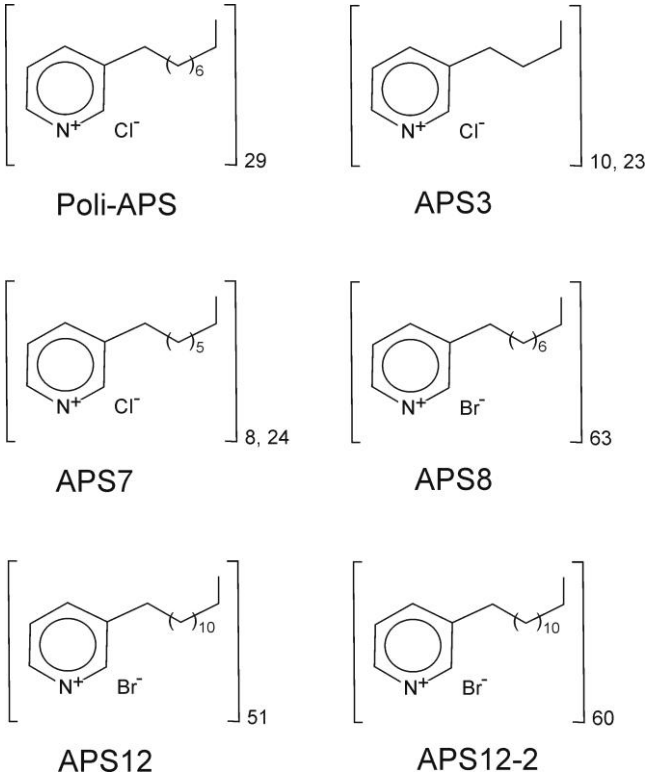
2.2 Sintetični analogi polimernih alkilpiridinijevih soli

Zelo zanimivi biološki učinki naravnih poli-APS in njihova potencialna uporaba v farmacevtski in kemijski industriji ter nezadostna količina naravnih poli-APS iz vodnih ekstraktov spužve so botrovali razvoju novih postopkov za sintezo analogov poli-APS. Sintetizirali so veliko analogov, s katerimi so spremenili lastnosti naravnih poli-APS in omogočili komercialno produkcijo. Leta 2004 so Mancini in sodelavci uspešno sintetizirali dimere in tetramere linearnih 3-alkilpiridinijevih soli. Leta 2010 je Houssen s sodelavci opisal nov postopek, ki omogoča sintezo velikih alkilpiridinijevih polimerov, saj so ugotovili, da so manjše spojine biološko manj aktivne kot tiste z višjo stopnjo polimerizacije. Da bi natančneje določili, kako struktura spojine vpliva na njene lastnosti, so sintetizirali spojine, ki so se med seboj razlikovale v dolžini alkilne verige, številu piridinijevih obročev in

nasprotnem ionu (bromov ali klorov), ki ima funkcijo nevtralizacije naboja na dušikovem atomu piridinijevega obroča.

2.2.1 Sinteza analogov polimernih alkilpiridinijevih soli

Razvili so metodo, ki omogoča enostavno, hitro in cenovno ugodno sintezo alkilpiridinijevih spojin z visoko stopnjo polimerizacije (Houssen in sod., 2010; Zovko in sod., 2012). V reakciji med 3-pikolinom in dibromoalkanom ali bromalkoholom nastane piridil alkohol. Bromidni monomeri nastanejo z nevtralizacijo alkohola, obdelanega z bromvodikom, kloridni monomeri pa nastanejo z reakcijo substrata in tionilklorida. Dobljeni monomeri se oligomerizirajo v prisotnosti acetonitrila ali metanola. Pri obsevanju z mikrovalovi nastanejo polimeri, katerih velikost je odvisna od časa obsevanja (Houssen in sod., 2010; Zovko in sod., 2012). Opisana metoda je hitra, varna, poceni in ekološko sprejemljiva (Kappe, 2004), hkrati pa omogoča sintezo večje količine produkta. Houssen in sodelavci so z opisano metodo izdelali številne sintetične analoge poli-APS, ki se med sabo razlikujejo v stopnji polimerizacije, nasprotnih ionih in dolžini alkilne verige. Nekatere spojine so mešanica več polimerov z različnimi molekulskimi masami in stopnjami polimerizacije. Analoge, ki smo jih proučevali v doktorski disertaciji, prikazuje slika 1.



Slika 1: Kemijska struktura poli-APS in njihovih sintetičnih analogov

Figure 1: Chemical structure of poly-APS and their synthetic analogues

Preglednica 1: Osnovne kemijske lastnosti poli-APS in njihovih sintetičnih analogov

Table 1: Basic chemical properties of poly-APS and their synthetic analogues

Spojina	Št. alkilnih C-atomov	Št. polimerov in molarno razmerje	Molekulska masa (kDa)	Stopnja polimerizacije	Nasprotni ion
Poli-APS	8	1	5,52	29	Cl^-
APS3	3	2 (9 : 1)	1,46 (1,2/3,8)	10 in 32	Cl^-
APS7	7	2 (2 : 1)	2,33 (1,4/4,2)	8 in 24	Cl^-
APS8	8	1	11,9	63	Br^-
APS12	12	1	12,5	51	Br^-
APS12-2	12	1	14,7	60	Br^-

2.3 Biološki učinki sintetičnih analogov polimernih alkilpiridinijevih soli

2.3.1 Hemolitična in protimikrobna aktivnost sintetičnih analogov polimernih alkilpiridinijevih soli

Tako kot naravni poli-APS imajo tudi sintetični analogi strukturo podobno kationskim detergentom (Malovrh in sod., 1999). Za oboje je značilno, da je hemolitična aktivnost premo sorazmerna z dolžino alkilne verige in stopnjo polimerizacije (Kondo in Tomizawa, 1969; Zaslavsky in sod., 1978). Rezultati raziskav na različnih sintetičnih analogih poli-APS so dokazali, da je hemolitična aktivnost največja pri analogih z najdaljšo alkilno verigo in največjo stopnjo polimerizacije, pri analogih z majhno molekulsko maso pa je hemolitična aktivnost izredno nizka (Houssen in sod., 2010; Zovko in sod., 2012). Ugotovili so, da nasprotni ioni nimajo vpliva na hemolitično aktivnost (Zovko in sod., 2012).

Različni sintetični analogi poli-APS so se v raziskavah pokazali kot bolj učinkoviti proti po Gramu pozitivnim bakterijam (*S. aureus*) kot proti po Gramu negativnim bakterijam (*E. coli*), ki so na delovanje sintetičnih analogov poli-APS odpornejše, verjetno zaradi dodatnega lipopolisaharidnega ovoja bakterijske celice (Mancini in sod., 2004; Zovko in sod., 2012). Ugotovili so da protibakterijska aktivnost narašča z višanjem števila pozitivnih nabojev in dolžine alkilne verige. Bolj aktivne so spojine z bromidnim nasprotnim ionom (Chelossi in sod., 2006; Zovko in sod., 2012). Zanimivo je, da so vsi sintetični analogi razen APS3, ki je najmanjši, bolj učinkoviti proti bakterijam kot naravnim poli-APS (Zovko in sod., 2012). V primerjavi s strukturno podobnimi snovmi, kot je cetilpridinijev klorid, ki ima minimalno inhibitorno koncentracijo (MIC) za *S. aureus* < 1,47 μM in za *E. coli* 470 μM, so sintetični analogi poli-APS dokaj učinkovita protibakterijska sredstva, saj imajo boljšo protibakterijsko aktivnost proti *E. coli* ($MIC_{APS12-2}$ = 34,01 μM) in primerljivo proti *S. aureus* ($MIC_{APS12-2}$ = 6,8 μM; Zovko in sod., 2012).

Sintetični poli-APS uspešno zavirajo rast patogenih gliv. Tudi v tem primeru igrata glavno vlogo dolžina alkilne verige in stopnja polimerizacije. Ugotovljeno je bilo, da ima največjo protiglivno aktivnost APS12-3, ki ima najdaljšo alkilno verigo in največjo stopnjo polimerizacije (Zovko in sod., 2012). Primerjali so učinkovitost sintetičnih analogov poli-APS s standardnimi protiglivnimi zdravili in ugotovili, da je učinkovitost analogov APS12-2 in APS12-3 podobna mikonazolu, medtem ko so ostala protiglivna zdravila od

deset- do stokrat bolj učinkovita (Zovko in sod., 2012). Sintetični analogi so učinkoviti tudi proti saprofitskim glivam. Tudi tukaj so bile učinkovitejše spojine z daljšimi alkilnimi repi. Ugotovili so, da kisikov atom v verigi spojine APS8-2 močno zmanjša njeno učinkovitost. Analog APS12-3 se je pokazal primeren kot biocid za zaščito lesa proti glivi *Gloeophyllum trabeum* (Zovko in sod., 2012).

2.3.2 Vpliv sintetičnih analogov polimernih alkilpiridinijevih soli na acetilholinesterazo

Za razliko od naravnih poli-APS je časovni potek zaviranja AChE pri sintetičnih analogih poli-APS linearen, kar kaže na reverzibilnost zaviranja. Sintetični APS delujejo kot kompetitivni in nekompetitivni zaviralci AChE. Nekompetitivni zaviralci AChE so APS12, APS12-2, APS12-3, APS7, APS7-2 in APS8, ki se vežejo na periferno anionsko mesto in tako preprečijo vezavo substrata v aktivno mesto žepa encima. Predvidevajo, da je ta vezava posledica velikosti spojin, ki tako ne morejo vstopiti v aktivni žep encima. Kompetitivna zaviralca AChE sta APS8-2 in APS3. Oba tekmujeta z ACh za vezavo na katalitično anionsko mesto, ki se nahaja na dnu aktivnega žepa. Način zaviranja AChE je tako odvisen od velikosti spojine, saj se večje vežejo nekompetitivno, manjše pa kompetitivno (Zovko, 2012). Ker so sintetični analogi poli-APS močni zaviralci AChE, bi lahko predvsem manjše polimere uporabili v medicini kot zdravila za terapijo bolezni, kjer je izločanje ACh zmanjšano (na primer Alzheimerjeva bolezen, očesni glavkom in *miasthenia gravis*; Cummings, 2000).

2.3.3 Protitumorska aktivnost sintetičnih analogov polimernih alkilpiridinijevih soli

Preliminarni poskusi s sintetičnim analogom APS8 so pokazali, da naj bi učinkovito zaviral nikotinske receptorje α7 že v koncentraciji 1 nM (W. Kem, neobjavljeni rezultati). Ker je omenjena koncentracija nižja od inhibicijske konstante za AChE (1,88 nM), je delovanje APS8 na celice najverjetneje posledica zaviranja receptorjev in ne AChE. APS8 učinkovito zavira razmnoževanje rakastih celičnih linij A549 in SKMES-1. Učinek na normalne pljučne fibroblaste je bistveno manjši. S pretočno citometrijo in diferencialnim barvanjem je bilo ugotovljeno, da APS8 v odvisnosti od koncentracije povzroči apoptozo rakastih celic (Zovko, 2012). V apoptozo rakastih celic, povzročeno z APS8, sta vpleteni notranja in zunanja pot apoptoze. Notranji poti rečemo tudi mitohondrijska pot, saj pride do permeabilizacije

mitohondrijske membrane. Aktivira jo celični stres. V zunanji poti apoptoze sodelujejo receptorji smrti, ki se aktivirajo po vezavi določenih ligandov. Sproži se vrsta reakcij, ki v končni fazi vodijo do apoptoze (Zovko, 2012).

Preglednica 2: Biološke aktivnosti poli-APS in njihovih sintetičnih analogov

Table 2: Biological activities of poly-APS and their synthetic analogues

Spojina	Zaviranje AChE – K_i (nM)*	Hemoliza (s^{-1} pri 500 nM)**	IC_{50} za NSCLC (μM)***
Poli-APS	ireverzibilni inhibitor	0,05	4,41
APS3	85	0	3.000
APS7	10	0,1	480
APS8	1,875	2,6	478
APS12-2	0,036	5,0	470

*Zovko in sod., 2012
**Grandič in sod., 2012
***Zovko, neobjavljeni rezultati

2.4 Tarče delovanja sintetičnih analogov polimernih alkilpiridinijevih soli

Sintetični analogi poli-APS imajo številne biološke aktivnosti, zaradi katerih bi lahko bili uporabni tako na področjih farmacije in medicine kot tudi v industriji. V nadaljevanju smo opisali farmakološko in toksikološko delovanje sintetičnih analogov poli-APS na tarčne organe oziroma organske sisteme. Na njih smo proučevali sistemske učinke izbranih sintetičnih analogov poli-APS, njihov vpliv na živčno-mišični prenos, vpliv na mišično kontrakcijo in vpliv na mišični tip nAChRs.

2.4.1 Živčno-mišični stik

Ena izmed najpogosteje proučevanih sinaps je prav stik med mišičnimi celicami in motoričnimi nevroni, ki ga imenujemo tudi motorična ploščica. Motorična ploščica je odgovorna za kemični prenos električnih impulzov z živčnega končiča na mišično vlakno. Sestavljena je iz treh delov: presinaptičnega dela, sinaptične špranje in postsinaptičnega dela.

Presinaptični del predstavlja betičasto zadebeljen, distalni, nemieliniziran del aksona motoričnega nevrona, v katerem potekata sinteza in skladiščenje ACh, ki je glavni živčni prenašalec v motorični ploščici. ACh se skladišči v veziklih, ki se pod vplivom akcijskega potenciala (AP) in posledičnega odpiranja kalcijevih kanalčkov in toka Ca^{2+} v živčni končič sprostijo v sinaptično špranjo. Sinaptična špranja je približno 50 nm široka špranja med pre- in postsinaptičnim delom, skozi katero molekule ACh difundirajo do postsinaptične membrane. V sinaptični špranji se nahaja AChE, nekaj pa je je tudi v področju izven motorične ploščice. AChE v manj kot 1 ms prekine delovanje ACh tako, da razcepi estersko vez med acetilnim in holinskim ostankom. Holin se transportira nazaj v živčni končič in se ponovno uporabi v sintezi ACh. AChE ima na svoji površini tako imenovani aktivni žep, globok 2 nm, kjer na katalitičnem anionskem mestu poteka hidroliza ACh (Šentjurc in sod., 1976; Sussman in sod., 1991). Na vhodu v aktivni žep se nahaja periferno anionsko mesto, ki predstavlja vezavno mesto za ACh in druge ligande. Postsinaptična membrana je močno nagubana. Plitvejše primarne in globlje sekundarne gube zelo povečajo površino membrane. Receptorska mesta za ACh so v največjem številu na vrhu gub, medtem ko je AChE največ v globini gub (Ghai in sod., 2006; Fagerlund in Eriksson, 2009; Martyn in sod., 2009).

Na postsinaptični membrani se ACh veže na nAChRs, ki se odprejo in omogočijo tok Na^{+} v mišično vlakno. Tako pride do depolarizacije in skrčenja mišičnega vlakna. Čas vezave ACh na nAChRs je izredno kratek (manj kot 1 ms), saj večino ACh takoj razgradi AChE, kar tudi prepreči desenzitizacijo nAChRs, ki bi lahko nastala zaradi dolgotrajne vezave ACh na receptor (Ghai in sod., 2006).

Elektrofiziološka osnova za krčenje mišice je nastanek AP. Depolarizacijo postsinaptične membrane imenujemo potencial motorične ploščice (PMP), ki je povzročen s sprostitvijo več sto veziklov ACh iz živčnega končiča in ima amplitudo več deset mV. Kationi, ki prehajajo skozi kanalčke aktiviranih nAChRs, sprožijo nastanek PMP, ki običajno preseže prag za nastanek AP. Ta lokalna sprememba MP odpre od napetosti odvisne natrijeve kanalčke in tako nastane AP, ki se širi vzdolž sarkoleme, kar v končni fazi povzroči kontrakcijo mišičnega vlakna (Fatt in Katz, 1951; Cohen-Cory, 2002). Ko opazujemo elektrofiziološko aktivnost sesalskih mišičnih vlaken v mirovanju, opazimo pojav majhnih, spontanih, depolarizirajočih potencialov na postsinaptični membrani s frekvenco ~$1s^{-1}$. To so tako imenovani miniaturni

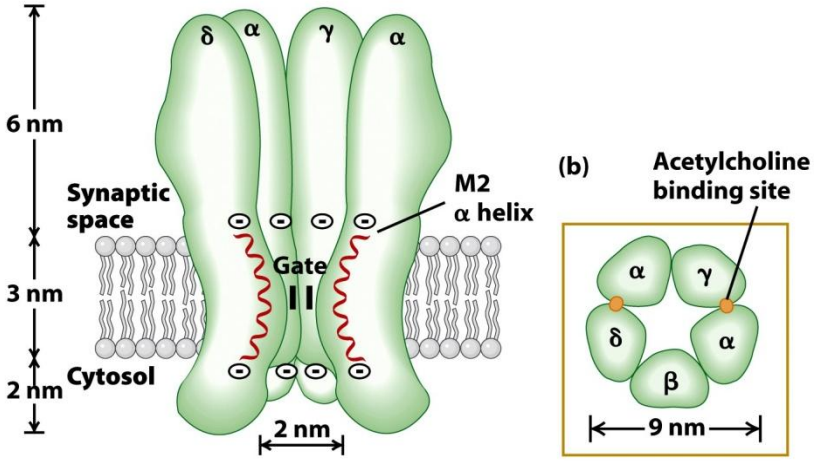
potenciali motorične ploščice (MPMP) z amplitudo 0,5–1 mV in so posledica občasnega sproščanja posamičnih veziklov z ACh (Castillo in Katz, 1954; Rich, 2006; Hammond, 2008; Martyn et al., 2009). Časovni potek je enak PMP s hitrim vzponom in počasnejšim upadanjem krivulje. V prvi fazi postaja potencial manj negativen, nato pa se polarizacija v drugi fazi vrne na vrednost mirovnega MP. Amplituda PMP je odvisna od števila sproščenih veziklov, ki vsebujejo ACh, in posledično od števila aktiviranih nAChRs. V primeru prisotnosti antagonistov nAChRs ali snovi, ki zmanjšujejo izločanje ACh, je amplituda PMP zmanjšana. Ob prisotnosti zaviralcev AChE je količina ACh v sinaptični špranji povečana, kar sprva povzroči povečanje amplitude PMP, pozneje pa ta zaradi desenzitizacije nAChRs začne padati (Katz in Thesleff, 1957a). Čas trajanja PMP je odvisen od delovanja AChE. Časovni potek druge faze PMP je odvisen od delovanja AChE, ki hidrolizira ACh in od pasivnih lastnosti membrane ($\tau = RC$). Če je aktivnost AChE dovolj zavrta, pride do posledičnega podaljšanja druge faze PMP, saj je ACh dlje časa prisoten v sinaptični špranji. Podobno velja tudi za drugo fazo MPMP (Katz in Thesleff, 1957b; Feltz in Trautmann, 1980; Molgo in Thesleff, 1982; Hammond, 2008).

2.4.1.1 Nikotinski acetilholinski receptorji

Nikotinski receptorji spadajo v skupino od liganda odvisnih ionskih kanalčkov. Nanje se veže fiziološki agonist ACh, ki aktivira receptor in povzroči prehod kationov skozi celično membrano. Mišični tip nAChRs posreduje prenos živčnih impulzov iz motoričnih nevronov na mišično vlakno skeletnih mišic. Živčni tipi nAChRs so odgovorni za sinaptične prenose v avtonomnih ganglijih živčnega sistema. Nikotinske receptorje najdemo tudi v možganih. Ti naj bi bili odgovorni za pojav odvisnosti od nikotina, vendar je o njihovi normalni fiziološki funkciji malo znanega (Colquhoun in sod., 2003; Gotti in Clementi, 2004; Hammond, 2008). Centralni in periferni nikotinski receptorji igrajo pomembno vlogo tudi pri različnih sistemskih motnjah, kot so Alzheimerjeva bolezen, *miasthenia gravis*, shizofrenija, nevropatična bolečina, depresija, hiperaktivnost, odvisnost od nikotina in različnih drog (Vallejo in sod., 2005; Ohno, 2011; Geerts, 2012; Mehta in sod., 2012).

Nikotinski receptorji so sestavljeni iz petih podenot, ki v obliki rozete oblikujejo kanalček. Vsaka podenota ima izvencelično aminoterminalno domeno, štiri transmembranske segmente, ki jih označimo z M1–M4, in dolgo hidrofilno citoplazemsko zanko med M3 in M4.

Receptorji mišičnega tipa so v embrionalnih celicah sestavljeni iz dveh α1-podenot, β-, ϒ- in δ-podenote. Kmalu po rojstvu v mišicah ϒ-podenoto zamenja ε-podenota. Receptorji so lahko glede na sestavo podenot homomerni, torej sestavljeni iz enakih ponavljajočih se podenot, ali heteromerni oziroma sestavljeni iz različnih podenot, kamor sodi tudi mišični tip nAChRs (Colquhoun in sod., 2003; Hammond, 2008).



Slika 2: Struktura nikotinskega acetilholinskega receptorja (vir: Lodish, 2008)

Figure 2: The structure of nicotinic acetylcholine receptor (from: Lodish, 2008)

Za aktivacijo nAChR se morata na receptor vezati dve molekuli ACh na vezavni mesti, ki sta na izvencelični strani obeh α-podenot. Ti vezavni mesti sta zelo blizu mejne ploskve s sosednjima γ- in δ-podenotama (Colquhoun in sod., 2003; Hammond, 2008). Pri tem pride do konformacijske spremembe, ki omogoči pretok kationov v notranjost celice (Albuquerque in sod., 1997; Hammond, 2008). Če je ACh v sinaptični špranji oziroma na področju receptorjev prisoten dlje časa in v velikih koncentracijah, se bodo receptorji desenzitizirali oziroma postali neobčutljivi za ligand. Pojavi se faza refraktarnosti, v kateri so receptorji neobčutljivi na aktivacijo z ACh. Kanalček se ne odpre, kljub temu da sta na receptor vezani dve molekuli ACh. Neobčutljivi receptorji imajo torej dve glavni lastnosti, in sicer visoko afiniteto za ACh in zaprt ionski kanalček. Proces desenzitizacije se pojavi počasi in je počasi reverzibilen (Katz in Thesleff, 1957a; Colquhoun in sod., 2003; Hammond, 2008).

Poznamo več tipov zaviralcev nAChRs. Prvi so kompetitivni zaviralci, ki z ACh tekmujejo za vezavno mesto na receptorju. Kompetitivni zaviralci po vezavi na receptor ne povzročijo aktivacije receptorja in odpiranja ionskega kanalčka, temveč ta ostane zaprt. Kompetitivni

zaviralci zmanjšajo število vezavnih mest za ACh in tako zmanjšajo ali popolnoma blokirajo nikotinski holinergični odgovor. Lahko so povratni ali nepovratni. Druga skupina nikotinskih zaviralcev so snovi, ki blokirajo ionske kanalčke tako, da se vežejo na odprt kanalček in fizično zaprejo poro ter preprečijo prehod ionov. Tretja skupina zaviralcev prek zaviranja AChE desenzitizira receptorje in prepreči ponovno vezavo ACh (Taylor, 2001). Četrta skupina zaviralcev nAChRs pa deluje alosterično, kar pomeni, da se veže na del receptorja, ki ni vezavno mesto za ACh. S to vezavo povzroči konformacijsko spremembo vezavnega mesta za ACh in blokado receptorja (Prinz, 2009).

2.4.1.2 Delovanje acetilholinesteraze

ACh je eden izmed prvih znanih živčnih prenašalcev (Dale, 1935). V telesu sodeluje pri posredovanju signalov med živčnimi celicami perifernega živčnega sistema in mišičnimi vlakni. Kot živčni prenašalec deluje tudi pri prenosu signalov med nevroni simpatičnega in parasimpatičnega dela vegetativnega živčnega sistema. Celice, ki kot prenašalec uporabljajo ACh, so holinergični nevroni. ACh se v motorični ploščici s procesom eksocitoze sprosti v sinaptično špranjo, kjer se, zasidrana na bazalni lamini, nahaja AChE (Fagerlund in Eriksson, 2009). Z difuzijo pride do postsinaptične membrane, kjer se veže na nAChRs in povzroči depolarizacijo postsinaptične membrane. Po depolarizaciji se mora ACh v zelo kratkem času, v manj kot 1 ms, odstraniti iz mesta vezave na receptorju in se razgraditi, saj pride v nasprotnem primeru do desenzitizacije receptorja. AChE po razgradnji ACh tako posredno prekine aktivacijo receptorjev in prenos signala. Holin ponovno privzame presinaptična membrana, kjer ga encim holin-acetiltransferaza ponovno uporabi za sintezo ACh (Zimmerman in Soreq, 2006). Razpad ACh poteka na dnu 2 nm globokega aktivnega žepa encima. Na vhodu v žep je vezavno mesto za substrat in druge ligande – periferno anionsko mesto, ki je tudi mesto vezave naravnih poli-APS. Vezava ligandov na to mesto oslabi vezavo substrata (Kitz in sod., 1970) in regulira aktivnost encima (Mooser in Sigman, 1974) zaradi nastalih konformacijskih sprememb (Barak in sod., 1995).

Delovanje encima AChE lahko zavrejo različne snovi (zaviralci AChE), ki se namesto na ACh vežejo na aktivno mesto AChE in zmanjšajo ali popolnoma ustavijo aktivnost encima. Posledično se v sinapsi kopiči ACh v velikih koncentracijah in povzroči aktivacijo, nato pa

desenzitizacijo nAChRs zaradi stalne aktivacije receptorjev. Zaviralce AChE delimo na povratne in nepovratne. Nepovratni zaviralci so izjemno toksične snovi, na primer organofosfati, ki se kovalentno vežejo v aktivni center encima in tvorijo stabilne komplekse (Taylor, 2001; Hotelier in sod., 2004). Povratni zaviralci se na encim vežejo šibkeje in upočasnijo ali popolnoma preprečijo vhod substrata v aktivno mesto. Kompetitivni zaviralci se vežejo na katalitično mesto na dnu aktivnega žepa, nekompetitivni pa na periferno anionsko mesto na vhodu v aktivni žep (Taylor, 2001). Povratni zaviralci se uporabljajo pri terapiji bolezni z zmanjšano količino ACh, kot so *miasthenia gravis*, očesni glaukom in Alzheimerjeva bolezen (Cummings, 2000). Nekateri zaviralci AChE imajo lahko dvojni učinek. Poleg zaviranja AChE namreč tudi modulirajo delovanje nAChRs. Primer takega zaviralca je povratni zaviralec AChE fizostigmin, ki v mikromolarnih koncentracijah aktivira nAChRs, v milimolarnih pa jih blokira (Sherby in sod., 1985; Okonjo in sod., 1991). Podoben primer je zaviralec AChE bis(7)-takrin, ki povratno blokira nAChRs. Ta učinek ni odvisen od napetosti in je posledica povečane densenzibilizacije nAChRs (Ros in sod., 2001). Zelo podobno kot bis(7)-takrin deluje tudi zaviralec AChE BW284c51 (Olivera-Bravo in sod., 2005). V doktorski disertaciji smo proučevali patofiziološke učinke dveh izbranih sintetičnih analogov poli-APS, od katerih je APS3 kompetitivni, APS12-2 pa nekompetitivni zaviralec AChE (Zovko in sod., 2012).

2.4.2 Obtočila

Kot morebitna tarča delovanja sintetičnih analogov poli-APS lahko nastopajo tudi obtočila oziroma njihovi sestavni deli, kot so srce in krvne žile. Zaradi sposobnosti poli-APS, da povzročajo nastanek prehodnih por v membranah celic (Sepčić in sod., 1997a), so eritrociti pomembna tarča delovanja, saj so poleg endotelijskih celic prvi, ki ob intravenski aplikaciji pridejo v stik s snovjo. Kot je že bilo opisano, imajo tako naravne kot sintetične poli-APS hemolitično aktivnost, torej povzročajo lizo eritrocitov zaradi detergentom podobnega delovanja in nastanka por v njihovih membranah (Sepčić in sod., 1997a; Malovrh in sod., 1999; Houssen in sod., 2010). Poleg propada fiziološke funkcije eritrocitov je pomembna posledica hemolize sproščanje velike količine kalija in ostalih znotrajceličnih snovi iz poškodovanih celic. Kalij je glavni znotrajcelični ion, saj je njegova koncentracija v celicah kar ~145 mM. Pri obsežni hemolizi pride do sproščanja velike količine kalija iz celic, kar

povzroči hiperkaliemijo. Ta ima številne negativne učinke na vzdražna tkiva, še posebej na delovanje srca. Posledica hiperkaliemije je depolarizacija celic, kar lahko povzroči srčno aritmijo. V odvisnosti od izvencelične koncentracije kalija (normalna je 3,5–5,5 mmol/l; Cunningham, 2002) se spreminja tudi elektrokardiogram (EKG). Prvi spremembi sta koničast, visok in ozek T-val ter skrajšan QT-interval. Sprememba T-vala je značilna pri koncentraciji kalija, večji od 6 mmol/l. Pri koncentracijah kalija, večjih od 7–8 mmol/l, pride do podaljšanja PR-intervala, razširjenja QRS-kompleksa ter zmanjšanja, razširjenja in izginotja P-vala. Končni spremembi sta "sinusni val", ko se razširjeni QRS-kompleks zlije s T-valom, in fibrilacija prekatov, kar pomeni hemodinamski zastoj (Parham in sod., 2006; Montague in sod., 2008).

Poli-APS in sintetični analogi lahko poleg učinkov na eritrocite in srce tudi neposredno poškodujejo endotelijske in gladke mišične celice krvnih žil, kjer lahko povzročajo vazodilatacijo ali vazokonstrikcijo. To je lahko zelo pomemben mehanizem v toksičnosti sintetičnih analogov poli-APS, saj lahko že majhne spremembe v premeru krvnih žil pomenijo velike spremembe v upornosti in pretoku skozi žile, kar popisuje Poiseuillova enačba (Sirs, 1991). Nastale hemodinamske motnje se lahko pojavijo tudi v koronarnih žilah srca, kjer bi arteriolokonstrikcija lahko privedla do ishemije kardiomiocitov in motenj v delovanju srca. Pomembno je poudariti, da pri nizkih koncentracijah uporabljenih sintetičnih analogov poli-APS lahko pričakujemo specifičen učinek na tarčne organe, medtem ko pri visokih koncentracijah sintetičnih analogov poli-APS specifične učinke zamaskirajo nespecifični učinki.

2.4.3 Dihala

Pljuča so pomembna tarča delovanja naravnih in sintetičnih poli-APS, saj te po intravenski aplikaciji pridejo s krvnim obtokom najprej v pljuča. Pljuča imajo specifično anatomsko zgradbo. So izredno dobro prekrvavljena, saj v njih poteka izmenjava kisika in ogljikovega dioksida med krvjo in vdihanim zrakom. Prav endotelne celice fine kapilarne mreže v pljučih bi lahko bile tudi tarča citolitičnega delovanja poli-APS in sintetičnih analogov. Pri tem lahko pride do poškodbe kapilarne mreže v pljučih, povečane prepustnosti stene kapilar ter do izhajanja tekočine in proteinov iz kapilar v intersticij, kar ima za posledico nastanek akutnega

pljučnega edema in oteženega dihanja (Guyton, 2006). Pljučni edem je lahko vzrok za stimulacijo na raztezanje občutljivih jukstapulmonalnih receptorjev (Paintal in sod., 1973). Njihova stimulacija s snovmi, kot so kapsaicin, serotonin ali fenil-digvanidin, vodi do apneje, bradikardije in hipotenzije (McCaffrey in Kern, 1980; Willette in sod., 1983). Ugotovljeno je bilo, da poli-APS povzročajo motnje v dihanju, ki so najverjetneje posledica zamašitve kapilar s čepki neznane sestave, ki nastanejo kot posledica toksičnega delovanja poli-APS (Bunc in sod., 2002a).

3 IZVIRNI ZNANSTVENI ČLANKI

3.1 *IN VIVO* TOXIC AND LETHAL CARDIOVASCULAR EFFECTS OF A SYNTHETIC POLYMERIC 1,3-DODECYLPYRIDINIUM SALT IN RODENTS

TOKSIČNI IN LETALNI KARDIOVASKULARNI UČINKI SINTETIČNIH POLIMERNIH 1,3-DODECILPIRIDINIJEVIH SOLI NA GLODAVCE *IN VIVO*

Grandič M[1], Sepčić K[2], Turk T[2], Juntes P[1], Frangež R[1]

[1] Univerza v Ljubljani, Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana

[2] Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za biologijo, Večna pot 111, 1000 Ljubljana

Objavljeno v: Toxicology and Applied Pharmacology, 2011, 255: 86–93

Abstract

APS12-2 is one in a series of synthetic analogs of the polymeric alkylpyridinium salts isolated from the marine sponge *Reniera sarai*. As it is a potential candidate for treating non small cell lung cancer (NSCLC), we have studied its possible toxic and lethal effects *in vivo*. The median lethal dose (LD_{50}) of APS12-2 in mice was determined to be 11.5 mg/kg. Electrocardiograms, arterial blood pressure and respiratory activity were recorded under general anesthesia in untreated, pharmacologically vagotomized and artificially ventilated rats injected with APS12-2. In one group, the *in vivo* effects of APS12-2 were studied on nerve-evoked muscle contraction. Administration of APS12-2 at a dose of 8 mg/kg caused a progressive reduction of arterial blood pressure to a mid-circulatory value, accompanied by bradycardia, myocardial ischemia, ventricular extrasystoles, and second degree atrio-ventricular block. Similar electrocardiogram and arterial blood pressure changes caused by APS12-2 (8 mg/kg) were observed in animals pretreated with atropine and in artificially ventilated animals, indicating that hypoxia and cholinergic effects do not play a crucial role in the toxicity of APS12-2. Application of APS12-2 at sublethal doses (4 and 5.5 mg/kg) caused a decrease of arterial blood pressure, followed by an increase slightly above control values. We found that APS12-2 causes lysis of rat erythrocytes *in vitro*, therefore it is reasonable to expect the same effect *in vivo*. Indeed, hyperkalemia was observed in the blood of experimental animals. Hyperkalemia probably plays an important role in APS12-2 cardiotoxicity since no evident changes in histopathology of the heart were found. However, acute lesions were observed in the pulmonary vessels of rats after application of 8 mg/kg APS12-2. Predominant effects were dilation of interalveolar blood vessels and lysis of aggregated erythrocytes within their lumina.

Izvleček

APS12-2 je eden izmed številnih sintetičnih analogov poli-APS, izoliranih iz morske spužve *Reniera sarai*. Ker je potencialni kandidat za zdravljenje nedrobnoceličnega pljučnega raka, smo ugotavljali njegove morebitne toksične in letalne učinke *in vivo*. Na miših smo določili srednji letalni odmerek, to je 11,5 mg/kg. Elektrokardiogrami, arterijski krvni tlak in respiratorna aktivnost so bili merjeni na podganah v splošni anesteziji, in sicer na netretirani,

farmakološko vagotomirani in umetno ventilirani skupini živali. Vsem skupinam živali smo intravensko aplicirali APS12-2. Pri eni skupini živali smo proučevali *in vivo* učinke APS12-2 na prek živca izzvano mišično kontrakcijo. Pri aplikaciji 8 mg/kg APS12-2 je prišlo do hitrega padca arterijskega krvnega tlaka na srednjo cirkulatorno vrednost, pojavile so se bradikardija, miokardna ishemija, prekatne ekstrasistole in atrio-ventrikularni blok druge stopnje. Podobne spremembe v EKG-ju in arterijskem krvnem tlaku, povzročene z APS12-2 (8 mg/kg), so se pojavile pri podganah, ki smo jim pred meritvami aplicirali atropin, in pri podganah, ki so bile umetno ventilirane. Iz tega sklepamo, da hipoksija in holinergični učinki ne igrajo ključne vloge pri toksičnosti APS12-2. Pri aplikaciji subletalnih odmerkov APS12-2 (4 in 5,5 mg/kg) je prišlo do padca arterijskega krvnega tlaka, ki je nato narastel malo nad kontrolno vrednost. Ker smo ugotovili, da APS12-2 povzroča lizo podganjih eritrocitov *in vitro*, je bilo smiselno pričakovati podoben učinek *in vivo*. V krvi poskusnih živali smo res ugotovili hiperkaliemijo. Ta najverjetneje igra pomembno vlogo pri kardiotoksičnosti APS12-2, saj pri histopatološkem pregledu srca nismo našli nobenih vidnih sprememb. Na pljučnih krvnih žilah podgan smo ugotovili akutne spremembe, kot sta dilatacija interalveolarnih žil in razpad agregiranih eritrocitov v njihovem lumnu.

3.2 TOXICITY OF THE SYNTHETIC POLIMERIC 3-ALKYLPYRIDINIUM SALT (APS3) IS DUE TO SPECIFIC BLOCK OF NICOTINIC ACETYLCHOLINE RECEPTORS

TOKSIČNOST SINTETIČNE POLIMERNE ALKILPIRIDINIJEVE SOLI (APS3) JE POSLEDICA SPECIFIČNEGA ZAVIRANJA NIKOTINSKIH ACETILHOLINSKIH RECEPTORJEV

Grandič M[1], Araoz R[2], Molgó J[2], Turk T[3], Sepčić K[3], Benoit E[2], Frangež R[1]

[1] Univerza v Ljubljani, Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana

[2] CNRS, Institut de Neurobiologie Alfred Fessard – FRC 2118, Laboratoire de Neurobiologie et Développement – UPR 3294, 91198 Gif-sur-Yvette Cedex, France

[3] Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za biologijo, Večna pot 111, 1000 Ljubljana

Objavljeno v: Toxicology, 2012, 303: 25–33

Abstract

The *in vivo* and *in vitro* toxic effects of the synthetic polymeric 3-alkylpyridinium salt (APS3), from the Mediterranean marine sponge *Reniera sarai*, were evaluated on mammals, with emphasis to determine its mode of action. The median lethal doses of APS3 were 7.25 and higher that 20 mg/kg in mouse and rat, respectively. Intravenous administration of 7.25 and 20 mg/kg APS3 to rat caused a significant fall followed by an increase in mean arterial blood pressure accompanied by tachycardia. In addition, cumulative doses of APS3 (up to 60 mg/kg) inhibited rat nerve-evoked skeletal muscle contraction *in vivo*, with a median inhibitory dose (ID_{50}) of 37.25 mg/kg. When administrated locally by intramuscular injection to mouse, APS3 decreased the compound muscle action potential recorded in response to *in vivo* nerve stimulation, with an ID_{50} of 0.5 mg/kg. *In vitro* experiments confirmed the inhibitory effect of APS3 on mouse hemidiaphragm nerve-evoked muscle contraction with a median inhibitory concentration (IC_{50}) of 20.3 μM, without affecting directly-elicited muscle contraction. The compound inhibited also miniature endplate and nerve-evoked endplate potentials with an IC_{50} of 7.28 μM in mouse hemidiaphragm. Finally, APS3 efficiently blocked acetylcholine-activated membrane inward currents flowing through *Torpedo* nicotinic acetylcholine receptors (nAChRs) incorporated to *Xenopus oocytes*, with an IC_{50} of 0.19 μM. In conclusion, our results strongly suggest that APS3 blocks muscle-type nAChRs, and show for the first time that *in vivo* toxicity of APS3 is likely to occur through an antagonist action of the compound on these receptors.

Izvleček

Na glodavcih smo z *in vivo* in *in vitro* poskusi ocenjevali toksične učinke APS3 – sintetičnega analoga poli-APS, izoliranih iz mediteranske morske spužve *Reniere sarai*, z namenom, da bi določili mehanizem delovanja proučevane snovi. Srednji letalni odmerek pri miših je 7,25 mg/kg in pri podganah nad 20 mg/kg. Intravenska aplikacija 7,25 in 20 mg/kg APS3 je pri podganah povzročila statistično značilen padec in nato porast srednjega arterijskega tlaka, ki ga je spremljala tudi tahikardija. Kumulativni odmerki APS3 do odmerka 60 mg/kg so pri podganah zavrli prek živca izzvano mišično kontrakcijo, merjeno *in vivo*. Določili smo srednji zaviralni odmerek (ID_{50}) 37,25 mg/kg. Pri lokalni intramuskularni aplikaciji mišim je APS3

povzročil padec sestavljenega AP mišice, registriranega kot odgovor na *in vivo* stimulacijo živca (ID_{50} = 0,5 mg/kg). Z *in vitro* poskusi smo potrdili zaviralni učinek APS3 na prek živca izzvano kontrakcijo mišje hemidiafragme (IC_{50} = 20,3 μM). Na neposredno izzvano mišično kontrakcijo ni bilo učinka. Proučevana snov je zmanjšala amplitudo MPMP in prek živca izzvane PMP (IC_{50} = 7,28 μM) na preparatih mišjih hemidiafragem. APS3 učinkovito zavira z ACh povzročeni tok skozi nAChRs električnega skata (*Torpedo marmorata*), vključene v membrano oocitov žabe *Xenopus laevis* (IC_{50} = 0,19 μM). Zaključimo lahko, da dobljeni rezultati potrjujejo, da APS3 blokira mišični tip nAChRs. Prvič smo pokazali, da je toksičnost APS3 *in vivo* najverjetneje posledica antagonističnega delovanja snovi na omenjene receptorje.

3.3 THE NON-COMPETITIVE ACETYLCHOLINESTERASE INHIBITOR APS12-2 IS A POTENT ANTAGONIST OF SKELETAL MUSCLE NICOTINIC ACETYLCHOLINE RECEPTORS

NEKOMPETITIVNI INHIBITOR ACETILHOLINESTERAZE APS12-2 JE MOČAN ANTAGONIST NIKOTINSKIH ACETILHOLINSKIH RECPETORJEV V SKELETNIH MIŠICAH

Grandič M[1], Araoz R[2], Molgó J[2], Turk T[3], Sepčić K[3], Benoit E[2], Frangež R[1]

[1] Univerza v Ljubljani, Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana

[2] CNRS, Institut de Neurobiologie Alfred Fessard – FRC 2118, Laboratoire de Neurobiologie et Développement – UPR 3294, 91198 Gif-sur-Yvette Cedex, France

[3] Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za biologijo, Večna pot 111, 1000 Ljubljana

Objavljeno v: Toxicology and Applied Pharmacology, 2012, 265: 221–228

Abstract

APS12-2, a non-competitive acetylcholinesterase inhibitor, is one of the synthetic analogues of polymeric alkylpyridinium salts (poly-APS) isolated from the marine sponge *Reniera sarai*. In the present work the effects of APS12-2 were studied on isolated mouse phrenic nerve-hemidiaphragm muscle preparations, using twitch tension measurements and electrophysiological recordings. APS12-2 in a concentration-dependent manner blocked nerve-evoked isometric muscle contraction (IC_{50} = 0.74 μM), without affecting directly-elicited twitch tension up to 2.72 μM. The compound (0.007–3.40 μM) decreased the amplitude of miniature endplate potentials until a complete block by concentrations higher than 0.68 μM, without affecting their frequency. Full size endplate potentials, recorded after blocking voltage-gated muscle sodium channels, were inhibited by APS12-2 in a concentration-dependent manner (IC_{50} = 0.36 μM) without significant change in the resting membrane potential of the muscle fibers up to 3.40 μM. The compound also blocked acetylcholine-evoked inward currents in *Xenopus* oocytes in which *Torpedo* ($\alpha 1_2\beta 1\gamma\delta$) muscle-type nicotinic acetylcholine receptors (nAChRs) have been incorporated (IC_{50} = 0.0005 μM) indicating a higher affinity of the compound for *Torpedo* ($\alpha 1_2\beta 1\gamma\delta$) than for the mouse ($\alpha 1_2\beta 1\gamma\varepsilon$) nAChR. Our data show for the first time that APS12-2 blocks neuromuscular transmission by a non-depolarizing mechanism through an action on postsynaptic nAChRs of the skeletal neuromuscular junction.

Izvleček

APS12-2, nekompetitvni zaviralec AChE, je eden izmed sintetičnih analogov poli-APS, izoliranih iz morske spužve *Reniere sarai*. Proučevali smo učinke APS12-2 na izolirane živčno-mišične preparate mišjih hemidiafragem, in sicer z meritvami izometrične mišične kontrakcije in elektrofizioloških kazalcev. APS12-2 je v odvisnosti od koncentracije blokiral prek živca izzvano izometrično mišično kontrakcijo (IC_{50} = 0,74 μM), in to brez vpliva na neposredno izzvano kontrakcijo do koncentracije 2,72 μM. Proučevana snov (0,007–3,40 μM) je zmanjšala amplitudo MPMP, dokler niso ti pri koncentraciji 0,68 μM popolnoma izginili. APS12-2 na frekvenco MPMP ni vplival. APS12-2 je, po blokadi od napetosti odvisnih natrijevih kanalčkov v mišičnih vlaknih, v odvisnosti od koncentracije blokiral PMP

(IC_{50} = 0,36 μM), ne da bi pri tem vplival na mirovni MP do koncentracije 3,40 μM. APS12-2 zavira tudi z ACh povzročeni tok prek membrane oocitov žabe *Xenopus laevis*, ki so na svoji površini izražali *Torpedo* ($\alpha 1_2\beta 1\gamma\delta$) mišični tip nAChRs ($IC_{50}$ = 0,0005 μM). To kaže na večjo afiniteto proučevane snovi na nAChR električnega skata ($\alpha 1_2\beta 1\gamma\delta$) kot na mišje ($\alpha 1_2\beta 1\gamma\varepsilon$) nAChR. Naši podatki tako prvič potrjujejo, da APS12-2 blokira živčno-mišični prenos prek nedepolarizirajočega mehanizma delovanja na postsinaptične nAChR v živčno-mišičnem stiku.

3.4 BINDING AND PERMEABILIZATION OF LIPID BILAYERS BY NATURAL AND SYNTHETIC 3-ALKYLPYRIDINIUM POLYMERS

VEZANJE IN PERMEABILIZACIJA LIPIDNIH DVOSLOJEV Z NARAVNIMI IN SINTETIČNIMI 3-ALKILPIRIDINIJEVIMI POLIMERI

Grandič M[1], Zovko A[2], Frangež R[1], Turk T[2], Sepčić K[2]

[1] Univerza v Ljubljani, Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana

[2] Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za biologijo, Večna pot 111, 1000 Ljubljana

Objavljeno v: Bioorganic & Medicinal Chemistry, 2012, 20: 1659–1664

Abstract

Naturally occurring 3-alkylpyridinium polymers from the marine sponge *Reniera sarai* are membrane-active compounds exerting a selective cytotoxicity towards non small cell lung cancer cells, and stable transfection of nucleated mammalian cells. In view of their possible use as chemotherapeutics and/or transfection tools, three poly-APS based synthetic compounds were tested on their activity using natural and artificial lipid membranes. Tested compounds were found to be very stable over a wide range of temperature, ionic strength, and pH, and to prefer the solid-ordered membrane state. Their membrane-damaging activity increases with the length of their alkyl chains and the degree of polymerization.

Izvleček

Naravni 3-alkilpiridinijevi polimeri, izolirani iz morske spužve *Reniera sarai*, so membransko aktivne snovi s citotoksično aktivnostjo proti nedrobnoceličnemu pljučnemu raku, hkrati pa omogočajo stabilno transfekcijo sesalskih celic. Ker obstaja možnost njihove uporabe kot kemoterapevtikov in/ali orodja za transfekcijo, smo testirali aktivnost treh sintetičnih analogov poli-APS na naravnih in umetnih lipidnih membranah. To je pomembno tudi zaradi interakcije s celičnimi membranami po aplikaciji snovi *in vivo* ter za razumevanje molekulskih mehanizmov delovanja sintetičnih analogov poli-APS na živalski organizem. Testirane snovi so se izkazale kot zelo odporne na širok temperaturni spekter ter na različne ionske jakosti in pH-vrednosti pufra. Izkazalo se je, da so bolj aktivne na membranah v trdni urejeni fazi. Membranska aktivnost testiranih snovi je naraščala z dolžino alkilne verige in s stopnjo njihove polimerizacije.

3.5 EFFECTS OF SYNTHETIC ANALOGUES OF POLY-APS ON CONTRACTILE RESPONSE OF PORCINE CORONARY ARTERIES

UČINKI SINTETIČNIH ANALOGOV POLI-APS NA KRČLJIVOST PRAŠIČJIH KORONARNIH ARTERIJ

Grandič M[1], Premrov Bajuk B[1], Sepčić K[2], Drobnič - Košorok M[1], Frangež R[1]

[1] Univerza v Ljubljani, Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana

[2] Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za biologijo, Večna pot 111, 1000 Ljubljana

Objavljeno v: Toxicology in vitro, 2013, 27; 627–631

Abstract

APS12-2 and APS3 are synthetic analogues of polymeric alkylpyridinium salts (poly-APS) isolated from the marine sponge *Reniera sarai*. The aim of the present study was to determine the possible direct contractile effects of these two synthetic molecules on coronary arteries, in order partly to explain hemodynamic and cardiotoxic effects of APS12-2 previously observed in *in vivo* studies and to reveal possible adverse effects on the organism in the case of their clinical use. In contrast to APS3, APS12-2 caused a concentration-dependent vascular smooth muscle contraction of isolated porcine coronary ring preparations in a concentration-range from 1.36 to 13.60 μM. Lanthanum chloride (5 mM) and verapamil (10 μM) completely abolished the APS12-2 evoked contraction of the coronary rings. Pre-incubation with indomethacin (10 μM) had no effect on the contractile responses of coronary ring preparations. These results indicate that APS12-2 contracts vascular smooth muscle in a concentration-dependent manner, due to an increase of Ca^{2+} influx through the voltage-gated Ca^{2+} channels. Our data show for the first time that APS12-2 induces concentration-dependent contraction of coronary ring preparations, which may contribute to the cardiotoxic effects of APS12-2, in addition to hyperkalemia.

Izvleček

APS12-2 in APS3 sta sintetična analoga poli-APS, izoliranih iz morske spužve *Reniere sarai*. Namen raziskave je bil ugotoviti možne neposredne vplive proučevanih analogov na krčljivost koronarnih arterij in s tem razjasniti hemodinamske in kardiotoksične učinke, ki jih je APS12-2 povzročil *in vivo*, ter pojasniti možne negativne učinke izbranih analogov na organizem v primeru njune klinične uporabe. Za razliko od APS3 je APS12-2 (1,36–13,60 μM) povzročil od koncentracije odvisno kontrakcijo gladkih mišičnih celic v izoliranih preparatih prašičjih koronarnih arterij. Lantanov klorid (5 mM) in verapamil (10 μM) sta preprečila kontrakcijo koronarnih žilnih obročkov, povzročeno z APS12-2. Preinkubacija z indometacinom (10 μM) ni imela učinka na krčljivost koronarnih žilnih obročkov. Iz rezultatov je razvidno, da APS12-2 v odvisnosti od koncentracije povzroči krčenje gladkih mišic v krvnih žilah zaradi vdora Ca^{2+} v gladke mišične celice prek napetostno odvisnih kanalčkov Ca^{2+}. S temi rezultati smo prvič pokazali, da APS12-2 povzroči od koncentracije

odvisno kontrakcijo preparatov žilnih obročkov, kar bi lahko poleg hiperkaliemije prispevalo h kardiotoksičnim učinkom APS12-2.

4 RAZPRAVA

V doktorski disertaciji smo proučevali farmakološke in patofiziološke učinke obeh izbranih substanc na nivoju organizma, organov, tkiv, celic in na molekulskem nivoju. V primerjavi z večjim sintetičnim analogom APS12-2 je manjši sintetični analog APS3 tudi farmakološko zanimiv, saj je do koncentracije 34 μM nehemolitičen in selektivno citotoksičen za celice pljučnega raka. Omenjena sintetična analoga poli-APS smo izbrali na podlagi preliminarnih poskusov ter zaradi njune različne velikosti in načina zaviranja AChE. Izbrali smo ju, ker na podlagi njunih kemijskih lastnosti in do sedaj opisanih bioloških aktivnosti pričakujemo razlike v mehanizmih njune toksičnosti, kar bi omogočilo lažjo razlago mehanizmov farmakološkega in toksikološkega delovanja.

4.1 Patofiziološki, farmakološki in toksikološki učinki sintetičnih analogov polimernih alkilpiridinijevih soli *in vivo*

Ker sta izbrana proučevana sintetična analoga potencialna kandidata za uporabo v medicini in farmaciji, je določanje njune toksičnosti bistvenega pomena. Z *in vivo* poskusi na podganah in miših smo želeli ugotoviti, kakšni so njuni negativni učinki na organe oziroma na organizem kot integrirano celoto, saj lahko s temi podatki ovrednotimo morebiten predklinični potencial izbrane substance. Poskusi so bili izvedeni na podlagi dovoljenja št. 34401-84/2008/6.

4.1.1 Toksičnost APS12-2 in APS3

Pri proučevanju toksičnosti izbranih sintetičnih analogov poli-APS smo najprej za oba analoga ocenili srednji letalni odmerek. Srednji letalni odmerek, določen na miših, je za APS12-2 11,5 mg/kg, za APS3 pa 7,25 mg/kg. Toksičnost APS3, ocenjena na podganah, je bila približno trikrat manjša kot pri miših in več kot sedemkrat manjša kot toksičnost naravnega poli-APS. To nakazuje, da sta APS12-2 in APS3 bistveno manj toksična kot naravni poli-APS, ki ima srednji letalni odmerek, določen na podganah, to je približno 2,7 mg/kg (Bunc in sod., 2002b). Kljub nizki toksičnosti APS3 je pri interpretaciji rezultatov potrebna previdnost, saj smo za razliko od podgan pri miših opazili znake akutne toksičnosti.

Intravenska aplikacija relativno visokih odmerkov APS12-2 (8 mg/kg) je pri podganah povzročila nastanek bradikardije, močan padec srednjega arterijskega tlaka in respiratorni zastoj ter pogin živali. Dvig S-T-segmenta na EKG-zapisu, ki se je pojavil 44 ± 8 s po aplikaciji APS12-2, in močan padec krvnega tlaka na vrednost pod 55 mmHg nakazujeta na pojav hipoksije miokarda, ki jo spremljata respiratorni zastoj in hipotenzija. Podobne spremembe so se pojavile tudi pri poskusih s poli-APS (Bunc in sod., 2000; Bunc in sod., 2002b). Respiratorni zastoj bi lahko bil posledica stimulacije jukstapulmonalnih receptorjev (J-receptorji ali C-vlakna) v pljučnem parenhimu. Na podlagi tega, da so bile v pljučih podgan, tretiranih z odmerkom 8 mg/kg, najdene multifokalne spremembe, lahko sklepamo, da imajo J-receptorji pomembno vlogo pri nastanku apneje, povzročene z letalnim odmerkom APS12-2. Da bi ugotovili učinek hipoksije, povzročene z APS12-2, na spremembe v krvnem tlaku in EKG-zapisu, smo podgane pred zastojem dihanja umetno ventilirali. Ker so se podobne spremembe v merjenih parametrih pojavljale tudi pri tej skupini živali, sklepamo, da spremembe v krvnem tlaku najverjetneje niso posledica hipoksije.

APS12-2 in APS3 sta zelo učinkovita zaviralca AChE, kar so ugotovili z *in vitro* poskusi na AChE, pridobljeni iz električnega skata (Houssen in sod., 2010). Bradikardija, ki se pojavi ob aplikaciji APS12-2, je lahko posledica zaviranja ganglijske AChE, saj je aktivnost ACh v holinergičnih kardialnih ganglijih prekinjena z AChE. Druga razlaga za nastanek bradikardije je neposreden vpliv APS12-2 na prevodni sistem srca, ki je posledica membranske aktivnosti APS12-2. APS3 je takoj po aplikaciji pri podganah povzročil padec, nato pa porast srednjega arterijskega tlaka. Ob tem se je pojavila tudi tahikardija, respiratorna aktivnost pa ni bila spremenjena. Ob aplikaciji enkratnega odmerka APS3 (7,25 mg/kg) je prišlo do nestabilnega padca krvnega tlaka iz bazalne vrednosti 80,38 ± 2,31 mmHg na 67,40 ± 1,89 mmHg, ki mu je sledil statistično značilen dvig 31 ± 1,94 s po aplikaciji substance. Statistično značilen padec srednjega arterijskega tlaka, povzročen ob aplikaciji APS3, ni neposredno posledica zmanjšanega utripnega volumna ali zmanjšane frekvence srca, saj se je ta ob aplikaciji substance celo povišala. Možna razlaga za začetno hipotenzijo, povzročeno z APS3, bi lahko bila, da snov, podobno kot naravni poli-APS (Turk in sod., 2008), deluje kot kompetitivni zaviralec AChE, saj sta bradikardija in hipotenzija pogosta klinična znaka ob zastrupitvah z zaviralci AChE (Kojima in sod., 1992; Dube in sod., 1993; Kassa in Fusek, 1998). Upoštevajoč to hipotezo, lahko povečano izločanje noradrenalina iz postganglijskih simpatičnih nevronov, ki je posledica povečane holinergične aktivnosti v simpatičnih

ganglijih zaradi posrednega nikotinskega učinka zaviralcev AChE, vodi v normalno ali celo povečano frekvenco srca (Bao in sod., 1997). V našem primeru bi povečana frekvenca srca lahko bila kompenzatorni odgovor na hipotenzijo, ki sledi aplikaciji APS3. To hipotezo lahko dodatno podkrepimo s podatkom, da je APS3 močan kompetitivni zaviralec AChE z inhibitorno konstanto 85 nM, kar je bilo dokazano z *in vitro* meritvami na AChE iz električnega skata (Zovko in Turk, neobjavljeni rezultati). Poleg tega je APS3 strukturno soroden kvartarnim amonijevim spojinam, kot so atrakurij, mivakurij in pankuronij, ter tako kot naštete snovi (Fisher, 1999) povzroči hudo arterijsko hipotenzijo in tahikardijo, povezano s sprostitvijo histamina. Kljub temu da bi zadnja možnost morala biti podrobneje raziskana, ne moremo izključiti dejstva, da je tahikardija, povzročena z APS3, lahko povezana z zaviralnim delovanjem APS3 na ganglijske $(\alpha 3)_2(\beta 4)_3$ nAChRs. Kljub temu v naših poskusnih pogojih nismo zasledili nobenega učinka APS3, povezanega z zaviranjem AChE, kot so mišične fascikulacije pri *in vivo* poskusih ali povečane amplitude mišične kontrakcije in podaljšane druge faze PMP pri *in vitro* poskusih.

APS12-2 in APS3 sta strukturno sorodna kvartarnim amonijevim spojinam, med katerimi so tudi zaviralci AChE, na primer dekametonij, ki prav tako povzroči nastanek bradikardije zaradi ganglijskih in postganglijskih učinkov akumuliranega ACh (Taylor, 2001). Zmanjšano frekvenco srca lahko povzroči tudi povečana količina ACh, ki stimulira muskarinske M2-receptorje (Dhein in sod., 2001). Vlogo parasimpatične stimulacije srca pri razvoju hipotenzije smo delno potrdili in njeno vlogo pri razvoju ostalih aritmij ovrgli s farmakološko blokado muskarinskih receptorjev z antagonistom muskarinskih receptorjev atropinom, ki smo ga podganam aplicirali 10 min pred aplikacijo APS12-2. Uporabili smo odmerek 1 mg/kg, ki blokira muskarinske M2-receptorje na srcu (Sawyer in Mundy, 1970; Wellstein in Pitschner, 1988). Ker muskarinski receptorji sodelujejo tudi pri živčnem prenosu skozi ganglije, atropin blokira nekatere ekscitatorne vplive zaviralcev AChE na avtonomne ganglije. Centralno delovanje APS12-2 na medularne vazomotorne centre ni pričakovano, saj APS12-2 zaradi svoje velikosti, naboja na kvartarnih dušikovih skupinah in dobre topnosti v vodi (Houssen in sod., 2010) najverjetneje ne more prehajati prek krvno-možganske bariere. Predvidevamo, da je bradikardija delno posledica zaviranja AChE, saj smo skladno z zaviranjem AChE *in vitro* ugotovili, da APS12-2 tudi *in vivo* zavira serumsko AChE pri podganah. Patofiziološki pomen teh učinkov je sicer še neznan. Za razliko od podgan, ki so prejele le subletalni odmerek APS12-2 in kjer je bila znižana frekvenca srca statistično

značilna, se pri podganah, ki smo jim pred aplikacijo APS12-2 aplicirali atropin, bradikardija v prvi minuti po aplikaciji APS12-2 ni pojavila. Pri enem poskusu je bila blaga. Bradikardijo lahko povzroči tudi hiperkaliemija, saj zmanjša hitrost prevodnosti v srcu (Parham in sod., 2006). Pri poskusih z APS12-2 *in vivo* nismo zaznali nobenih znakov, kot so fascikulacije, tremor mišic, zmanjšana amplituda kontrakcije ali blokada mišične kontrakcije, ki so sicer povezani z zaviranjem AChE v živčno-mišičnem stiku. Pri poskusih na podganah z APS3 pa smo ugotovili, da APS3 zavira krčenje mišic (ID_{50} = 37,25 mg/kg). Pri živalih, ki so bile umetno ventilirane in smo jim aplicirali letalni odmerek APS12-2, se je pojavila akutna hipotenzija, za katero ni bila odgovorna hipoksija. Frekvenca srca je po začetni bradikardiji postala normalna ali pa se je povečala, kar ni presenetljivo, saj je to značilen kompenzatorni mehanizem. Histopatološke spremembe, najdene multifokalno v pljučnem parenhimu, bi lahko prek refleksnih lokov vagalnega živca povzročile bradikardijo in hipotenzijo. Subletalni odmerki APS12-2 so povzročili prehodni padec in nato porast krvnega tlaka. Možno je, da je za padec krvnega tlaka odgovorna bradikardija, povzročena s hiperkaliemijo, ki je najverjetneje posledica hemolitične aktivnosti APS12-2, ali prek pljučnih refleksov. Porast krvnega tlaka je lahko delno posledica kompenzatorne stimulacije simpatikusa, lahko pa je posledica neposrednega ali posrednega delovanja APS12-2 na upor v perifernem ožilju. Ta ugotovitev je podprta z dejstvom, da med porastom krvnega tlaka nismo izmerili statistično značilno povečane frekvence srca.

Hemolitična aktivnost APS12-2 in naravnega poli-APS pri intravenski aplikaciji je verjetno vzrok za nastanek hiperkaliemije, ki vodi v aritmije in kardiorespiratorni zastoj ter končno v pogin poskusnih živali (Bunc in sod., 2002a; Grandič in sod., 2011). V povezavi z nehemolitičnim delovanjem APS3 nismo opazili nobenih aritmij ali prizadetosti vitalnih funkcij, povzročenih z intravensko aplikacijo APS3, kljub zvišani koncentraciji kalija v serumu. Čeprav je bila koncentracija kalija v serumu statistično značilno povišana, je bila v primerjavi s hiperkaliemijo, povzročeno z APS12-2 (Grandič in sod., 2011), nizka. Koncentracije natrija, kalcija in klora v serumu ter hematokrit niso bili bistveno spremenjeni, razen rahlega padca koncentracije serumskega natrija in kalcija pri visokih odmerkih APS12-2. Odmerki APS12-2 nad 4 mg/kg so povzročili statistično značilen dvig koncentracije kalija v krvi, kar je lahko pomemben vzrok kardiotoksičnosti substance. Odpoved srca in pogin podgan sta posledica visoke koncentracije kalija (nad 10 mM) v krvi (Emberson in Muir, 1969; Van der Meer in sod., 1986; Parham in sod., 2006). Hiperkaliemija je najverjetneje

posledica hemolitične aktivnosti APS12-2, ki smo jo dokazali s poskusi na podganjih eritrocitih *in vitro*. Hemolizo smo pri poskusih z APS12-2 ugotovili tudi na podlagi rdečkaste barve seruma podgan. Kljub temu vrednosti hematokrita niso bile statistično značilno spremenjene in niso bile znižane, kot bi pričakovali. Hematokrit je bil celo nekoliko povišan, predvsem pri višjih uporabljenih odmerkih APS12-2, kar je lahko posledica prehoda znotrajžilne tekočine v izvenžilni prostor. To smo še dodatno potrdili, ko smo predvsem v pljučih podgan našli znake serozne transudacije (ekstravazacije) v obliki blagega alveolarnega, srednje močnega interalveolarnega in perivazalnega edema. V dilatiranih interalveolanih venulah smo našli agregate razpadlih ali na pol razpadlih eritrocitov, s čimer smo še dodatno potrdili hemolitično aktivnost APS12-2 *in vivo*.

S poskusi na podganjih eritrocitih in umetnih lipidnih membranah različne sestave smo dodatno razjasnili molekulske mehanizme vezave in toksičnosti APS12-2 in nekaterih drugih sintetičnih analogov poli-APS (APS7, APS8 in naravnih poli-APS). Še posebej smo želeli razjasniti mehanizme hemolitične aktivnosti, ki neposredno ali posredno zaradi hiperkaliemije privede do kardiotoksičnosti in pogina poskusnih živali. Mehanizme vezave sintetičnih analogov poli-APS in njihove učinke smo še dodatno razjasnili s poskusi v različnih pogojih okolja. Ugotovili smo, da so se trije testirani sintetični analogi poli-APS izkazali kot zelo stabilni v širokem spektru temperatur, ionske jakosti in pH-raztopine. Njihova membranska aktivnost je odvisna predvsem od njihove strukture in narašča z dolžino alkilne verige in s stopnjo polimerizacije, kar je značilno za membransko aktivnost tudi drugih amfifilnih snovi (Zarif in sod., 1993; Kuroda in DeGrado, 2005; Kleszczynska in sod., 2006). Najbolj litično aktiven, tako na eritrocitnih membranah kot na lipidnih veziklih, je bil APS12-2. Večjo hemolitično aktivnost APS12-2 lahko pojasnimo tudi z naravo nasprotnih ionov, saj so tiste piridinijeve soli, ki vsebujejo bromidni ion, bolj membransko aktivne kot tiste s kloridnim ali drugim ionom (Sarapuk in sod., 1998; Sarapuk in sod., 1999). Vsi uporabljeni sintetični analogi poli-APS so pokazali membransko aktivnost v koncentracijah, podobnih tistim, uporabljenim na eritrocitnih membranah. Ugotovili smo, da imajo pri delovanju na celične membrane sintetičnih analogov poli-APS večjo afiniteto do lipidov v trdni urejeni fazi. To bi lahko bil vzrok za njihovo relativno nizko citotoksičnost na netarčne celične membrane, katerih membrane so sestavljene kot mozaik tekočih neurejenih in tekočih urejenih domen (McConnell in Vrljič, 2003).

Aritmije, ki se pojavljajo ob aplikaciji visokih odmerkov APS12-2, imajo lahko več vzrokov. Lahko so posledica hiperkaliemije ali neposrednega delovanja snovi na miokard ali prevodni sistem srca, vendar je za nastanek aritmij najverjetneje odgovorna hiperkaliemija. To je podprto s pojavom značilnih sprememb na EKG-zapisu, kot so bradikardija, atrioventrikularni blok druge stopnje in prekatne ekstrasistole. Take spremembe se običajno pojavijo pri napredovani hiperkaliemiji, ko so v krvi različno visoke koncentracije kalija (Kuwahara in sod., 1992; Parham in sod., 2006). To, da bi lahko bile aritmije posledica neposrednega delovanja sintetičnih analogov poli-APS na miokard in prevodni sistem srca, posredno podpirajo rezultati, iz katerih je razvidno, da APS12-2 povzroči koncentracijsko odvisno permeabilizacijo membran, torej bi lahko prišlo tudi do depolarizacije membran kardiomiocitov. Membranska aktivnost APS12-2 je najverjetneje posledica njihove surfaktantom podobne narave. To so že dokazali za strukturno sorodni poli-APS, ki povzročita detergentski tip lize eritrocitnih membran (Malovrh in sod., 1999). Podobne spremembe v časovnem poteku krvnega tlaka in EKG-zapisa so opisali za dva hemolitična toksina, to je ekvinatoksin II in ostreolizin, ki povzročata hiperkaliemijo enake stopnje in tudi pogin podgan (Šuput in sod., 2001; Žužek in sod., 2006).

Histološki pregled src podgan, tretiranih z letalnimi odmerki APS12-2, je pokazal, da so bile vse štiri srčne votline razširjene in napolnjene s krvjo, kar kaže na akutno odpoved srca. Ker na srcu in koronarnih žilah nismo našli nobenih morfoloških sprememb, predvidevamo, da so za odpoved srca krive motnje v električni prevodnosti srca, kar je posledica velikih koncentracij kalija ob aplikaciji visokih odmerkov APS12-2. Rezultati histopatološke analize se skladajo s tistimi v *in vivo* poskusih. Neposrednega učinka APS12-2 na kardiomiocite oziroma na njihovo mehanično disfunkcijo in na prevodni sistem srca za zdaj ne moremo izločiti, kljub temu da na srcih podgan nismo našli vidnih lezij.

Predpostavka, da APS3 antagonistično deluje na nAChRs, je v skladu z zaviralnim delovanjem proučevane substance na prek živca izzvano mišično kontrakcijo *in vivo* (ID_{50} = 37,25 mg/kg) in na sestavljeni AP mišice (angl. compound muscle action potential – CMAP; ID_{50} = 0,5 mg/kg). Omenjeno hipotezo smo dodatno potrdili s poskusi na izoliranih živčno-mišičnih preparatih *in vitro*. V preglednici 3 je pregled najpomembnejših učinkov APS12-2 in APS3 *in vivo.*

Preglednica 3: Učinki APS12-2 in APS3 *in vivo*

Table 3: Effects of APS12-2 and APS3 *in vivo*

Merjeni parameter	**APS12-2**	**APS3**
LD_{50} (miši)	11,5 mg/kg	7,25 mg/kg
EKG (podgane)	• bradikardija • AV-blok II. stopnje • prekatne ekstrasistole	• prehodna tahikardija
Arterijski tlak	močan padec takoj po aplikaciji	najprej padec, nato dvig nad bazalno vrednost
Dihanje	respiratorni zastoj kmalu po aplikaciji	ni vpliva
Biokemijski parametri	statistično značilno povišan K^+ (10,44 ± 0,44 mM)	statistično značilno povišan K^+ (5,66 ± 0,37 mM)
Mišična kontrakcija	ni vpliva (do odmerka 8,6 mg/kg)	ID_{50} = 37,25 mg/kg

4.2 Vpliv sintetičnih analogov polimernih alkilpiridinijevih soli *in vitro*

4.2.1 Vpliv APS12-2 in APS3 na živčno-mišični prenos

Proučevana sintetična analoga poli-APS sta strukturno sorodna kvartarnim amonijevim spojinam, med katerimi nekatere blokirajo mišični tip nAChRs (Fisher, 1999). APS3 in APS12-2 sta oba zaviralca AChE (Houssen in sod., 2010; Zovko, neobjavljeni rezultati). Nekateri zaviralci AChE imajo lahko celo dva učinka. Poleg zaviranja AChE lahko namreč tudi modulirajo delovanje nAChR. Primer takega zaviralca AChE je fizostigmin (Sherby in sod., 1985), ki v mikromolarnih koncentracijah aktivira nAChR, v milimolarnih koncentracijah pa jih neposredno blokira (Okonjo in sod., 1991). Glede na strukturo in antiacetilholinesterazno aktivnost izbranih sintetičnih analogov smo pričakovali učinke APS3 in APS12-2 na živčno-mišični prenos. Iz tega razloga smo proučevali vpliv obeh analogov na kontrakcijo mišic na izoliranem preparatu mišje diafragme. Da bi natančneje določili mehanizem delovanja proučevanih sintetičnih analogov poli-APS, smo z mikroelektrodno tehniko na izoliranih mišjih diafragmah proučevali vpliv APS3 in APS12-2 na MP, MPMP in

PMP. Poleg tega smo preverili tudi neposreden učinek obeh proučevanih snovi na nAChR, izraženih v plazmalemi žabjih oocitov.

Poskusi *in vitro* na izoliranih živčno-mišičnih preparatih mišjih diafragem so pokazali, da APS12-2 in APS3 v odvisnosti od koncentracije zavirata posredno izzvano enostavno mišično kontrakcijo in posredno izzvano tetanično kontrakcijo. V primerjavi z nedepolarizirajočim kompetitivnim antagonistom mišičnih nAChRs D-tubokurarinom (DTC; IC_{50} = 1,42 μM; Ridtitid in sod., 1998; Nguyen-Huu in sod., 2005) je sposobnost zaviranja živčno-mišičnega prenosa z APS12-2 približno dvakrat večja (IC_{50} = 0,74 μM), z APS3 pa približno petnajstkrat manjša (IC_{50} = 20,3 μM). Ugotovili smo, oba izbrana analoga v odvisnosti od koncentracije (APS3: 0,07–6,85 μM in APS12-2: 0,07–2,72 μM) zmanjšata amplitudo PMP, hkrati pa ne vplivata bistveno na MP in na frekvenco MPMP. Ti rezultati nakazujejo na to, da APS12-2 in APS3 v živčno-mišičnem stiku delujeta postsinaptično na nAChRs. Glede na rezultate lahko sklepamo, da APS12-2 in APS3 zmanjšata število nAChRs, razpoložljivih za aktivacijo z ACh, ne da bi pri tem spremenila pasivne lastnosti membrane mišičnih vlaken in kakor koli vplivala na proces sproščanja ACh. Z uporabo neostigmina (1 μM), ki je povratni zaviralec AChE, nismo uspeli preprečiti živčno-mišične blokade. Če bi proučevana sintetična analoga poli-APS imela antiacetilholinesterazni učinek v živčno-mišičnem stiku, bi pričakovali, da bo neostigmin blokado, povzročeno s sintetičnimi analogi poli-APS, še poglobil. Pri izvedenih poskusih nismo opazili nobenega dokaza o morebitnem antiacetilholinesteraznem delovanju APS12-2 in APS3. Omenjeni učinek bi se sicer pokazal kot povečana amplituda posamičnih krčenj mišjih hemidiafragem in kot podaljšana druga (padajoča) faza sinaptičnih potencialov (MPMP in PMP), kar bi bila posledica zaviranja in manjše aktivnosti AChE ter s tem povečane količine ACh v sinaptični špranji, ki bi vedno znova aktiviral nAChRs na postsinaptični membrani motorične ploščice (Van der Kloot in sod., 1994). Ta mehanizem bi lahko bil zamaskiran zaradi blokade nAChRs z APS12-2 in APS3, ki zmanjšata število receptorjev, ki so na voljo za vezavo z ACh. Za razliko od nekaterih drugih študij (Houssen in sod., 2010) bi lahko bila odsotnost antiacetilholinesteraznega delovanja APS12-2 in APS3 tudi posledica njune polikationske strukture, ki omeji njuno sposobnost, da dosežeta tarče delovanja. Blokada mišične kontrakcije z APS12-2 in APS3 je bila povratna ob dodatku blokatorja kalijevih kanalčkov, to je 3,4-diaminopiridina, ki je delno zavrl učinek APS12-2 in APS3 na posredno (preko živca) izzvano mišično kontrakcijo. 3,4-diaminopiridin poveča sproščanje ACh iz presinaptičnega

dela motorične ploščice tako, da zavre kalijeve kanalčke in posredno poveča tok kalcija v živčni končič ter sproščanje ACh (Molgó in sod., 1980; Hong in sod., 1990). Na ta način 3,4-diaminopiridin prek tekmovanja med ACh in sintetičnimi analogi poli-APS za vezavno mesto na nAChRs poveča moč mišične kontrakcije (Khan in Lemeignan, 1983; Van Lunteren in sod., 2008).

Da bi ugotovili morebiten neposreden učinek APS12-2 in APS3 na sarkolemo ali mišične procese sklopitve vzdražnosti in kontrakcije, smo v istih poskusih z neposredno stimulacijo mišje diafragme opazovali časovni potek in amplitudo tetaničnih in enostavnih mišičnih kontrakcij. Pri neposredni stimulaciji mišice se moč enostavne mišične in tetanične kontrakcije ni spremenila niti pri velikih koncentracijah APS12-2 in APS3. Neposreden učinek obeh proučevanih sintetičnih analogov poli-APS na vzdražnost membrane mišičnega vlakna je tako malo verjeten, saj pri neposredni stimulaciji na istih preparatih nobeden od njiju ni zmanjšal amplitude in časovnega poteka krčenja mišice. Na podlagi tega lahko sklepamo, da APS12-2 in APS3 nimata neposrednega vpliva na sarkolemo in fiziološke procese sklopitve vzdražnosti in kontrakcije, kar bi povzročilo blokado kontrakcije. Mišični odgovor na neposredno stimulacijo hemidiafragme se je zmanjšal le pri najvišji uporabljeni koncentraciji APS12-2 (2,72 μM). Ker APS12-2 lahko povzroči permeabilizacijo naravnih in umetnih lipidnih membran v nanomolarnih koncentracijah (Grandič in sod., 2012), predvidevamo, da lahko v koncentracijah, višjih od 2,72 μM, APS12-2 depolarizira in inaktivira od napetosti odvisne natrijeve kanalčke v mišičnih vlaknih. To domnevo smo kasneje ovrgli z znotrajceličnimi meritvami MP mišičnih celic hemidiafragme. Kljub temu da so bili mišični preparati različno dolgo izpostavljeni različnim koncentracijam APS12-2, se mirovni MP ni spremenil pri nobeni od uporabljenih koncentracij APS12-2.

Ker tako APS3 kot APS12-2 zmanjšata amplitudo posredno izzvane mišične kontrakcije, smo želeli preveriti, ali vplivata na nekatere fiziološke parametre, pomembne za živčno-mišični prenos. Pokazali smo, da APS12-2 in APS3 v odvisnosti od koncentracije zmanjšata amplitudo MPMP in PMP, kar nakazuje na postsinaptični učinek obeh izbranih analogov na nAChRs, saj je znižanje amplitude postsinaptičnih potencialov proporcionalno številu blokiranih nAChRs. V koncentracijah, ki niso bile zadostne za blokado živčno-mišičnega prenosa, sta APS12-2 in APS3 statistično značilno znižala amplitudo MPMP, ne da bi pri tem vplivala na mirovni MP in frekvenco MPMP. Iz tega sklepamo, da APS12-2 in APS3

zmanjšata število razpoložljivih nAChRs, vendar ne prek sprememb v pasivnih lastnostih membrane ali prek količine sproščenega ACh.

Da bi potrdili neposredni zaviralni učinek APS12-2 in APS3 na mišični tip nAChRs, smo izvedli poskuse na oocitih žabe vrste *Xenopus laevis*. Oociti so v svoji membrani izražali mišični tip nAChR, pridobljenih iz električnega skata *Torpedo marmorata*. Ugotovili smo, da APS12-2 in APS3 učinkovito zavirata ionski tok skozi mišični tip nAChRs, izraženih v membrani oocitov, kar nakazuje na blokado nAChRs. Razlike v dobljenih IC_{50} za vpliv APS12-2 na mišično kontrakcijo (IC_{50} = 0,74 μM) in na PMP (IC_{50} = 0,36 μM) so pričakovane in so lahko povezane z varnostnim pragom za živčno-mišični prenos (Wood in Slater, 2001). Dejstvo, da je pri vplivu APS12-2 na električni tok, povzročen z ACh, izračunani IC_{50} (0,0005 μM) manjši kot IC_{50}, dobljen pri meritvah mišične kontrakcije in PMP, nakazuje na možnost, da ima APS12-2 večjo afiniteto za vezavo na *Torpedo* ($\alpha 1_2\beta 1\gamma\delta$) nAChRs kot za vezavo na mišje ($\alpha 1_2\beta 1\gamma\varepsilon$) nAChRs. IC_{50}, izračunan za vpliv APS3 na nAChRs, izražene v oocitni membrani, je 0,19 μM. Blokado nAChRs lahko snovi povzročijo na več različnih načinov. Najenostavnejši med njimi je vezava molekule toksina v kanalček, ko je ta v odprtem stanju. Tako se ta kanalček »zamaši« in prekine oziroma se zmanjša tok ionov skozenj (Adams, 1976; Pascual in sod., 1998; Rozman in sod., 2010). Drugi možni mehanizem je vezava kompetitivnega antagonista na nAChR v motorični ploščici in preprečitev vezave ACh na vezavno mesto. Primer za to je vezava DTC v nizkih koncentracijah (Bowman, 2006). Pri večjih koncentracijah DTC blokira nAChR neposredno na nekompetitivni način (Colquhoun in sod., 1979). Iz koncentracijsko odvisne krivulje za APS12-2 smo izračunali Hillov koeficient za mišični tip nAChRs ($\alpha 12\beta 1\Upsilon\delta$) 3,82, iz koncentracijsko odvisne krivulje za APS3 pa je izračunan Hillov koeficient 1,65. To kaže, da se APS12-2 na nAChRs veže v molekulskem razmerju ~ 4 : 1, APS3 pa v razmerju ~ 2 : 1. Visok Hillov koeficient je lahko tudi pokazatelj možnega alosteričnega učinka APS12-2 in APS3, pri čemer pride do interakcij substance z regijami na nAChR, ki niso vezavno mesto za ACh (Prinz, 2009). Ugotovili smo, da inhibicija ionskega toka prek membran oocitov, povzročena z APS12-2 in APS3, ni napetostno odvisna, kar močno nakazuje na to, da se APS12-2 in APS3 ne vežeta le na odprte nikotinske receptorje, temveč tudi na zaprte.

Iz vseh dobljenih rezultatov lahko povzamemo, da APS12-2 v submikromolarnih koncentracijah in APS3 v mikromolarnih koncentracijah blokirata mišični tip nAChR, saj

zmanjšata prek živca izzvano mišično kontrakcijo, hkrati pa ne vplivata na neposredno izzvano mišično kontrakcijo, znižata amplitudo MPMP in PMP ter ne spremenita MP.

APS12-2 najverjetneje deluje kot počasi disociirajoči antagonist nikotinskih receptorjev. Ti zaviralni učinki APS12-2 na posredno izzvano kontrakcijo diafragme bi lahko prispevali k respiratornemu zastoju, ki se pojavi v *in vivo* poskusih. Poleg tega se – ko APS12-2 *in vivo* apliciramo mišim intramuskularno in ob tem z elektrodami dražimo repni živec – pojavi izrazita blokada CMAP-a, merjenega na repni mišici, pri čemer ne pride do bistvenih sprememb v ostalih parametrih vzdraženja, kot je pričakovano pri snoveh, ki so zaviralci nAChRs. Pomembno je dodati, da bi vsi opisani učinki lahko omejili uporabo APS12-2 kot protitumorske snovi, saj se pojavljajo v podobnih koncentracijah kot antiproliferativna aktivnost substance.

Za APS3 sklepamo, da deluje kot počasno disociirajoči zaviralec nAChRs v motorični ploščici. Toksični in celo letalni učinki APS3, ki smo jih ugotovili pri poskusih *in vivo,* bi torej lahko bili povezani z blokado nAChRs pri zelo visokih odmerkih (> 20 mg/kg). Razlike med odmerkom LD_{50} določenim na miših in letalnimi odmerki določenimi na podganah ne moremo pojasniti.

4.2.2 Vpliv APS12-2 in APS3 na žilne obročke

APS12-2 je v subletalnih odmerkih (4 in 5,5 mg/kg) pri podganah povzročil padec arterijskega tlaka, ki mu je sledil dvig nad bazalno vrednost. V letalnem odmerku (11 mg/kg) je APS12-2 povzročil progresivni, nepovratni padec arterijskega tlaka, ki so ga spremljali znaki miokardialne hipoksije in aritmije. Za razliko od APS12-2 je APS3 povzročil povratni in prehodni padec arterijskega tlaka brez spremljajočih aritmij. APS3 v koncentraciji do 20 mg/kg in kumulativno do 60 mg/kg ni kazal nobenih kardiotoksičnih učinkov. Z namenom proučevanja učinkov APS12-2 in APS3 in boljše razlage specifičnih mehanizmov kardiotoksičnih učinkov APS12-2 smo izvedli *in vitro* poskuse na izoliranih preparatih prašičjih koronarnih arterij. APS12-2 je povzročil od koncentracije odvisno krčenje žilnih obročkov, kar bi lahko pomagalo razložiti njegov kardiotoksični učinek *in vivo*. Ker so koncentracije 4,1–13,6 μM, uporabljene v študiji žilne kontrakcije, povzročile maksimalni

kontraktilni odgovor na žilnih obročkih, lahko sklepamo, da APS12-2 prispeva k hemodinamskim spremembam in celo kardiorespiratornemu zastoju pri podganah. Vendar interpretacija teh rezultatov zahteva nekoliko previdnosti, saj so kot model za proučevanje učinkov APS12-2 bili uporabljeni prašičji žilni obročki. Za razliko od APS12-2 je APS3 pri *in vivo* poskusih povzročil le majhno in prehodno spremembo arterijskega tlaka, in to tako pri odmerku 20 mg/kg kot tudi pri kumulativnih odmerkih vse do 60 mg/kg. Pri poskusih na žilnih obročkih APS3 v visokih koncentracijah (41,1–137 μM) ni imel učinka. Kot smo že omenili, je aktivnost alkilpiridinijevih soli premo sorazmerna z dolžino alkilne verige in s stopnjo polimerizacije ter je večja ob prisotnosti bromidnega iona. Prav zato ni presenetljivo, da je APS12-2 učinkovit v bistveno nižjih koncentracijah kot APS3.

Da bi še dodatno razjasnili mehanizme arteriokonstrikcije z APS12-2, smo naredili še poskuse z različnimi zaviralci gladkomišične kontrakcije. Glavni sprožilec kontrakcije gladkih mišičnih celic je porast citosolnega Ca^{2+} zaradi vdora Ca^{2+} iz izvenceličnega prostora in v manjši meri iz celičnih zalog (Elmoselhi in Grover, 1997; Kuriyama in sod., 1998). Mišična kontrakcija, ki se pojavi ob aplikaciji APS12-2, je lahko posledica vdora Ca^{2+} v celico ali nastanka velikih prehodnih por v membrani, ki so prav tako posledica delovanja APS12-2 (McClelland in sod., 2003; Houssen in sod., 2010). Da bi preizkusili te hipoteze, smo v gladkih mišicah krvnih žil blokirali kalcijeve kanalčke tako, da smo žilne obročke pred aplikacijo APS12-2 inkubirali z neselektivnim zaviralcem kationskih kanalčkov, to je z lantanovim kloridom. $LaCl_3$ neselektivno prepreči vdor kalcija iz izvenceličnega prostora tako, da blokira napetostno odvisne kalcijeve kanalčke tipa-L (VDCC) in neselektivne kationske kanalčke (NSCC; Hogestatt in Anderson, 1984; Kasai in Neher, 1992). Ker je bilo ugotovljeno, da naravni poli-APS in sintetični APS12-2 povečata aktivnost Ca^{2+} v človeških embrionalnih ledvičnih celicah (HEK 293) ter v hipokampusnih nevronih in DRG (angl. dorsal root ganglia) podgane zaradi nastanka velikih prehodnih por v celični membrani (McClelland in sod., 2003; Houssen in sod., 2010), smo predvidevali, da bi APS12-2 lahko povzročil permeabilizacijo membran gladkih mišičnih celic ter tako povzročil vdor kalcija v celice in posledično kontrakcijo. $LaCl_3$ je popolnoma preprečil kontrakcijo žilnih obročkov, izzvano z APS12-2. To lahko nakazuje na nastanek konduktivnih por z APS12-2, ki jih $LaCl_3$ blokira, ali pa nakazuje na aktivacijo NSCC in/ali VDCC, kar lahko prispeva h kontraktilnemu učinku APS12-2. Iz tega razloga smo v nadaljnjih poskusih žilne obročke inkubirali z verapamilom, ki je selektivni zaviralec L-tipa VDCC (Triggle, 1999). Ker v teh

pogojih APS12-2 ni izzval kontrakcije, sklepamo, da poteka mehanizem krčenja gladkih mišičnih celic z APS12-2 prek aktivacije L-tipa VDCC. Eden izmed možnih mehanizmov delovanja APS12-2 bi lahko bil depolarizacija membrane in aktivacija VDCC. Kljub temu neposrednih učinkov APS12-2 na L-tip VDCC ne moremo izključiti. Pomembno je omeniti, da je APS12-2 v poskusih izničil vazorelaksacijsko delovanje substance P, ki povzroči sproščanje gladkih mišičnih celic prek NK1-receptorjev (Cathieni in sod., 1999), kar kaže, da APS12-2 onemogoči ta mehanizem. Da bi ugotovili morebitno vazokonstrikcijsko vlogo eikozanoidov v kontrakciji žilnih obročkov, povzročeni z APS12-2, smo žilne obročke preinkubirali z indometacinom, neselektivnim zaviralcem ciklooksigenaz (COX-1, 2), ki so encimi, ki pretvarjajo arahidonsko kislino v prostaglandine. COX-1 je encim, ki sodeluje pri kontrakciji, odvisni od endotelija, v velikih krvnih žilah podgan in miši tako, da povzroči sintezo endotelijskih kontraktilnih faktorjev (EDCF) ali kontraktilnih prostaglandinov, ki se lahko sintetizirajo tudi v gladkih mišičnih celicah (Wong in sod., 2010). Ker inkubacija žilnih obročkov z indometacinom ni imela učinka na kontrakcijo, povzročeno z APS12-2, sklepamo, da prostanoidi pri tem nimajo pomembne vloge.

Največja uporabljena koncentracija APS12-2 (13,6 μM), ki je povzročila statistično značilno kontrakcijo žilnih obročkov *in vitro*, je primerljiva z maksimalno izračunano koncentracijo APS12-2 v krvni plazmi *in vivo* po aplikaciji srednjega letalnega odmerka APSS12-2, ki je povzročil nastanek aritmij in kardiorespiratornega zastoja. Kontrakcija mišic v venčnih žilah vodi do vazospazma in zmanjšuje pretok krvi, posledica česar je hipoksija miokarda. Poleg tega je tudi relaksacija gladkih mišic v arterijah ob delovanju APS12-2 zmanjšana. Vsi ti mehanizmi imajo verjetno pomembno vlogo v kardiotoksičnosti APS12-2. V preglednici 4 je podan pregled najpomembnejših učinkov APS12-2 in APS3 *in vitro*.

Preglednica 4: Učinki APS12-2 in APS3 *in vitro*

Table 4: Effects of APS12-2 and APS3 *in vitro*

Merjeni parametri		**APS12-2**		**APS3**	
		učinek	**IC_{50}**	**učinek**	**IC_{50}**
Mišična kontrakcija	posredno izzvana	zaviranje	0,74 μM	zaviranje	20,3 μM
	neposredno izzvana	ni vpliva do konc. 2,72 μM	/	ni vpliva (do konc. 20,55 μM)	/
Farmakološki vpliv	atropin	ni vpliva (80 μM)	/	ni vpliva (80 μM)	/
	neostigmin	ni vpliva (1 μM)	/	ni vpliva (1 μM)	/
	3,4-DAP	prekine blokado kontrakcije (300 μM)	/	prekine blokado kontrakcije (300 μM)	/
Vpliv na	MP	ni vpliva do konc. 3,40 μM	/	ni vpliva do konc. 68,49 μM	/
	MEPP	znižanje amplitude, nad 0,68 μM izginejo	/	znižanje amplitude, nad 6,85 μM izginejo	/
	EPP	znižanje amplitude	0,36 μM	znižanje amplitude	7,28 μM
Inhibicija nAChRs		zaviranje	0,0005 μM	zaviranje	0,19 μM
Vpliv na žilne obročke		kontrakcija (4,1–13,6 μM)	/	ni vpliva do konc. 137 μM	/

5 ZAKLJUČKI

1. Toksičnost APS12-2 in APS3 za miši in podgane je razmeroma nizka; ocenjena LD_{50} za APS12-2 je 11,5 mg/kg, za APS3 pa 7,25 mg/kg.

2. Za razliko od APS3 je APS12-2 povzročil koncentracijsko odvisno hemolizo podganjih eritrocitov *in vitro* in *in vivo*, kar je verjetno vzrok za pojav hiperkaliemije *in vivo*.

3. Po parenteralni aplikaciji APS12-2 so podgane poginile zaradi srčnega zastoja, ki je verjetno posledica hiperkaliemije zaradi hemolize eritrocitov. Toksičnost APS3 je najverjetneje posledica antagonističnega delovanja na mišični tip nAChRs.

4. APS12-2 in APS3 povzročita na izoliranih mišjih diafragmah *in vitro* od koncentracije odvisno zaviranje posredno izzvane izometrične mišične kontrakcije, kar je verjetno posledica blokade nAChRs. Prehodne potenciacije mišične kontrakcije, ki bi bila posledica antiacetilholinesterazne aktivnosti proučevanih substanc, nismo ugotovili, iz česar lahko sklepamo, da drugi mehanizmi delovanja APS12-2 in APS3 (na primer zaviranje nAChRs) zamaskirajo učinke, ki so posledica antiacetilholinesterazne aktivnosti.

5. APS12-2 in APS3 zmanjšata amplitudo MPMP in PMP, kar nakazuje na blokado nAChRs.

6. APS12-2 in APS3 sta antagonista nAChRs, saj povzročita koncentracijsko odvisno prekinitev ionskega toka, povzročenega z ACh, skozi mišični tip $\alpha 1_2\beta 1\gamma\delta$ nAChRs električnega skata (*Torpedo marmorata*), izraženih v membrani oocitov žabe *Xenopus laevis*.

7. Za razliko od APS3, ki na žilne obročke nima vpliva, APS12-2 povzroči kontrakcijo žilnih obročkov, najverjetneje zaradi vstopa Ca^{2+} skozi od napetosti odvisne kalcijeve kanalčke, saj ob prisotnost selektivnega zaviralca le-teh APS12-2 ne povzroči kontrakcije.

8. APS12-2 v podobnih koncentracijah, kot se nahaja v plazmi podgan po intravenski aplikaciji letalnega odmerka, povzroči skrčenje žilnih obročkov, zato sklepamo, da je to eden izmed mehanizmov kardiotoksičnega delovanja APS12-2.

9. Za razliko od APS3 je APS12-2 hemolitično aktiven, saj je hemolitična aktivnost sintetičnih analogov poli-APS odvisna od stopnje polimerizacije. Na njegovo aktivnost vplivajo lastnosti okolja in sestava lipidnih membran, saj aktivnost APS12-2 narašča s padajočo ionsko jakostjo in padanjem pH-raztopine, najbolj aktivno pa APS12-2 deluje na lipidne membrane, ki so v trdni urejeni fazi.

6 POVZETEK

Poli-APS spadajo med najpogosteje proučevane alkilpiridinijeve spojine. Izolirane so iz vodnega ekstrakta sredozemske morske spužve *Reniera sarai*. Spužvi omogočajo nemoteno rast in razvoj ter jo varujejo pred zunanjim okoljem. Poli-APS so amfifilne snovi, sestavljene iz hidrofilnih piridinijevih obročev in hidrofobnih alkilnih verig (Sepčić in sod., 1997a; Sepčić in sod. 1997b). So visoko polimerizirane snovi s številnimi biološkimi aktivnostmi. Njihova biološka aktivnost je odvisna od stopnje polimerizacije, narave nasprotnega iona in dolžine alkilne verige (Mancini in sod., 2004; Turk in sod., 2008). Poli-APS imajo hemolitično, protimikrobno in protivegetativno aktivnost, uporabne so lahko kot orodje za transfekcijo celic, zavirajo encim AChE in rast celic pljučnega raka (Turk in sod., 2008; Zovko in sod., 2012).

Zaradi naštetih bioloških aktivnosti so naravni poli-APS zanimivi za potencialno uporabo v medicini, farmacevtski in kemijski industriji. Ker je njihova količina pridobljena iz naravnega ekstrakta majhna, so razvili nove metode za sintezo sintetičnih analogov poli-APS, ki se med seboj razlikujejo po stopnji polimerizacije (od 3 do 12 C atomov), dolžini alkilne verige in naravi nasprotnih ionov (kloridni ali bromidni ion; Houssen in sod., 2010; Zovko in sod., 2012). Sinteza strukturno točno opredeljenih analogov poli-APS z različnimi kemijskimi lastnostmi in z različno stopnjo polimerizacije omogoča nadzor in uravnavanje stopnje njihovih bioloških aktivnosti.

V doktorski disertaciji smo proučevali patofiziološko delovanje dveh sintetičnih analogov poli-APS. APS12-2 je sintetični analog z večjo stopnjo polimerizacije in daljšo alkilno verigo, kot nasprotni ion ima vezan bromid. Deluje kot nekompetitivni zaviralec AChE in je močno hemolitičen. APS3 pa je manjši in krajši analog, v svoji strukturi ima vezan kloridni ion, je kompetitivni zaviralec AChE in ni hemolitičen. Zaradi potencialne uporabe sintetičnih analogov poli-APS v medicini je določitev toksičnosti in patofiziološkega delovanja izbranih sintetičnih analogov zelo pomembna. Z raziskavami smo dobili odgovore na vprašanje, ali imata APS12-2 in APS3 v koncentracijski odvisnosti *in vivo* in *in vitro* morebitne neželene učinke na nivoju organov, tkiv, celic in na molekulskem nivoju ter ali vplivata na vitalne funkcije v sesalskem organizmu. Ugotavljali smo, ali izbrane snovi zavirajo encim AChE tudi v živčno-mišičnem stiku. Z ugotavljanjem molekulskih mehanizmov njihove vezave in interakcije z umetnimi in naravnimi lipidnimi membranami smo lahko dodatno razložili

mehanizme učinkov izbranih sintetičnih analogov na nivoju celic, tkiv in organov ter na nivoju organizma kot integrirane celote.

Pri *in vivo* poskusih smo najprej ocenili srednji letalni odmerek obeh snovi na miškah. Za APS12-2 smo ocenili LD_{50} 11,5 mg/kg, za APS3 pa 7,25 mg/kg. V primerjavi z naravnim poli-APS, ki ima LD_{50}, ocenjeno na podganah, 2,7 mg/kg (Bunc in sod., 2002b), sta APS12-2 in APS3 razmeroma malo toksična. Sledili so poskusi *in vivo* na podganah, kjer smo podganam intravensko aplicirali različne odmerke sintetičnih analogov poli-APS. Ugotovili smo, da so podgane bolj občutljive na izbrana analoga kot miši. APS12-2 je povzročil pogin podgan zaradi srčno-respiratornega zastoja. Srčni zastoj lahko povzroči hiperkaliemija (Emberson in Muir, 1969; Van der Meer in sod., 1986; Parham in sod., 2006), ki nastane zaradi močne hemolitične aktivnosti APS12-2 (Grandič, 2012). Vzrok respiratornega zastoja bi lahko bila stimulacija jukstapulmonalnih kapilarnih receptorjev v pljučnem parenhimu (McCaffrey in Kern, 1980; Willette in sod., 1983). Ti receptorji so mehano-senzitivni, kar pomeni, da jih aktivirajo stanja, kot so pljučni edem, kongestija pljuč in pljučni mikroembolizem (Paintal in sod., 1973), kar smo ugotovili tudi pri podganah, injiciranih z letalnim odmerkom APS12-2. Pri aplikaciji APS ni prišlo do pogina podgan pri odmerkih do 20 mg/kg oziroma kumulativnih odmerkih do 60 mg/kg. Pojavile so se le prehodne spremembe v tlaku. Zaradi odsotnosti hemolitične aktivnosti APS3 se koncentracija kalija v serumu po pričakovanjih ni bistveno povišala.

Glede na strukturo in antiacetilholinesterazno aktivnost izbranih sintetičnih analogov smo pričakovali učinke APS3 in APS12-2 na živčno-mišični prenos. Proučevali smo vpliv obeh analogov na kontrakcijo mišic na izoliranem preparatu mišje diafragme. Da bi natančneje določili mehanizem delovanja proučevanih sintetičnih analogov poli-APS, smo z mikroelektrodno tehniko na izoliranih mišjih diafragmah proučevali vpliv APS3 in APS12-2 na MP, MPMP in PMP. Poleg tega smo preverili tudi neposreden učinek obeh proučevanih snovi na nAChR, izražene v membrani žabjih oocitov. Ugotovili smo, da oba analoga v odvisnosti od koncentracije zmanjšata moč posredno izzvane izometrične mišične kontrakcije, kar je najverjetneje posledica zaviranja nAChRs. Pokazali smo tudi, da APS12-2 in APS3 v odvisnosti od koncentracije zmanjšata amplitudo MPMP in PMP, kar nakazuje na postsinaptični učinek obeh izbranih analogov na nAChRs, saj je znižanje amplitude postsinaptičnih potencialov proporcionalno številu blokiranih nAChRs. Da bi potrdili možen

neposredni zaviralni učinek APS12-2 in APS3 na mišični tip nAChRs na živčno-mišičnem stiku, smo izvedli poskuse na oocitih žabe vrste *Xenopus laevis*. Oociti so na svoji površini izražali mišični tip nAChR, pridobljenih iz električnega skata *Torpedo marmorata*. Ugotovili smo, da APS12-2 (IC_{50} = 0,0005 μM) in APS3 (IC_{50} = 0,19 μM) učinkovito zavirata ionski tok skozi mišični tip nAChRs, izraženih v membrani oocitov, kar tudi nakazuje na blokado nAChRs.

Z namenom proučevanja učinkov APS12-2 in APS3 ter boljše razlage specifičnih mehanizmov kardiotoksičnih učinkov APS12-2 smo izvedli še *in vitro* poskuse na izoliranih preparatih prašičjih koronarnih arterij. Ugotovili smo, da APS3 za razliko od APS12-2 v koncentracijah do 137 μM nima učinka na žilne obročke. APS12-2 je v odvisnosti od koncentracije povzročil kontrakcijo žilnih obročkov. S tretiranjem žilnih obročkov z različnimi zaviralci gladkomišične kontrakcije smo dodatno proučili mehanizme delovanja APS12-2 na žilne obročke. Ugotovili smo, da neselektivni zaviralec kalcijevih kanalčkov lantanov klorid (Hogestatt in Anderson, 1984; Kasai in Neher, 1992) in selektivni zaviralec kalcijevih kanalčkov verapamil (Triggle, 1999) popolnoma preprečita kontrakcijo koronarnih žilnih obročkov, povzročeno z APS12-2. Ti rezultati kažejo, da APS12-2 v odvisnosti od koncentracije povzroči krčenje gladkih mišic v krvnih žilah zaradi povečanega vdora Ca^{2+} v gladke mišične celice prek napetostno odvisnih kanalčkov Ca^{2+}. S temi rezultati smo prvič pokazali, da APS12-2 povzroči od koncentracije odvisno kontrakcijo preparatov žilnih obročkov, kar bi lahko poleg hiperkaliemije prispevalo h kardiotoksičnim učinkom APS12-2. Pomembno je omeniti tudi to, da je največja uporabljena koncentracija APS12-2 v poskusih na žilnih obročkih primerljiva s (izračunano) koncentracijo APS12-2, ki se pojavi v serumu podgan, tretiranih s srednjim letalnim odmerkom APS12-2.

7 SUMMARY

Polymeric 3-alkylpyridinium salts (poly-APS) are one of the most studied alkylpyridinium compounds. They were isolated from the water extract of the Mediterranean marine sponge *Reniera sarai*. They enable undisturbed growth and protect the sponge from predatory environment. Poly-APS are amphipatic molecules expressing hydrophilic cationic pyridinium headgroups and hydrophobic alkyl chains (Sepčić et al., 1997a; Sepčić et al., 1997b). They are highly polymerized and exert a wide range of biologic activities. Their biologic activity is correlated to the polymerization degree, length of alkyl chains, and the nature of their counter ions (Mancini et al., 2004; Turk et al., 2008). Poly-APS exert hemolytic, antimicrobial, antifouling, and anti-acetycholinesterase (anti-AChE) activity. They can be used as transfection tools, and are cytotoxic for lung cancer cells (Turk et al., 2008; Zovko et al., 2012).

Because of wide range of biologic activities, poly-APS are possible candidates for use in medicine, pharmacy and industry. Due to the small quantities of natural poly-APS, new methods for synthesis of poly-APS analogues were developed. Synthetic analogues differ in degree of polymerization (from 3 to 12 C atoms), length of the alkyl chains, and in counter ions (chloride or bromide ion) present in their structures (Houssen et al., 2010; Zovko et al., 2012). Synthesis of structurally defined analogues with different chemical properties and degrees of polymerization enables the control of biologic activities.

In the present work we studied pathophysiological actions of two synthetic poly-APS analogues. APS12-2 is an analogue with higher degree of polymerization and longer alkyl chain, bearing a bromine counter ion. It is very hemolytic and acts as a non-competitive AChE inhibitor. APS3 is smaller and shorter, with a chloride counter ion. APS3 is non-hemolytic and acts as a competitive AChE inhibitor. Because of potential use of these two analogues in medicine, the assessment of their toxicity and pathophysiologic actions is very important. With *in vitro* and *in vivo* experiments we obtained significant data on possible adverse effects of APS12-2 and APS3 in organs, tissues, cells, as well as on the molecular level. We also obtained information about their effects on vital functions in mammal organism in concentration-dependent manner. We also studied whether APS12-2 and APS3 inhibit AChE in neuro-muscular junction. Additionally, we explained mechanisms of effects of APS12-2- and APS3 at cellular, tissue and organ level, and at the level of the whole

organism by studying molecular mechanisms of their interactions with natural and artificial lipid membranes.

The estimated median lethal dose for APS12-2 and APS3 in mice was 11.5 and 7.25 mg/kg, respectively. In comparison with natural poly-APS, with estimated an LD_{50} in rats of 2.7 mg/kg (Bunc et al., 2002b), the toxicity of APS12-2 and APS3 is relatively low. In *in vitro* experiments, where we applied different doses of APS12-2 and APS3 into rats, we found out that the rats are more sensitive than mice to both analogues. In rats, APS12-2 caused death due to the cardiorespiratory arrest. This is most likely the consequence of hyperkalemia (Emberson and Muir, 1969; Van der Meer et al., 1986; Parham et al., 2006), which is the consequence of APS12-2´s hemolytic activity. The reason for respiratory arrest could be a stimulation of jukstapulmonary capillary receptors in lung parenchyma (McCaffrey and Kern, 1980; Willette et al., 1983). Those receptors are mechano-sensitive, which means that they are activated by conditions like pulmonary edema, congestion or pulmonary microembolism (Paintal et al., 1973). APS3 did not cause death of experimental rats in doses up to 20 mg/kg and in cumulative doses up to 60 mg/kg. Only transient changes in arterial blood pressure were observed. The serum potassium concentration was, as we expected, not significantly altered, due to absence of hemolytic activity of APS3.

Based on the structure and anti-AChE activity of APS12-2 and APS3 we expected effects on neuro-muscular transmission. The effect of both analogues on muscular contraction of mouse hemidiaphragm preparations was studied. To better determine the mechanism of action of APS12-2 and APS3 we used microelectrode technique on mouse hemidiaphragm preparations to study the effects of APS12-2 and APS3 on resting membrane potential (RP), miniature endplate potential (MEPP) and endplate potential (EPP). We also studied direct influence of the analogues on nAChRs expressed on *Xenopus* oocytes. In a concentration-dependent manner, APS12-2 and APS3 blocked nerve-evoked isometric muscle contraction, most likely due to the inhibition of nAChRs. APS12-2- and APS3 in concentration-dependent manner decrease the amplitude of EPPs and MEPPs, which indicates the postsynaptic action of both analogues on nAChRs. Decrease of the amplitude is proportional to the number of blocked nAChRs. To confirm possible direct effect of APS12-2 and APS3 on muscle type of nAChRs on neuro-muscular junction, experiments on *Xenopus laevis* oocytes in which *Torpedo* ($\alpha 12\beta 1\gamma\delta$) muscle-type nicotinic acetylcholine receptors (nAChRs) had been incorporated,

were performed. APS12-2 (IC_{50} = 0.0005 μM) and APS3 (IC_{50} = 0.19 μM) effectively blocked acetylcholine current through muscle type of nAChRs expressed in oocyte membranes due to block of nAChRs.

In order to study the effects of APS12-2 and APS3, and to better reveal the mechanisms of APS12-2 in cardiovascular effects and to provide more data on mechanistic specificity, experiments were performed on isolated porcine coronary vessels. In contrast to APS3, having no effect, APS12-2 induced contraction of coronary ring preparations in the concentration-dependent manner. For further evaluation of mechanism of action of APS12-2, we pre-treated coronary ring preparations with different muscle contraction inhibitors. The non-selective cation channel blocker, lanthanum chloride (Hogestatt and Anderson, 1984; Kasai and Neher, 1992), and selective antagonist of L-type of voltage dependent calcium channels, verapamil (Triggle, 1999), completely abolished the contraction of coronary rings induced with APS12-2. These results indicate that APS12-2 contracts vascular smooth muscle in a concentration-dependent manner due to the increase of Ca^{2+} influx through the voltage-gated Ca^{2+} channels. Our data show for the first time that APS12-2 induces a concentration-dependent contraction of coronary ring preparations, which may contribute to the cardiotoxic effects of APS12-2 in addition to hyperkalemia. It is worth mentioning that the final maximum concentration of APS12-2 (13.60 μM), producing a significant increase in coronary ring tension *in vitro*, is comparable to the maximal calculated concentration of APS12-2 in the blood (plasma) *in vivo* after injection of one LD_{50}, which produced arrhythmias and cardiorespiratory arrest.

8 ZAHVALA

Zahvaljujem se mentorju prof. dr. Robertu Frangežu za več kot odlično opravljeno mentorstvo, ki je nastanek doktorske disertacije močno olajšalo, za pogovore, posvetovanje in številne še kako koristne nasvete ter za vso podporo in pomoč tudi izven okvirov podiplomskega študija.

Zahvaljujem se somentorici prof. dr. Kristini Sepčić za vso pomoč, podporo in svetovanje pri poskusih, opravljenih na Biotehniški fakulteti, za vse hitre odgovore in preglede tekstov ter za odlično sodelovanje.

Zahvaljujem se predsednici in članoma komisije, prof. dr. Silvesti Kobal, prof. dr. Petru Mačku in prof. dr. Dušanu Šuputu, za pregled doktorske disertacije ter za vse pripombe in napotke, ki so moje delo še izboljšali.

Zahvaljujem se vsem sodelavcem na Inštitutu za fiziologijo, farmakologijo in toksikologijo za vso pomoč pri nastajanju doktorske disertacije. Hvala Katarini Babnik in Boštjanu Drolcu za izvrstno tehnično pomoč.

Zahvaljujem se vsem zaposlenim na Katedri za biokemijo Oddelka za biologijo Biotehniške fakultete v Ljubljani, še posebej Kristini Sepčić, Ireni Pavešič, Katji Ota in Ani Zovko za vso neprecenljivo pomoč pri izvajanju poskusov.

Zahvaljujem se dr. Jordiju Molgóju, dr. Evelyne Benoit, dr. Romulu Araozu in ostalim zaposlenim v Laboratoriju za nevrofiziologijo Inštituta CNRS v Gif-sur-Yvette v Franciji, ki so mi omogočili izvedbo dela poskusov v njihovem laboratoriju.

Zahvaljujem se doc. dr. Alenki Nemec Svete in Aleksandru Jenku iz Klinike za kirurgijo in male živali za biokemijske analize.

Zahvaljujem se prof. dr. Poloni Juntes iz Inštituta za patologijo, sodno in upravno veterinarstvo za histološke analize in za pomoč pri njihovi interpretaciji.

Zahvaljujem se mag. Brigiti Grecs Smole za pregled literature.

Hvala moji družini: mojemu zlatemu možu Iztoku, mami, tatotu, sestri, Oliverju in Cofku za dvigovanje motivacije, kadar je to bilo potrebno.

9 LITERATURA

Adams PR. Drug blockade of open end-plate channels. J Physiol 1976; 260: 531–52.

Albuquerque EX, Alkondon M, Pereira EF et al. Properties of neuronal nicotinic acetylcholine receptors: pharmacological characterization and modulation of synaptic function. J Pharmacol Exp Ther 1997; 280(3): 1117–36.

Almeida E, Diamantino TC, de Sousa O. Marine paints: the particular case of antifouling paints. Progr Org Coating 2007; 59(1): 2–20.

Baier RE. Initial events in microbial film formation. In: Costlow JD, Tipper RC, eds. Marine biodeterioration: an interdisciplinary study. London: E & FN Spon, 1984: 57–62.

Bao G, Randhawa PM, Fletcher EC. Acute blood pressure elevation during repetitive hypocapnic and eucapnic hypoxia in rats. J Appl Physiol 1997; 82(4): 1071–8.

Barak D, Ordentlich A, Bromberg A et al. Allosteric modulation of acetylcholinesterase activity by peripheral ligands involves a conformational transition of the anionic subsite. Biochemistry 1995; 34(47): 15444–52.

Bowman WC. Neuromuscular block. Br J Pharmacol 2006; 147: 277–86.

Bunc M, Sepčić K, Turk T, Šuput D. *In vivo* effects of head-to-tail 3-alkylpiridinium polymers isolated from the marine sponge *Raniera sarai*.
Pflugers Arch 2000; 440 (suppl. 5): 173–4.

Bunc M, Šarc L, Rozman J, Turk T, Sepčić K, Šuput D. Intravascular plug formation induced by poly-APS is the principal mechanism of the toxin's lethality in rats/rat tissues.
Cell Mol Biol Lett 2002a; 7(1): 106–8.

Bunc M, Strupi - Šuput J, Vodovnik A, Šuput D. Toxic effects of head-to-tail 3-alkylpyridinium polymers isolated from the marine sponge *Reniera sarai* in rat.
Toxicon 2002b; 40(7): 843–9.

Castillo J, Katz B. Quantal components of the end-plate potential.
J Physiol 1954; 124(3): 560–73.

Cathieni MM, Taban CH, Sors P, Mastrangelo D. Binding of gold– protein ligand complexes to neurokinin receptors. Visualization with the electron microscope on pig coronary artery and rat portal vein. J Vasc Res 1999; 36: 59–67.

Chelossi E, Mancini I, Sepčić K, Turk T, Faimali M. Comparative antibacterial activity of polymeric 3-alkylpyridinium salts isolated from the Mediterranean sponge *Reniera sarai* and their synthetic analogues. Biomol Eng 2006; 23(6): 317–23.

Cohen-Cory S. The developing synapse: construction and modulation of synaptic structures and circuits. Science 2002; 298: 770–76.

Colquhoun D, Dreyer F, Sheridan RE. The actions of tubocurarine at the frog neuromuscular junction. J Physiol 1979; 293: 247 – 84.

Colquhoun D, Unwin N, Shelley C, Hatton C, Sivilotti L. Nicotinic acetylcholine receptors. In: Abraham D, ed. Burger´s medicinal chemistry. 6th ed. Vol. 2: Drug discovery and drug developement. New York: Willy, 2003: 357–405.

Cummings JL. Cholinesterase inhibitors: A new class of psychotropic compounds.
Am J Psychiatry 2000; 157(1): 4–15.

Cunningham JG. Textbook of veterinary physiology. 4th ed. St. Louis: Saunders Elsevier, 2002: 13.

Dale H. Pharmacology and nerve-endings (Walter Ernest Dixon Memorial Lecture): (Section of Therapeutics and Pharmacology). Proc R Soc Med 1935; 28(3): 319–32.

De Muynck W, Ramirez AM, De Belie N, Verstraete W. Evaluation of strategies to prevent algal fouling on white architectural and cellular concrete.
Int Biodeter Biodegr 2009; 63(6): 679–89.

Dhein S, Van Koppen CJ, Brodde OE. Muscarinic receptors in the mammalian heart. Pharmacol Res 2001; 44(3): 161–82.

Dube SN, Kumar P, Kumar D, Das Gupta S. Route-specific cardiorespiratory and neuromuscular changes following organophosphorous poisoning in rats.
Arch Int Pharmacodyn Ther 1993; 321: 112–22.

Eleršek T, Kosi G, Turk T, Pohleven F, Sepčić K. Influence of polymeric 3-alkylpyridinium salts from the marine sponge *Reniera sarai* on the growth of algae and wood decay fungi. Biofouling 2008; 24: 137–43.

Elmoselhi AB, Grover AK. Endothelin contraction in pig coronary artery: receptor types and Ca(2+)-mobilization. Mol Cell Biochem 1997; 176: 29–33.

Emberson JW, Muir AR. Changes in the ultrastructure of rat myocardium induced by hyperkalaemia. J Anat 1969; 104(3): 411–21.

Fagerlund MJ, Eriksson LI. Current concepts in neuromuscular transmission.
Br J Anaesth 2009; 103(1): 108–114.

Faimali M, Falugi C, Gallus L, Piazza V, Tagliafierro G. Involvement of acetyl choline in settlement of *Balanus amphitrire*. Biofouling 2003a; 19: 213–20.

Faimali M, Sepčić K, Turk T, Geraci S. Non-toxic antifouling activity of polymeric 3-alkylpyridium salts from the Mediterranean sponge *Reniera sarai* (Pulitzer Finali).
Biofoulig 2003b; 19: 47–56.

Fatt P, Katz B. An analysis of the end-plate potential recorded with an intracellular electrode.
J Physiol 1951; 115(3): 320–70.

Feltz A, Trautmann A. Interaction between nerve-related acetylcholine and bath applied agonists at the frog end-plate. J Physiol 1980; 299: 533–52.

Fisher DM. Clinical pharmacology of neuromuscular blocking agents. Am J Health Syst Pharm 1999; 56(11): S4–9.

Geerts H. α7 Nicotinic receptor modulators for cognitive deficits in schizophrenia and Alzheimer's disease. Expert Opin Investig Drugs 2012; 21(1): 59–65.

Ghai B, Makkar JK, Wig J. Neuromuscular monitoring: a review.
J Anaesth Clin Pharmacol 2006; 22(4): 347–356.

Gotti C, Clementi F. Neuronal nicotinic receptors: from structure to pathology.
Prog Neurobiol 2004; 74(6): 363–96.

Grandič M, Sepčić K, Turk T, Juntes P, Frangež R. *In vivo* toxic and lethal cardiovascular effects of a synthetic polymeric 1,3-dodecylpyridinium salt in rodents.
Toxicol Appl Pharmacol 2011; 255(1): 86–93.

Grandič M, Zovko A, Frangež R, Turk T, Sepčić K. Binding and permeabilization of lipid bilayers by natural and synthetic 3-alkylpyridinium polymers.
Bioorg Med Chem 2012; 20(5): 1659–64.

Guyton AC, Hall JE. Textbook of medical physiology. 11th ed. Philadelphia: Elsevier Saunders, 2006: 488–9.

Hammond C. Cellular and molecular neurophysiology. 3rd ed. London: Elsevier, 2008: 160–85.

Hogestatt ED, Andersson KE. Mechanisms behind the biphasic contractile response to potassium depolarization in isolated rat cerebral arteries. J Pharmacol Exp Ther 1984; 228, 187–95.

Hong SJ, Chang CC. Facilitation by 3,4-diaminopyridine of regenerative acetylcholine release from mouse motor nerve. Br J Pharmacol 1990; 101: 793–8.

Hotelier T, Renault L, Cousin X, Negre V, Marchot P, Chatonnet A. ESTHER, the database of the alpha/beta-hydrolase fold superfamily of proteins. Nucleic Acids Res 2004; 32: 145–7.

Houssen WE, Lu Z, Edrada-Ebel RA et al. Chemical synthesis and biological activities of 3-alkyl pyridinium polymeric analogues of marine toxins. J Chem Biol 2010; 3(3): 113–25.

Jin QH, He HY, Shi YF, Lu H, Zhang XJ. Overexpression of acetylcholinesterase inhibited cell proliferation and promoted apoptosis in NRK cells.
Acta Pharmacol Sin 2004; 25(8): 1013–21.

Kappe CO. Controlled microwave heating in modern organic synthesis.
Angew Chem Int Ed Engl 2004; 43(46): 6250–84.

Kassa J, Fusek J. The positive influence of a cholinergic-anticholinergic pretreatment and antidotal treatment on rats poisoned with supralethal doses of soman.
Toxicology 1998; 128: 1–7.

Kasai H, Neher E. Dihydropyridine-sensitive and omega-conotoxin-sensitive calcium channels in a mammalian neuroblastoma-glioma cell line. J Physiol 1992; 448: 161–88.

Katz B, Thesleff S. A study of the desensitization produced by acetylcholine at the motor end-plate. J Physiol 1957a; 138(1): 63–80.

Katz B, Thesleff S. On the factors which determine the amplitude of the miniature end-plate potential. J Physiol 1957b; 137(2): 267–78.

Kawabata N, Nishiguchi M. Antibacterial activity of soluble pyridinium-type polymers. Appl Environ Microbiol 1988; 54(10): 2532–35.

Khan RA, Lemeignan M. Effects of 3,4-diaminopyridine on mechanical and electrical responses of frog single muscle fibres. Acta Pharmacol Toxicol 1983; 52(3): 181–7.

Kitz RJ, Braswell LM, Ginsburg S. On the question: is acetylcholinesterase an allosteric protein? Mol Pharmacol 1970; 6(2): 108–21.

Kleszczyńska H, Bielecki K, Sarapuk J et al. Biological activity of new N-oxides of tertiary amines. Z Naturforsch C 2006; 61(9/10): 715–20.

Kojima T, Tsuda S, Shirasu Y. Non-cholinergic mechanisms underlying the acute lethal effects of P = S type organophosphorus insecticides in rats. J Vet Med Sci 1992; 54: 529–33.

Kondo T, Tomizawa M. Mechanism of hemolysis by cationic surface-active agents. J Pharm Sci 1969; 58(11): 1378–81.

Koss DJ, Hindley KP, David K et al. A comparative study of the actions of alkylpyridinium salts from a marine sponge and related synthetic compounds in rat cultured hippocampal neurones. BMC Pharmacology 2007; 7: e 1–17. http://www.biomedcentral.com/1471-2210/7/1/ (24.11.2012)

Kuriyama K, Kitamura T, Itoh R. Physiological features of visceral smooth muscle cells, with special reference to receptors and ion channels. Physiol Rev 1998; 78: 811–920.

Kuroda K, DeGrado WF. Amphiphilic polymethacrylate derivatives as antimicrobial agents. J Am Chem Soc 2005; 127(12): 4128–29.

Kuwahara M, Chiku K, Shiono T, Tsubone H, Sugano S. ECG changes under hyperkalemia with nephrectomy in the rat. J Electrocardiol 1992; 25(3): 215–9.

Lodish H. Molecular cell biology. 6th ed. New York: W. H. Freeman and Company, 2008.

Madaan P, Tyagi VK. Quaternary pyridinium salts: a review.
J Oleo Sci 2008; 57(4): 197–215.

Malovrh P, Sepčić K, Turk T, Maček P. Characterization of haemolytic activity of 3-alkylpyridinium polymers from the marine sponge *Reniera sarai*.
Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1999; 124(2): 221–6.

Mancini I, Sicurelli A, Guella G, Turk T, Maček P, Sepčić K. Synthesis and bioactivity of linear oligomers related to polymeric alkylpyridinium metabolites from the Mediterranean sponge *Reniera sarai*. Org Biomol Chem 2004; 2(9): 1368–75.

Martyn JA, Fagerlund MJ, Eriksson LI. Basic principles of neuromuscular transmission. Anaesthesia 2009; 64(1): 1–9.

McCaffrey TV, Kern EB. Laryngeal regulation of airway resistance. II. Pulmonary receptor reflexes. Ann Otol Rhinol Laryngol 1980; 89(5): 462–66.

McClelland D, Evans RM, Abidin I et al. Irreversible and reversible pore formation by polymeric alkylpyridinium salts (poly-APS) from the sponge *Reniera sarai*.
Br J Pharmacol 2003; 139(8): 1399–1408.

McConnell HM, Vrljic M. Liquid-liquid immiscibility in membranes.
Annu Rev Biophys Biomol Struct 2003; 32: 469–492.

Mehta M, Adem A, Kahlon MS, Sabbagh MN. The nicotinic acetylcholine receptor: smoking and Alzheimer's disease revisited. Front Biosci 2012; 1(4): 169–80.

Molgó J, Lundh H, Thesleff S. Potency of 3,4-diaminopyridine and 4-aminopyridine on mammalian neuromuscular transmission and the effect of pH changes.
Eur J Pharmacol 1980; 61: 25–34.

Molgó J, Thesleff S. Electronic properties of motor nerve terminals.
Acta Physiol Scand 1982;114(2): 271–75.

Moni RW, Parsons PG, Quinn RJ, Willis RJ. Critical micelle concentration and hemolytic activity - a correlation suggested by the marine sterol, halistanol trisulfate.
Biochem Biophys Res Commun 1992; 182(1): 115–20.

Montague BT, Ouellette JR, Buller GK. Retrospective review of the frequency of ECG changes in hyperkalemia. Clin J Am Soc Nephrol 2008; 3(2): 324–30.

Mooser G, Sigman DS. Ligand binding properties of acetylcholinesterase determined with fluorescent probes. Biochemistry 1974; 13(11): 2299–307.

Nguyen-Huu T, Dobbertin A, Barbier J et al. Cholinesterases and the resistance of the mouse diaphragm to the effect of tubocurarine. Anesthesiology 2005; 103: 788–95.

Ohno K. Genetic defects and disorders at the neuromuscular junction.
Brain Nerve 2011; 63(7): 669–78.

Okonjo KO, Kuhlmann J, Maelicke A. A second pathway of activation of the Torpedo acetylcholine receptor channel. Eur J Biochem 1991; 200: 671–7.

Olivera-Bravo S, Ivorra I, Morales A. The acetylcholinesterase inhibitor BW284c51 is a potent blocker of Torpedo nicotinic AchRs incorporated into the Xenopus oocyte membrane. Br J Pharmacol 2005;144(1): 88–97.

Paintal AS, Damodaran VN, Guz A. Mechanism of excitation of type J receptors. Acta Neurobiol Exp (Wars) 1973; 33(1): 15–9.

Paleari L, Trombino S, Falugi C et al. Marine sponge-derived polymeric alkylpyridinium salts as a novel tumor chemotherapeutic targeting the cholinergic system in lung tumors.
Int J Oncol 2006; 29(6): 1381–8.

Parham WA, Mehdirad AA, Biermann KM, Fredman CS. Hyperkalemia revisited.
Tex Heart Inst J 2006; 33(1): 40–7.

Pascual JM, Karlin A. Delimiting the binding site for quaternary ammonium lidocaine derivatives in the acetylcholine receptor channel. J Gen Physiol 1998; 112: 611–21.

Prinz H. Hill coefficients, dose-response curves and allosteric mechanisms.

J Chem Biol 2009; 3: 37–44.

Qian PY, Dobretsov S, Dahms HU, Pawlik J. Antifouling activity and microbial diversity of two congeneric sponges *Callyspongia* spp. From Hong Kong and the Bahamas.
Marine Ecology Progress Series 2006; 324: 151–65.

Rich MM. The control of neuromuscular transmission in health and disease.
Neuroscientist 2006; 12(2): 134–42.

Ridtitid W, Rattanaprom W, Thaina P, Chittrakarn S, Sunbhanich M. Neuromuscular blocking activity of methanolic extract of *Piper sarmentosum* leaves in the rat phrenic nerve-hemidiaphragm preparation. J Ethnopharmacol 1998; 6: 135–42.

Ros E, Aleu J, Gomez de Aranda I et al. Effects of bis(7)-tacrine on spontaneous synaptic activity and on the nicotinic ACh receptor of Torpedo electric organ.
J Neurophysiol 2001; 86(1): 183–189.

Rozman KB, Araoz R, Sepčić K, Molgo J, Šuput D. Parazoanthoxanthin A blocks Torpedo nicotinic acetylcholine receptors. Chem Biol Interact 2010; 187: 384–87.

Sarapuk J, Kleszczyńska H, Rózycka-Roszak B. The role of counterions in the interaction of bifunctional surface active compounds with model membranes.
Biochem Mol Biol Int 1998; 44(6): 1105–10.

Sarapuk J, Kleszczyńska H, Pernak J, Kalewska J, Rózycka-Roszak B. Influence of counterions on the interaction of pyridinium salts with model membranes.
Z Naturforsch C 1999; 54(11): 952–955.

Sawyer N, Mundy R. Autonomic responses in the mouse. Pharmacologist 1970; 12: 246.

Sepčić K, Batista U, Vacelet J, Maček P, Turk T. Biological activities of aqueous extracts from marine sponges and cytotoxic effects of 3-alkylpyridinium polymers from *Reniera sarai*. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1997a; 117(1): 47–53.

Sepčić K, Guella G, Mancini I et al. Characterization of anticholinesterase-active 3-alkylpyridinium polymers from the marine sponge *Reniera sarai* in aqueous solutions. J Nat Prod 1997b; 60(10): 991–9.

Sepčić K, Marcel V, Klaebe A, Turk T, Šuput D, Fournier D. Inhibition of acetylcholinesterase by an alkylpyridinium polymer from the marine sponge *Reniera sarai*. Biochim Biophys Acta 1998; 1387: 217–25.

Sepčić K, Muševič I, Lahajnar G, Turk T, Maček P. AFM imaging of surface adsorbed polymeric 3-alkylpyridinium salts from the marine sponge *Reniera sarai*.
Int J Biol Macromol 1999; 26(5): 353–56.

Sepčić K, Turk T. 3-Alkylpyridinium compounds as potential non-toxic antifouling agents. Prog Mol Subcell Biol 2006; 42: 105–24.

Sherby SM, Eldefrawi AT, Albuquerque EX, Eldefrawi ME. Comparison of the actions of carbamate anticholinesterases on the nicotinic acetylcholine receptor.
Mol Pharmacol 1985; 27: 343–8.

Shirai A, Sumitomo T, Yoshida M, et al. Synthesis and biological properties of gemini quaternary ammonium compounds, 5,5'-[2,2'-(alpha,omega-polymethylnedicarbonyldioxy)diethyl]bis-(3-alkyl-4-methylthiazolium iodide) and 5,5'-[2,2'-(p-phenylenedicarbonyldioxy)diethyl]bis(3-alkyl-4-methylthiazolium bromide). Chem Pharm Bull (Tokyo) 2006; 54(5): 639–45.

Sirs JA. The flow of human blood through capillary tubes. J Physiol 1991; 442: 569–83.

Sussman JL, Harel M, Frolow F et al. Atomic structure of acetylcholinesterase from *Torpedo californica*: A prototypic acetylcholine binding protein. Science 1991; 253: 872–9.

Šentjurc M, Štalc A, Zupancic AO. On the location of active serines of membrane acetylcholinesterase studied by the ESR method. Biochim Biophys Acta 1976; 438(1): 131–7.

Šuput D, Frangež R, Bunc M. Cardiovascular effects of equinatoxin III from the sea anemone *Actinia equina (L.)*. Toxicon 2001; 9: 1421–27.

Taylor P. Agents acting at the neuromuscular junction and autonomic ganglia. In: Goodman Gilman A, Hardman JG, Limbird LE, eds. Goodman and Gilman`s the pharmacological basis of therapeutics, 10th ed. New York: McGraw Hill, 2001: 193–206.

Thorsteinsson T, Masson M, Kristinsson KG, Hjalmarsdottir MA, Hilmarsson H, Loftsson T. Soft Antimicrobial Agents: Synthesis and Activity of Labile Environmentally Friendly Long Chain Quaternary Ammonium Compounds. J Med Chem 2003; 46(19): 4173–81.

Triggle DJ. The pharmacology of ion channels: with particular reference to voltage-gated Ca2+ channels. Eur J Pharmacol 1999; 375(1-3): 311–25.

Tsoukatou M, Hellio C, Vagias C, Harvala C, Roussis V. Chemical defense and antifouling activity of three mediterranean sponges of the genus *Ircinia*.
Z Naturforsch C 2002; 57: 161–71.

Tucker SJ, McClelland D, Jaspars M, Sepčić K, MacEwan DJ, Scott RH. The influence of alkyl pyridinium sponge toxins on membrane properties, cytotoxicity, transfection and protein expression in mammalian cells. Biochim Biophys Acta 2003; 1614(2): 171–81.

Turk T, Sepčić K, Mancini I, Guella G. 3-akylpyridinium and 3-alkylpyridine compounds from marine sponges, their synthesis, biological activities and potential use. In: Atta-ur-Rahman, eds. Studies in natural products chemistry. Vol. 35 (Part O). Bioactive natural products. Amsterdam: Elsevier, 2008: 355–97.

Vallejo YF, Buisson B, Bertrand D, Green WN. Chronic nicotine exposure upregulates nicotinic receptors by a novel mechanism. J Neurosci 2005; 25(23): 5563–72.

Van der Kloot W, Balezina OP, Molgó J, Naves LA. 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 1994; 428(2): 114–26.

Van der Meer C, Valkenburg PW, Snijders PM. Studies on hyperkalemia as a cause of death in intestinal ischemia shock in rats. Circ Shock 1986; 19(3): 329–45.

Van Lunteren E, Moyer M, Pollarine J. Long-lasting *in vivo* inotropic effects of the K(+) channel blocker 3,4-diaminopyridine during fatigue-inducing stimulation.
Muscle Nerve 2008; 38(6): 1616–22.

Wahl M. Marine epibiosis. Fouling and antifouling: some basic aspects.
Mar Ecol Prog Ser1989; 58: 175–89.

Wainwright M, Crossley KB. Photosensitising agents—circumventing resistance and breaking down biofilms. Int Biodeter Biodegrad 2004; 53: 119–26.

Wang Y, Ge X, Xu XF, Wang XY. Acetylcholinesterase inhibitor is a potentially useful therapeutic agent for nicotine-induced periodontal disease.
Med Hypotheses 2009; 74(4): 604–5.

Wellstein A, Pitschner HF. Complex dose-response curves of atropine in man explained by different functions of M1- and M2-cholinoceptors.
Naunyn Schmiedebergs Arch Pharmacol 1988; 338(1): 19–27.

Willette RN, Barcas PP, Krieger AJ, Sapru HN. Pulmonary resistance and compliance changes evoked by pulmonary opiate receptor stimulation.
Eur J Pharmacol 1983; 91(2-3): 181–8.

Wood SJ, Slater CR. Safety factor at the neuromuscular junction.
Prog Neurobiol 2001; 64(4): 393–429.

Wong MS, Vanhoutte PM. COX-mediated endothelium-dependent contractions: from the past to recent discoveries. Acta Pharmacol Sin 2010; 31: 1095–102.

Ye W, Gong X, Xie J et al. AChE deficiency or inhibition decreases apoptosis and p53 expression and protects renal function after ischemia/reperfusion.
Apoptosis 2010; 15(4): 474–87.

Zarif L, Riess JG, Pucci B, Pavia AA. Biocompatibility of alkyl and perfluoroalkyl telomeric surfactants derived from THAM.
Biomater Artif Cells Immobilization Biotechnol 1993; 21(5): 597–608.

Zaslavsky BY, Ossipov NN, Rogozhin SV. Action of surface-active substances on biological membranes III. Comparison of hemolytic activity of ionic and nonionic surfactants.
Biochim Biophys acta 1978; 510(1): 151–9.

Zimmerman G, Soreq H. Termination and beyond: acetylcholinesterase as a modulator of synaptic transmission. Cell Tissue Res 2006; 326(2): 655–69.

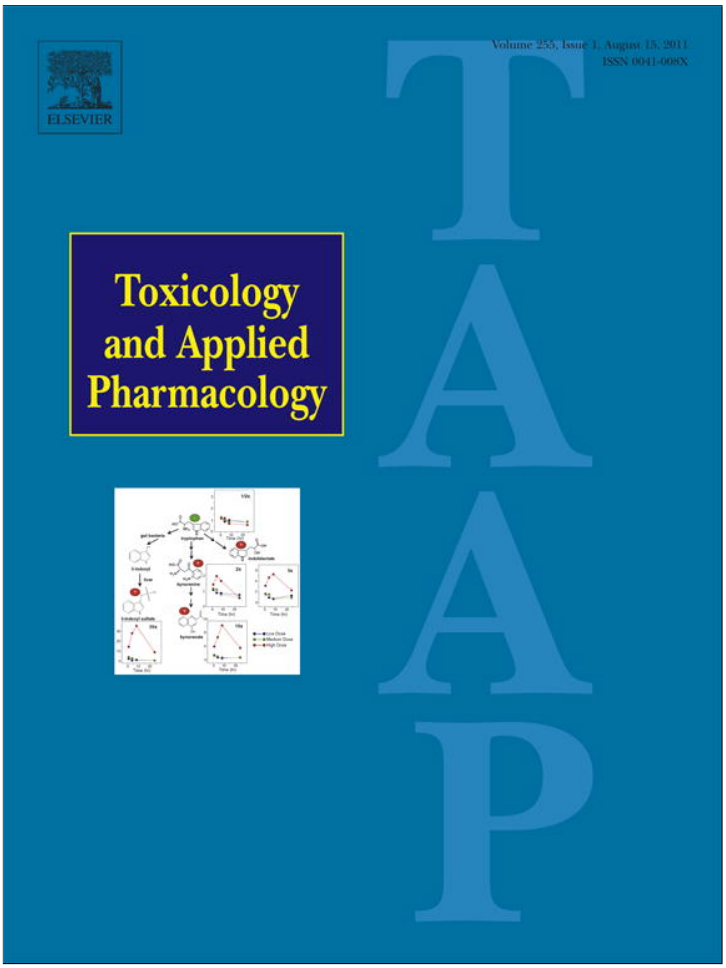
Zovko A. Biološke aktivnosti sintetičnih alkilpiridinijevih polimerov. Ljubljana: Univerza v Ljubljani, Biotehniška fakulteta, 2012. Doktorska disertacija.

Zovko A, Vaukner Gabrič M, Sepčić K et al. Antifungal and antibacterial activity of 3-alkylpyridinium polymeric analogues of marine toxins.
Int Biodeter Biodegrad 2012; 68: 71–7.

Žužek MC, Maček P, Sepčić K, Cestnik V, Frangež R. Toxic and lethal effects of ostreolysin, a cytolytic protein from edible oyster mushroom (*Pleurotus ostreatus*), in rodents.
Toxicon 2006; 48: 264–71.

10 PRILOGE

PRILOGA A: Objavljeni znanstveni članki



Toxicology and Applied Pharmacology 255 (2011) 86–93

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

In vivo toxic and lethal cardiovascular effects of a synthetic polymeric 1,3-dodecylpyridinium salt in rodents

Marjana Grandič [a], Kristina Sepčić [b], Tom Turk [b], Polona Juntes [c], Robert Frangež [a,*]

[a] Institute of Physiology, Pharmacology and Toxicology, 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] Institute of Pathology, Forensic and Administrative Veterinary Medicine, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 9 February 2011
Revised 23 May 2011
Accepted 2 June 2011
Available online 17 June 2011

Keywords:
APS12-2
Cardiorespiratory effects
Hemolysis
LD_{50}
Mice
Rats

ABSTRACT

APS12-2 is one in a series of synthetic analogs of the polymeric alkylpyridinium salts isolated from the marine sponge *Reniera sarai*. As it is a potential candidate for treating non small cell lung cancer (NSCLC), we have studied its possible toxic and lethal effects *in vivo*. The median lethal dose (LD_{50}) of APS12-2 in mice was determined to be 11.5 mg/kg. Electrocardiograms, arterial blood pressure and respiratory activity were recorded under general anesthesia in untreated, pharmacologically vagotomized and artificially ventilated rats injected with APS12-2. In one group, the *in vivo* effects of APS12-2 were studied on nerve-evoked muscle contraction. Administration of APS12-2 at a dose of 8 mg/kg caused a progressive reduction of arterial blood pressure to a mid-circulatory value, accompanied by bradycardia, myocardial ischemia, ventricular extrasystoles, and second degree atrio-ventricular block. Similar electrocardiogram and arterial blood pressure changes caused by APS12-2 (8 mg/kg) were observed in animals pretreated with atropine and in artificially ventilated animals, indicating that hypoxia and cholinergic effects do not play a crucial role in the toxicity of APS12-2. Application of APS12-2 at sublethal doses (4 and 5.5 mg/kg) caused a decrease of arterial blood pressure, followed by an increase slightly above control values. We found that APS12-2 causes lysis of rat erythrocytes *in vitro*, therefore it is reasonable to expect the same effect *in vivo*. Indeed, hyperkalemia was observed in the blood of experimental animals. Hyperkalemia probably plays an important role in APS12-2 cardiotoxicity since no evident changes in histopathology of the heart were found. However, acute lesions were observed in the pulmonary vessels of rats after application of 8 mg/kg APS12-2. Predominant effects were dilation of interalveolar blood vessels and lysis of aggregated erythrocytes within their lumina.

Introduction

3-alkylpyridiniums and 3-alkylpyridines with various degrees of polymerization are a group of biologically active compounds found in several marine sponges of the order Haplosclerida (Almeida and Berlinck, 1997; Sepčić, 2000; Sepčić and Turk, 2006; Turk et al., 2008). Among them, the water-soluble polymeric 3-alkylpyridinium salts (poly-APS), isolated from crude extracts of the Mediterranean marine sponge *Reniera sarai* (Sepčić et al., 1997), show the highest degree of polymerization. They were found to be a mixture of two polymers. MALDI TOF analysis revealed a major peak of molecules with molecular weight of 5520 Da and a minor peak of molecules with molecular weight of 18,900 Da, corresponding to 29–30 and 99–100 N-butyl-3-butyl pyridinium monomers, respectively (Sepčić et al., 1997). However, latest MALDI TOF experiments revealed only one population of poly-APS molecules centered around 5520 Da (Mancini and Jaspars, unpublished). Poly-APS also exert the broadest spectrum of biological activities, including hemolysis and cytotoxicity, strong inhibition of acetylcholinesterase (AChE), antimicrobial effects against marine bacteria and non-toxic antifouling properties against representatives of marine zoo- and phytoplankton (reviewed in Turk et al., 2008). In particular, their ability to form transient pores in biological membranes can be used for stable transfection of various mammalian cells with heterologous DNA, and has a potential in gene therapy (Tucker et al., 2003). Finally, poly-APS exert selective cytotoxicity towards non small cell lung cancer (NSCLC) cells, which are the most common form of lung cancer, and which express α7-nicotinic receptors (Catassi et al., 2008; Egleton et al., 2008; Paleari et al., 2006). The cytotoxic doses of poly-APS against NSCLC cells are in the nanomolar range (Paleari et al., 2006), and are significantly lower than those inducing toxic and lethal effects in experimental animals after intravenous (*i.v.*) administration. In the latter case, toxic effects directly ascribable to interference with cholinergic system are only observed after administration of lower doses (0.7 mg/kg) of poly-APS, while at higher doses (above 1 mg/kg) such effects are masked by

* Corresponding author.
E-mail address: robert.frangez@vf.uni-lj.si (R. Frangež).

0041-008X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2011.06.003

other, more pronounced and faster developing lethal effects of the toxin, such as hemolysis and platelet aggregation. The rat half-lethal dose for poly-APS was estimated to be 2.7 mg/kg (Bunc et al., 2002; reviewed in Turk et al., 2007).

In a view of the possible use of poly-APS based synthetic compounds in the fields of industry (as components of environment-friendly antifouling paints) and medicine (as new transfection or chemotherapeutic agents), several 3-alkylpyridinium oligomers and polymers have recently been synthesized, and their hemolytic, AChE-inhibitory, antibacterial, antifouling, and transfection potencies tested (Faimali et al., 2005; Houssen et al., 2010; Mancini et al., 2004). A couple of large synthetic polymeric 1,3-dodecylpyridinium salts, APS12 and APS12-2, of 12.5 and 14.7 kDa respectively, were shown to be more potent transfecting agents than the natural poly-APS (Houssen et al., 2010), APS12-2 being particularly active. The antitumor potency of these compounds against NSCLC cells is currently being tested, showing promising results and acting in nanomolar to micromolar range (T. Turk and A. Zovko, unpublished results). In a view of their possible use as chemotherapeutics, it is important to evaluate the mechanism of their toxicity *in vivo*. The aim of this work was thus mainly to determine the mechanism of *in vivo* toxicity and lethality of APS12-2 in rodents.

Materials and methods

2.1. Materials. The synthetic polymer 1,3-dodecylpyridinium bromide (APS12-2, Fig. 1) was synthesized from 3-(12-bromododecyl) pyridine using microwave-assisted polymerization, as described in Houssen et al., 2010. Its molecular weight, determined by ESI-MS analysis, was 14.7 kDa. Prior to use, APS12-2 was dissolved in sterile 0.9% saline solution at a concentration of 10 mg/mL.

2.2. Experimental animals. Male Balb/C mice, body weight 22–28 g, were purchased from the animal breeding house (Veterinary faculty, University of Ljubljana, Slovenia). To estimate the approximate LD_{50} of APS12-2 (according to the 3R concept of Russell and Burch) 24 animals were used. For the study of cardiorespiratory effects of APS12-2, 33 male Wistar albino rats, weighing 220–310 g, were purchased from the Medical Faculty, University of Ljubljana. The experiments were approved by the Veterinary administration of the Republic of Slovenia (Permit no. 34401-84/2008/6) and followed the ethical standards.

2.3. Determination of hemolytic activity in vitro. Rat erythrocytes were centrifuged from freshly collected citrated blood, and washed twice with excess 0.9% saline solution and once with 140 mM NaCl, 20 mM Tris–HCl buffer, pH 7.4. Hemolytic activity was measured by a turbidimetric method as described previously by Maček and Lebez (1981). APS12-2 was serially diluted in 140 mM NaCl, 20 mM Tris–HCl buffer, pH 7.4 and 100 μL of the resulting solutions were combined with 100 μL of erythrocyte suspension to give an initial absorbance of 0.5 at 630 nm. Hemolysis was recorded for 30 min as the decrease of apparent absorbance at 630 nm, using a Kinetic Microplate Reader MRX (Dynex Technologies, USA). At the end of the experiment, the time required for 50% hemolysis, t_{50}, was determined. All experiments were performed at 25 °C.

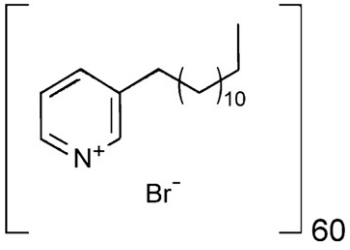


Fig. 1. Chemical structure of 1,3-dodecylpyridinium bromide (APS12-2).

2.4. In vivo experiments. 2.4.1. Lethality determination in mice. APS12-2 was dissolved in sterile 0.9% NaCl. Into the right tail vein of the male mice which received doses of 2, 4, 8, 10, 11, 12, 14 or 16 mg/kg, respectively, 100 μL of solution were injected. With each dose three animals were injected. The control group ($n=3$) received 100 μL of saline solution. Following the 3R principles (Russell and Burch) the LD_{50} was determined according to the reasonably adopted OECD 425 directive (OECD Guideline 425 'Acute oral toxicity; up and down procedure'). The mice were observed for 24 h for signs of intoxication and lethality. LD_{50} was estimated according to the method described by Reed and Muench (1938).

2.4.2. Cardiorespiratory effects in spontaneously breathing anesthetized rats. The anesthetics Chanazine [5,6-dihydro-2-(2,6-xylidino)-4H-1,3-thiazin] (Bayer AG, Leverkusen), at a dose of 15 mg/kg, and Bioketan (ketamin hydrochloride, Parke-Davis, Berlin), at a dose of 100 mg/kg of body weight, were simultaneously injected *i.p.* to introduce general anesthesia in rats. Body temperature was kept at 37 ± 0.5 °C with the aid of a heating blanket. An incision was made in the ventral cervical region, and cannulae were placed into the jugular vein and carotid artery. In the experimental group of animals where artificial ventilation was required, a tracheotubus for artificial ventilation was introduced into the trachea. The right common carotid artery was cannulated with a polyethylene tube and connected to a mechano-electrical transducer previously calibrated with a mercury manometer, in order to measure arterial blood pressure (aBP). The voltage signal from the transducer was amplified and digitized at a sampling rate of 1 kHz using a data acquisition system (Digidata 1440A, Molecular Devices, USA) and corresponding software Axoscope (Molecular Devices, USA). Mean arterial pressure (MAP) was calculated as described (Žužek et al., 2006). The electrocardiogram (ECG) signal was registered using needle electrodes (standard lead II of ECG) connected to the A/D converter noted above, amplified and digitized at 1 kHz sampling frequency. Respiratory activity was recorded using a mechano-electrical transducer placed to the sternum. The voltage signal was amplified by a DC amplifier, digitized and stored on an IBM-compatible PC. APS12-2, dissolved in sterile 0.9% saline solution, was administered *i.v.* through a heparinized (50 I.U./mL saline solution) cannula inserted into the left *vena jugularis* at a dose of 4 mg/kg ($n=7$), 5.5 mg/kg ($n=6$) and 8 mg/kg ($n=6$), and solution left in the dead space of the cannula flushed out with 100 μL of 0.9% saline solution. The same volume of saline solution was injected into control rats ($n=6$).

2.4.3. Experiments in pharmacologically vagotomized anesthetized rats. Atropine sulfate (Belupo, Macedonia), which abolishes cholinergic effects on heart (Sawyer and Mundy, 1970), was injected *i.v.* through the cannulated *v. jugularis* into rats ($n=3$) at a dose of 1 mg/kg, 10 min before the application of 8 mg/kg APS12-2. The compound was injected in the same way and at the same dose as described for spontaneously breathing rats.

2.4.4. Experiments in artificially ventilated anesthetized rats. An Anesthesia EMC WorkStation (Hallowell, USA) was connected to the tracheal tube and rats were artificially ventilated by pure oxygen with the following settings: tidal volume 3–4 mL, frequency of ventilation 54/min, and intrapulmonary pressure <10 mm H_2O. APS12-2 at dose of 8 mg/kg was injected after the artificial ventilation had started, and 15 min after the blood pressure and heart rate had stabilized. Furthermore, three animals which were injected with atropine prior to the administration of APS12-2 were also artificially ventilated.

2.5. Effects on nerve-evoked muscle twitch tension. Under general anesthesia, the left sciatic nerve was prepared in the gluteal region and embraced with bipolar platinum electrodes connected to the stimulator. The distal tendon of the left *tibialis cranialis* muscle was exposed, dissected, and attached to an isometric force transducer (Itis, Slovenia). The load applied to transducers was optimized to obtain maximal contractile response. Supramaximal single stimulation pulses of typically 5–10 V, 0.1 ms, were supplied at a frequency of 0.1 Hz by an S48 stimulator (Grass Instruments, West Warwick, RI, USA). Electrical signals from the force transducer were amplified and digitized at a sampling rate of 1 kHz, using a data acquisition system (Digidata 1440A; Molecular Devices, Sunnyvale, CA, USA). The following successive doses of APS12-2: 0.1, 0.5, 1, 2, 3 and 2 mg/kg, were used in three animals and administered *i.v.* at 5-min intervals.

2.6. Blood chemistry. Rat blood samples (0.8 mL per animal) were collected from the right sublingual vein under deep general anesthesia, 10–15 min after the injection of anesthetics (control). Blood samples were also taken from the *v. cava caudalis*, 5 min after injecting 4, 5.5 or 8 mg/kg APS12-2. Electrolyte composition (Na^+, K^+, Ca^{2+}, Cl^-) and hematocrit were measured on the day of sampling. Serum calcium concentration was measured using an RA-XT analyzer (Bayer, Germany), and sodium, potassium and chloride using an Analyzer Ilyte Na/K/Cl (Instrumentation Laboratory, Lexington, USA). The micro-hematocrit method was used for hematocrit determination. The activity of AChE in blood serum was determined colorimetrically by Ellman's method (Ellman et al., 1961), using acetylthiocholine iodide (0.5 mM) as a substrate in 100 mM potassium phosphate buffer (pH 7.4 at 25 °C). Hydrolysis of the substrate was followed for 5 min on a Kinetic Microplate Reader MRX (Dynex Technologies, U.S.A.) at 412 nm. In the test, 25 μL of blood serum samples were added to a final 200 μL of the reaction mixture. All readings were corrected for appropriate blanks (in which 25 μL of 100 mM potassium phosphate buffer, pH 7.4, were used instead of serum samples), and each measurement was repeated at least three times.

2.7. Tissue collection, storage and histological examination. Heart and lungs were removed from control and APS12-2-treated animals immediately after death. Organs were fixed by direct immersion in 10% neutral buffered formalin and paraffin-embedded after fixation. Control rats were euthanized using carbon dioxide. Following tissue blocks were chosen for histopathology: fixed hearts were cut through the median plane in the direction from cranial to caudal border of the heart, which enable comparable visual estimation of all four chambers and their walls including septum, and two transversal sections of the left caudal lobe of the lung. Histological examination was performed under light microscope on 4 μm thick haematoxylin-eosin (HE) stained tissue sections. HE staining was made according to the standard protocol which includes: deparaffinisation and rehydration (xylene, series of decreasing concentrations of ethanols — 100%, 96%, 70%, distilled water), staining with Mayer's haematoxylin, washing and bluing in running tap water, staining with eosin, dehydration in increasing concentrations of ethanols (70%, 96%, 100%), clearing with xylene and mounting with synthetic resin and cover glass.

2.8. Statistical analysis. Data were analyzed statistically using SigmaPlot for Windows 11.0 (Systat software, inc., Germany). The results are presented as average ± S.E. The Student two-tailed test was used for the statistical analysis. A P value ≤0.05 was considered as statistically significant.

Results

3.1. Hemolytic effect of APS12-2 on rat erythrocytes

APS12-2 showed a considerable hemolytic activity on rat erythrocytes *in vitro* (Fig. 2). The rate of hemolysis ($1/t_{50}$) increased with increasing doses of the compound. In the presence of 1 μg/mL of APS12-2 the rate was 0.035 s^{-1} indicating that rat erythrocytes are 3.4-fold more sensitive to the compound than bovine ones (Houssen et al., 2010). The hemolytic potential of APS12-2 on rat erythrocytes was approximately 100-fold stronger as compared with the natural polymer, poly-APS (Grandič, unpublished results).

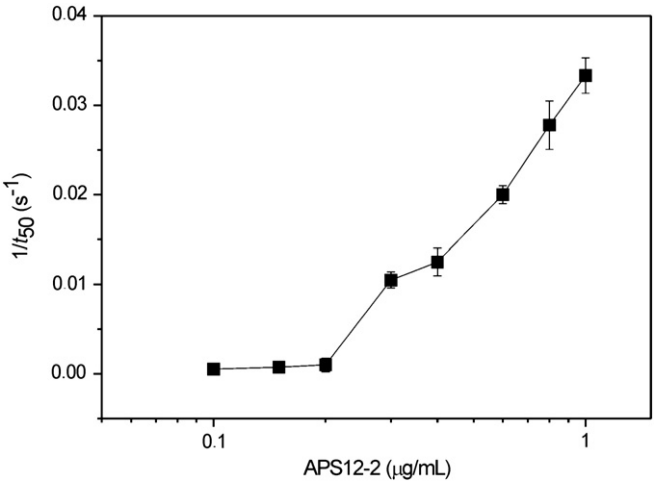


Fig. 2. Hemolytic activity of APS12-2 on rat erythrocytes *in vitro*. The symbols represent the dependence of the reciprocal half time of hemolysis, ($1/t_{50}$), on the dose of APS12-2 (0.1–1 μg/mL). Each point represents the mean of three measurements with corresponding standard errors.

3.2. Effects of APS12-2 on serum acetylcholinesterase activity

In vivo, APS12-2 caused inhibition of serum AChE (sAChE). The activity of this enzyme in serum samples of control rats was 55.0 ± 2.1 dA412/min. In rats injected with 8 mg/kg of APS12-2 the activity was 37.0 ± 3.9 dA412/min, a value significantly lower compared with the controls ($P \leq 0.05$).

3.3. Determination of LD_{50} in mice

Toxicity and LD_{50} were determined by a mouse lethality assay. At doses of 2, 4, and 8 mg/kg of APS12-2 no signs of intoxication were observed. At doses of 10 and 11 mg/kg APS12-2 all mice survived but, shortly after administration (5–10 min), showed only few signs of intoxication such as cyanosis, cessation of movement and, in some cases, movement difficulties. The animals recovered completely within 10–30 min. At a dose of 12 mg/kg APS12-2 or higher, all animals died in less than 10 min, after exhibiting the same signs described above.

3.4. Effect of APS12-2 on arterial blood pressure, ECG and respiratory activities in spontaneously breathing anesthetized rats

Our preliminary experiments showed that rats were more sensitive to APS12-2 than mice. Therefore, a lower dose (8 mg/kg) than that estimated in mice (11.5 mg/kg) was used in rats in order to study lethal effects of APS12-2 *in vivo*. Intravenous application of APS12-2 (8 mg/kg) produced immediate effects, as shown in a typical experiment (Fig. 3). Respiratory activity stopped within 5.9 ± 0.7 s and, at the same time, ECG revealed bradycardia (trace A_2 in Figs. 3, 4). The heart rate significantly decreased from the basal value of 217.2 ± 14.5 beats/min (BPM) to 182.4 ± 21.3 BPM (Fig. 4). The heart rate gradually recovered, and some ventricular extrasystoles appeared as widened QRS complexes (trace A_3 in Fig. 3). Heart rate increased to a maximal value of 307.5 ± 57.0 BPM (Fig. 4), while the respiratory

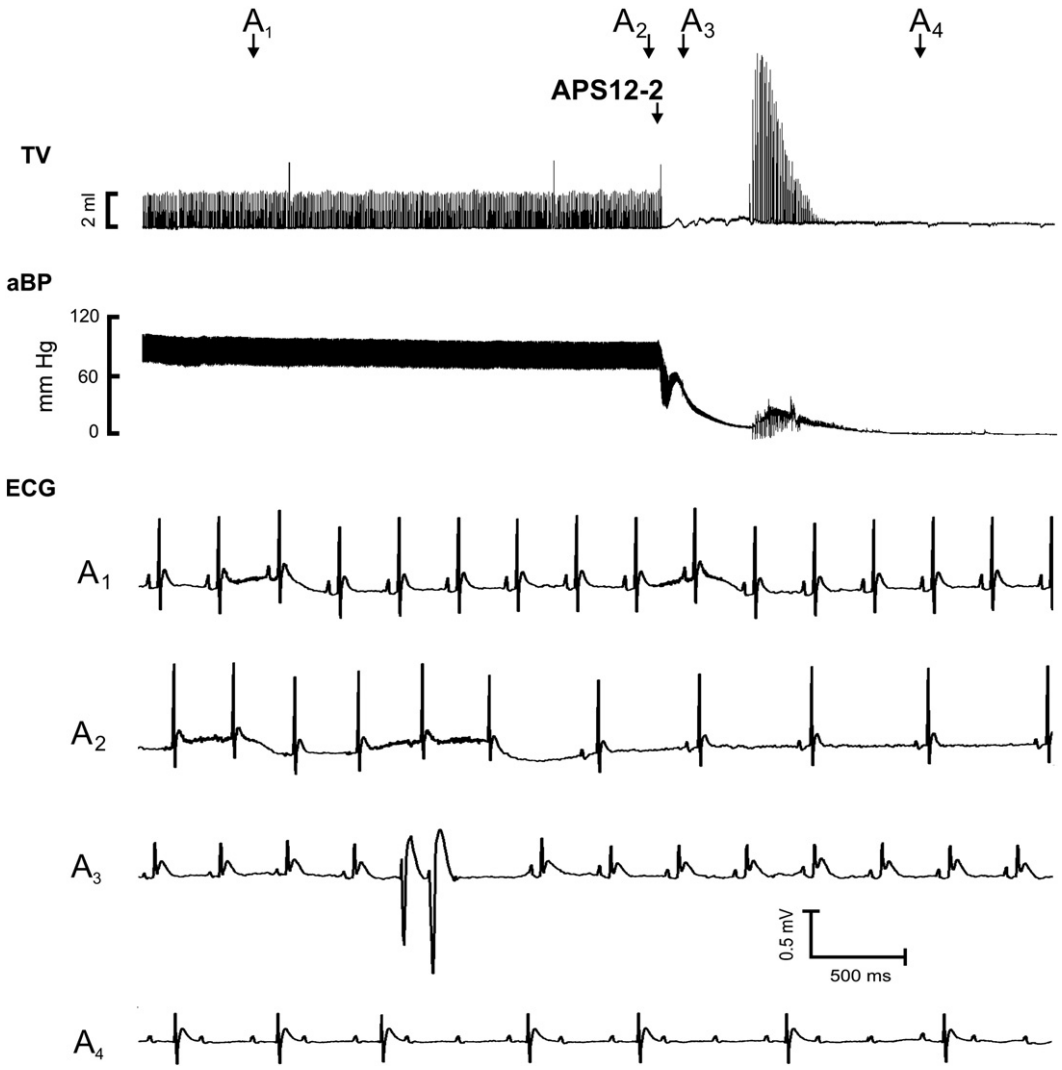


Fig. 3. Effect of an *i.v.* injection of 8 mg/kg APS12-2 on arterial blood pressure, ECG and respiratory activities in anesthetized, spontaneously breathing rats. TV = tidal volume, aBP = coronary arterial blood pressure, ECG = electrocardiogram. The ECG recordings A_1–A_4 represent corresponding time intervals marked during the whole experimental recording. The ECG on outline A_1 represents rhythmic control of ECG activity before APS12-2 administration. Traces in A_2–A_4 represent changes in ECG time course after the injection of 8 mg/kg APS12-2.

activity reappeared. Heart rate then dropped progressively to 173.1 ± 19.6 min^{-1} ($n = 6$).

APS12-2 at lethal dose (8 mg/kg, n = 6) produced progressive, irreversible drop of the aBP (Fig. 4). When aBP fell from 79 ± 4 mm Hg towards a mid-circulatory pressure of 10 ± 4 mm Hg (Fig. 4), ECG revealed an atrioventricular conduction block of the second degree (trace A_4 in Fig. 3).

In order to study sublethal effects of APS12-2 *in vivo*, to better reveal the mechanism of APS12-2 effects and to provide more mechanistic specificity, doses of 4 and 5.5 mg/kg were used. After application of both doses of APS12-2 ECG revealed slight, statistically not significant bradycardia ($P > 0.05$). After injection of 4 mg/kg, the heart rate decreased from basal value of 231.8 ± 9.3 BPM to 206.3 ± 10.0 BPM in 9.2 ± 2.0 s ($n = 7$). Similar effects were observed when 5.5 mg/kg of APS12-2 has been applied. In both cases heart rate gradually recovered.

Immediately after application of sublethal doses of APS12-2 statistically significant decrease of aBP was observed followed by a significant increase and gradual decrease of aBP back to its basal value ($P < 0.05$). After application of 4 mg/kg, MAP decreased from its basal value of 76 ± 0 mm Hg to 51 ± 3 mm Hg in 16.3 ± 1.8 s, followed by a significant increase to 124 ± 6 mm Hg in 127.5 ± 21.6 s. When 5.5 mg/kg of APS12-2 was administered, MAP decreased from its basal value of 86 ± 0 mm Hg to 47 ± 7 mm Hg in 21.8 ± 0.0 s. This was followed by significant increase to 124 ± 14 mm Hg in 93.0 ± 11.4 s after injection Fig. 5.

For a short period after injection of APS12-2 cessation of respiratory activity was evident in 3 out of 7 and 4 out of 6 animals at applied doses of 4 and 5.5 mg/kg, respectively. Respiratory activity reappeared in 6.0 ± 0.0 s and 12.3 ± 4.5 s after it has stopped at dose of 4 and 5.5 mg/kg, respectively.

Injection of 8 mg/kg APS12-2 caused death of all animals ($n = 6$) within minutes. Blood samples collected 5 min after the injection of APS12-2 revealed significantly increased serum potassium concentration ($P \leq 0.05$, $n = 6$), and red-colored appearance of collected serum. These data were accompanied by the slight, though not significant, increase of hematocrit (Table 1).

Blood samples collected from animals treated with sublethal doses of 4 and 5.5 mg/kg revealed significantly increased potassium concentration. In all animals treated with sublethal doses of APS12-2 the serum was reddish and hematocrit was not significantly decreased or increased (Table 1).

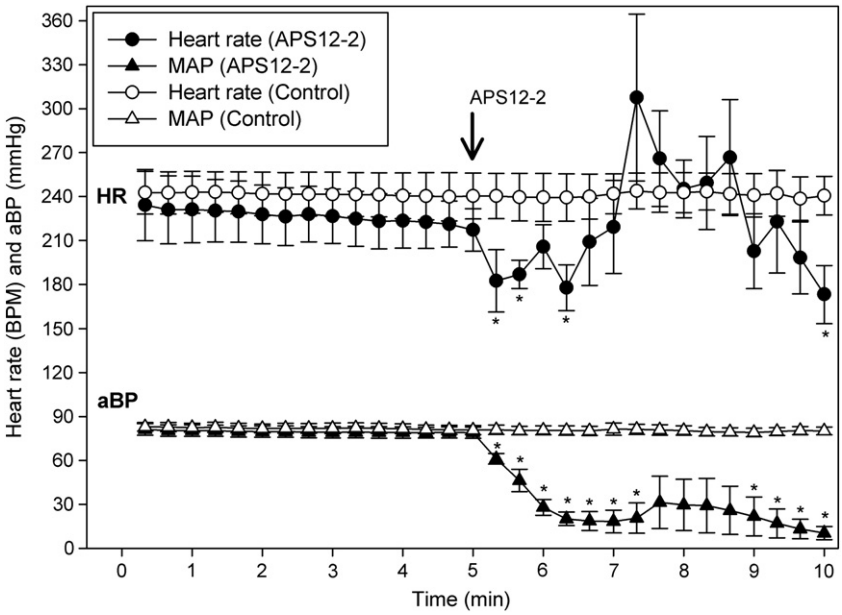


Fig. 4. Effect of APS12-2 (8 mg/kg) on the heart frequency and mean arterial blood pressure (MAP). APS12-2 was administered in one group of rats ($n=6$) while another group (control) was treated only with saline solution ($n=6$). Note the progressive drop of MAP and heart rate following APS12-2 administration. Time 5 min: injection of APS12-2 or saline solution. * indicates statistically significant differences ($P\leq 0.05$).

3.5. Effect of APS12-2 on arterial blood pressure, ECG and respiratory activities in pharmacologically vagotomized anesthetized rats

As a non-selective anticholinergic drug, atropine was administered to artificially ventilated animals at a dose of 1 mg/kg, which prevents cholinergic effects on the heart (Sawyer and Mundy, 1970). Atropine did not significantly change the time course of ECG and MAP after administration of APS12-2. Administration of atropine resulted in a significant increase ($P\leq 0.05$) in heart rate and in MAP from 198.3 ± 18.5 BPM and 76 ± 1 mm Hg to 257.4 ± 6.5 BPM and 94 ± 7 mm Hg,

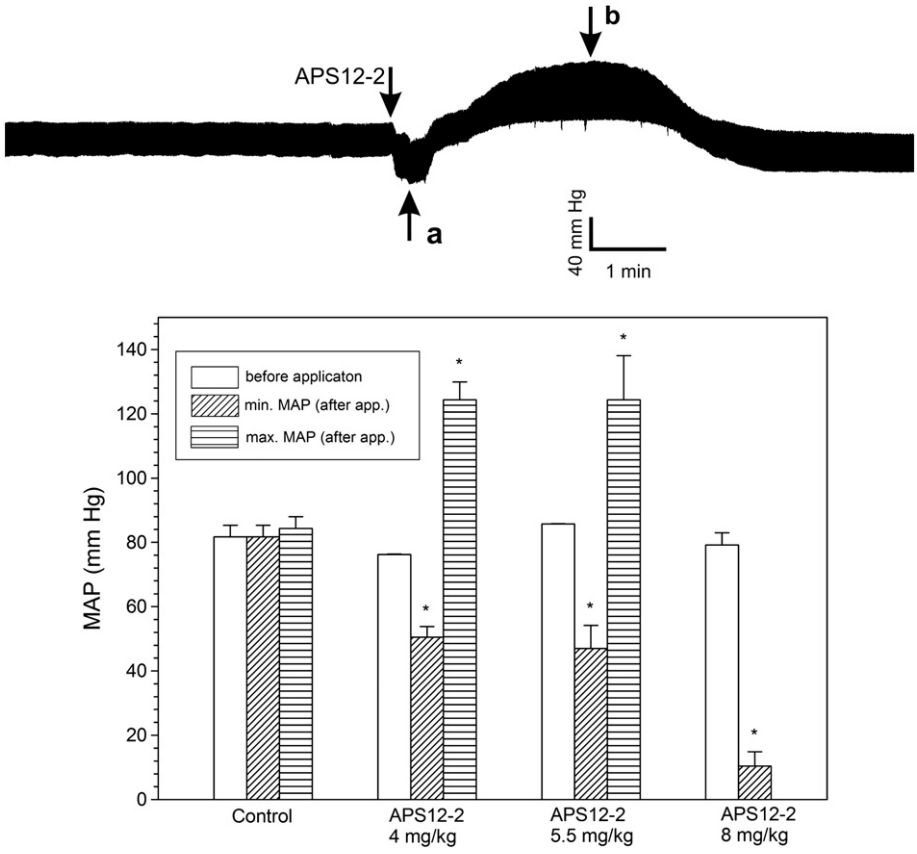


Fig. 5. Effects of sublethal doses of APS12-2 on mean arterial blood pressure (MAP). Upper panel: intravenous application of APS12-2 caused instant drop of aBP followed by transient increase in MAP. Lower panel: bar graph shows minimal and maximal values of MAP in control and treated groups of animals after injection of APS12-2. First bar of each group indicates MAP before application of APS12-2, second bar indicates maximal drop of MAP and third bar shows maximal increase in MAP. Group of animals treated with APS at dose of 8 mg/kg does not have third bar, since MAP progressively dropped to the middle circulatory pressure after application of APS 12–2. * indicates statistically significant differences ($P\leq 0.05$).

Table 1
Effects of APS12-2 (4, 5.5 and 8 mg/kg) on hematocrit and some blood serum electrolytes (mM) in rats.

	Control (n = 6)		4 mg/kg (n = 7)	5.5 mg/kg (n = 6)	8 mg/kg (n = 6)
Time after introduction of anesthesia (min)	10–15	≈50–60	≈50–60	≈50–60	≈50–60
K^+	4.24 ± 0.22	4.47 ± 0.33	6.06 ± 0.53*	6.22 ± 0.33*	10.40 ± 0.44*
Na^+	149.06 ± 0.82	145.06 ± 0.82	140.77 ± 0.39	140.08 ± 0.98*	138.16 ± 2.56
Cl^-	100.72 ± 0.89	100.42 ± 1.29	98.71 ± 0.88	97.03 ± 1.26	95.23 ± 2.16
Ca^{2+}	2.71 ± 0.07	2.79 ± 0.04	2.75 ± 0.11	3.02 ± 0.14	3.12 ± 0.23
Hematocrit	48.80 ± 0.58	43.40 ± 0.60	43.14 ± 1.30	45.17 ± 1.83	47.20 ± 3.65

Mean values of data obtained from the control (first column in bold) and treated (other columns in bold) groups were compared by the Student t-test. Control group of animals received only 0.3 mL of saline solution *i.v.* Blood samples were collected 10–15 min after the injection of anesthetics and 5 min after injecting 4, 5.5 or 8 mg/kg APS12-2 (≈50–60 min after introduction of anesthesia). Values are presented as mean ± SE.
* $P \leq 0.05$.

respectively. In all experiments ($n = 3$), the time-course of ECG and MAP were very similar in amplitude and duration as compared with the intact, spontaneously breathing rats injected with 8 mg/kg APS12-2, except for a marked, transient increase in MAP (from 79 ± 2 to 115 ± 19 mm Hg), 35.4 ± 8.6 s after application of APS12-2 (not shown).

3.6. Effect of APS12-2 on arterial blood pressure, ECG and respiratory activities in artificially ventilated anesthetized rats

In order to determine whether hypoxia, caused by respiratory arrest after APS12-2 administration (8 mg/kg), was responsible for changes of blood pressure and ECG, artificial ventilation was performed, starting before the administration of APS12-2. In artificially ventilated animals ($n = 3$), the time courses of blood pressure and ECG were similar to those observed in spontaneously ventilated rats injected with APS12-2. Progressive reduction of MAP to mid-circulatory pressure (10 ± 4 mm Hg), decrease of heart rate and arrhythmias were noted after *i.v.* injection of APS12-2.

3.7. Histological changes induced by APS12-2

Histopathology revealed moderate lesions in the lungs of treated rats, which developed within 5 min after application of 8 mg/kg APS12-2 ($n = 6$). The most prominent lesions were mild serous alveolar and interalveolar edema, perivasal edema of middle-sized blood vessels and dilated interalveolar veins. Few veins were filled with aggregated erythrocytes, while majority were filled with eosinophilic, fine granular substance, probably mixture of lysed erythrocytes and fibrin (Fig. 6). There was no evidence of lesions in the hearts of treated animals.

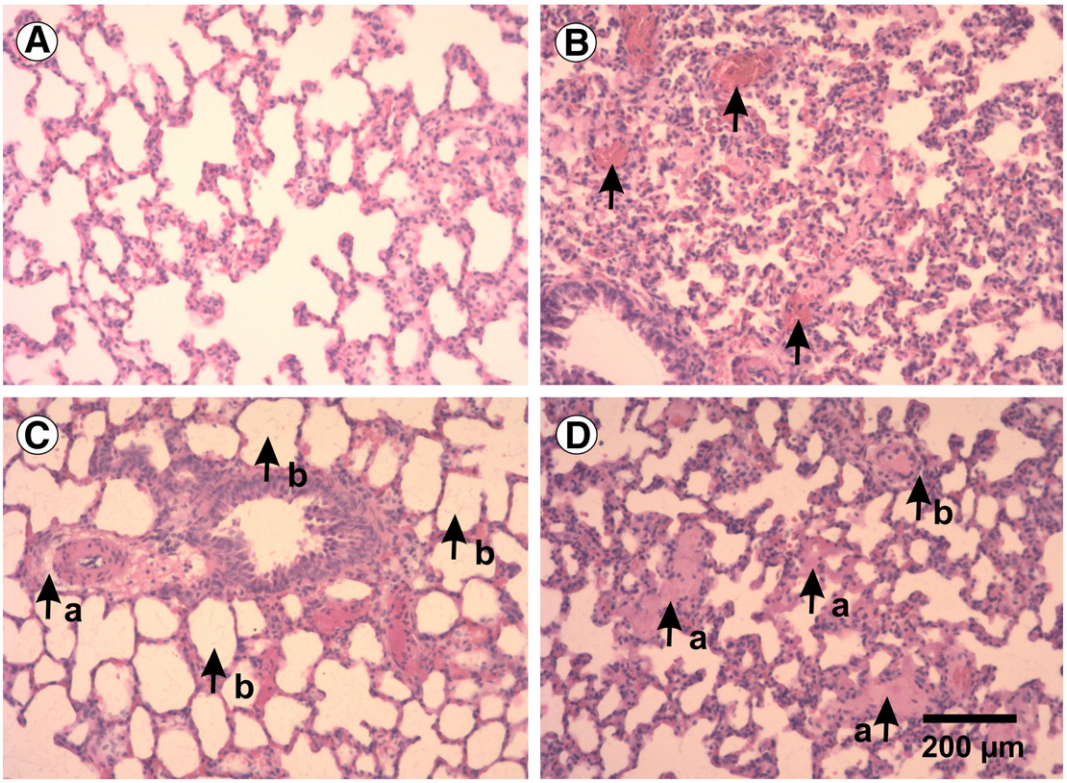


Fig. 6. Representative histopathological changes in the lungs of animals treated with APS12-2. Upper left panel (A) represents lungs of control animal. Panels B, C and D represent lungs of treated animals. On panel B, mild parenchymal atelectasis and several intravascular aggregates of erythrocytes are evident (arrows). Panel C represents mild serous perivascular (a) and alveolar (b) edema. On panel D eosinophilic substance formed of lysed erythrocytes and acute fibrin deposits is evident within the lumina of several dilated interalveolar venules (marked by arrows a). Arrow b shows mild periarteriolar edema. Scale bars = 200 µm apply to all panels.

Discussion

In this work, the toxicity and lethal effects of APS12-2 have been studied in anesthetized rats, and the approximate LD_{50} was estimated in mice. These data are important in a view of the toxicity of selected substance, as well as in a view of possible adverse or even lethal effects on organs and organism, as an integrated system, which may limit the preclinical potential of the studied substance. The LD_{50} of APS12-2, at 11.5 mg/kg in mice suggests that the compound is much less toxic than natural poly-APS, for which a lethal dose of ~2.7 mg/kg was estimated in rats (Bunc et al., 2002). The time-course of ECG (bradycardia, S-T segment elevation, ventricular extrasystoles), and the progressive drop in aBP were similar for APS12-2 (this work, dose of 8 mg/kg) and poly-APS (Bunc et al., 2000; Bunc et al., 2002). When injected in relatively high doses (8 mg/kg) intravenously into rats, APS12-2 caused respiratory arrest and death. At a dose of 8 mg/kg, the compound led to bradycardia and to a progressive drop of MAP. The S–T elevation observed in all experiments on the ECG trace, 44 ± 8 s after APS12-2 administration accompanied with MAP drop under 55 mm Hg, indicates myocardial hypoxia accompanied by respiratory arrest. Respiratory arrest may be produced by strong stimulation of juxtapulmonary capillary receptors (J-receptors; C-fibers) in lung parenchyma. Stimulation of J-receptors after intravenous injection of certain drugs like phenyl diguanidine, serotonine or capsaicin leads to apnea, bradycardia and hypotension (McCaffrey and Kern, 1980; Willette et al., 1983) Moreover, J-receptors are mechanosensitive and are strongly stimulated in pathophysiological conditions like pulmonary congestion, pulmonary edema and pulmonary microembolism (Paintal et al., 1973). Since all this changes were found multifocally in the lungs of all the rats treated by APS12-2 at a dose of 8 mg/kg, we can conclude that activation of J-receptors might have an important role in apnea produced by lethal doses of APS12-2.

In order to determine the effect of hypoxia caused by APS12-2 on the changes in aBP and ECG, artificial ventilation was performed before the respiratory arrest. However, changes in the time-course of blood pressure did not appear to depend on hypoxia only, since the same changes in measured parameters were observed in artificially ventilated animals. APS12-2 is also a very potent AChE inhibitor, as revealed by *in vitro* measurements on electric eel AChE (Houssen et al., 2010). Bradycardia produced by APS12-2 can be caused by ganglionic AChE inhibition, since in the cholinergic cardiac ganglia the action of acetylcholine (ACh) is completed by AChE. Bradycardia produced by APS12-2 can also be the consequence of direct effect of APS12-2 on the conduction system of the heart. APS12-2 is structurally related to quaternary ammonium compounds, some of which are also AChE inhibitors, for example decamethonium which also produces bradycardia (Goodman Gilman, 2001). Increased ACh levels also affect muscarinic M_2-receptors, which result in a decrease of heart rate (Dhein et al., 2001). Pharmacological blockade of muscarinic receptors with a muscarinic antagonist atropine 10 min prior to the APS12-2 (8 mg/kg) administration resulted in a significant, sustained increase in blood pressure and heart rate, indicating elimination of parasympathetic tone on the heart. Atropine also blocks some of the excitatory effects of anti-AChE agents on autonomic ganglia since muscarinic receptors are also involved in ganglionic neurotransmission. A central action of APS12-2 on the medullary vasomotor centers is not expected, because of the size of the compound, charged quaternary nitrogen groups and solubility in water (Houssen et al., 2010), so it almost certainly does not pass the blood brain barrier. We can hypothesize that the bradycardia is partly a result of AChE inhibition since, in accordance with the inhibition of electric eel AChE *in vitro*, APS12-2 also inhibited serum AChE in rats, but the pathophysiological importance of this effect is uncertain. Additionally, bradycardia during the first minute after APS12-2 administration was not observed in two rats pretreated with atropine, and in one experiment it was mild in contrast to the animals injected only with APS12-2, in which bradycardia was marked. However, hyperkalemia can also slow down the conduction velocity in the heart producing bradycardia (Parham et al., 2006). On the other hand, we did not register any effect on nerve-evoked muscle contraction (i.e. fasciculation, amplitude of muscle contraction or block of muscle contraction) related to the inhibition of AChE in the neuromuscular junction *in vivo* (not shown). The resulting systemic hypoxemia as a result of apnea can both exaggerate the sympathetic tone and augment epinephrine release from the adrenal medulla. This release is a strong stimulus, causing acute MAP elevation and tachycardia (Bao et al., 1997). In animals artificially ventilated and injected with lethal dose of APS12-2 (8 mg/kg), acute hypotension was observed with progressive decrease of arterial blood pressure, for which acute hypoxia was not responsible. Therefore, it is not surprising that normal or even increased heart rate as a compensatory mechanism was observed after bradycardia in rats receiving the lethal dose of APS12-2. Additionally, histopathological changes found in the lung parenchyma of rats injected with lethal doses of APS12-2 can also contribute *via* vagal reflexes to the bradycardia and hypotension. Sublethal doses of APS12-2 cause short transient drop followed by a sustained increase in arterial blood pressure. Bradycardia produced by hyperkalemia or lung reflexes may be responsible for the drop of arterial blood pressure. For the following sustained increase of blood pressure may be partly responsible compensatory increase in sympathetic tone as a response to the hypotension or direct or indirect effects of the substance on peripheral blood vessel resistance. This view is supported by the fact that no significant increase in heart frequency was registered during the period of increased blood pressure.

APS12-2 at dose above 4 mg/kg causes significant elevation in blood potassium level *in vivo*, which could be an important cause of the cardiorespiratory toxicity. Heart failure and death of experimental rats are due to high (>10 mM) blood potassium (Van der Meer et al., 1986). Hyperkalemia might be interpreted as a consequence of the APS12-2 hemolytic activity (or other cellular damage) observed with hemolytic measurements *in vitro* on rat erythrocytes (Fig. 2), and semiquantitatively as the collected serum was reddish, although we did not observed a statistically significant decrease in hematocrit value. Hematocrit values were not decreased as expected. Hematocrit even increased although non-significantly, especially at the highest doses of APS12-2 due to fluid escape from vascular space. This fact is supported by the serous extravasation (transudation), especially in the lungs of treated animals (8 mg/kg) evident as mild alveolar, multifocal interalveolar and perivasal edema. Hemolytic activity is supported by the finding of semi-lysed or lysed aggregates of erythrocytes within dilated interalveolar venules (Fig. 6).

Arrhythmias produced by high doses of APS12-2 may be partly due to hyperkalemia, as well as to the direct effect of APS12-2 on the myocardium or heart conduction system. Hyperkalemia is most probably responsible for the arrhythmia (Emberson and Muir, 1969). This assumption is supported by the changes in ECG (bradycardia, second degree atrioventricular block, ventricular extrasystole, etc.), usually produced by concentration-dependent, high plasma potassium levels in advanced hyperkalemia (Kuwahara et al., 1992; Parham et al., 2006). Additionally, it was shown that APS12-2 causes dose-dependent permeabilization of erythrocytes (Houssen et al., 2010; this work) and artificial lipid membranes with different compositions (Grandič, unpublished results). The membrane-disrupting activity is most likely reflecting the surfactant character of the compound, as it was already shown for structurally related natural poly-APS which induce a detergent-like lysis of erythrocyte membranes (Malovrh et al., 1999). Similar time-course changes in MAP and ECG were described for two hemolytic toxins producing hyperkalemia approximately of the same level and death of experimental rats (Šuput et al., 2001; Žužek et al., 2006).

Histological examination of the hearts revealed that all four chambers were dilated and filled with blood indicating acute systolic

heart failure. Since no morphological changes were found in the heart including coronary vessels, this suggests mechano-electrical discoupling which can certainly be produced by high blood potassium level at the highest applied dose of APS12-2.

In conclusion, our results are consistent with the cardiotoxic effects of APS12-2 observed *in vivo* at relatively high doses (8 mg/kg) being caused mainly by the high blood potassium levels. However, the direct functional effects (mechanical dysfunction) of APS12-2 on cardiomyocytes and the heart conduction system cannot be excluded at present, although no evidence of lesions in the hearts of treated animals was found.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors gratefully acknowledge the financial support of the Slovenian Research Agency, by grant P4-0053-0406-04. We thank Katarina Babnik and Boštjan Drolc for excellent technical support, Professor Roger Pain for critical reading of the manuscript and Evelyne Benoit for reading and commenting on the manuscript.

References

Almeida, A.M.P., Berlinck, R.G.S., 1997. Alcalóides alquilpiridínicos de esponjas marinhas. Quim. Nova. 20, 170–185.

Bao, G., Randhawa, P.M., Fletcher, E.C., 1997. Acute blood pressure elevation during repetitive hypocapnic and eucapnic hypoxia in rats. J. Appl. Physiol. 82, 1071–1078.

Bunc, M., Sepčić, K., Turk, T., Šuput, D., 2000. In vivo effects of head-to-tail 3-alkylpiridinium polymers isolated from the marine sponge *Reniera sarai*. Pflugers. Arch. 440, 173–174.

Bunc, M., Strupi-Šuput, J., Vodovnik, A., Šuput, D., 2002. Toxic effects of head-to-tail 3-alkylpiridinium polymers isolated from the marine sponge *Reniera sarai* in rat. Toxicon 40, 843–849.

Catassi, A., Servent, D., Paleari, L., Cesario, A., Russo, P., 2008. Multiple roles of nicotine on cell proliferation and inhibition of apoptosis: implication on lung carcinogenesis. Mutat. Res. 659, 221–231.

Dhein, S., van Koppen, C.J., Brodde, E., 2001. Muscarinic receptors in the mammalian heart. Pharmacol. Res. 44, 161–182.

Egleton, R.D., Brown, K.C., Dasgupta, P., 2008. Nicotinic acetylcholine receptors in cancer: multiple roles in proliferation and inhibition of apoptosis. Trends. Pharmacol. Sci. 29, 151–158.

Ellman, G.L., Courtney, D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Emberson, J.W., Muir, A.R., 1969. Changes in the ultrastructure of rat myocardium induced by hyperkalaemia. J. Anat. 104, 411–421.

Faimali, M., Garaventa, F., Mancini, I., Sicurelli, A., Guella, G., Piazza, V., Greco, G., 2005. Antisettlement activity of synthetic analogues of polymeric 3-alkylpyridinium salts isolated from the sponge *Reniera sarai*. Biofouling 21, 49–57.

Goodman Gilman, A., 2001. Goodman and Gilman's the Pharmacological Basis of Therapeutics, tenth ed. McGraw Hill, New York, pp. 175–215.

Houssen, W.E., Lu, Z., Edrada-Ebel, R., Chatzi, C., Tucker, S., Sepčić, K., Turk, T., Zovko, A., Shen, S., Mancini, I., Scott, R.H., Jaspars, M., 2010. Chemical synthesis and biological activities of 3-alkyl pyridinium polymeric analogues of marine toxins. J. Chem. Biol. 3, 113–125.

Kuwahara, M., Chiku, K., Shiono, T., Tsubone, H., Sugano, S., 1992. ECG changes under hyperkalemia with nephrectomy in the rat. J. Electrocardiol. 25, 215–219.

Maček, P., Lebez, D., 1981. Kinetics of hemolysis induced by equinatoxin, a cytolytic toxin from the sea anemone *Actinia equina*. Effects of some ions and pH. Toxicon 19, 233–244.

Malovrh, P., Sepčić, K., Turk, T., Maček, P., 1999. Characterization of hemolytic activity of 3-alkylpyridinium polymers from the marine sponge *Reniera sarai*. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 124, 221–226.

Mancini, I., Sicurelli, A., Guella, G., Turk, T., Maček, P., Sepčić, K., 2004. Synthesis and bioactivity of linear oligomers related to polymeric alkylpyridinium metabolites from the Mediterranean sponge *Reniera sarai*. Org. Biomol. Chem. 2, 1368–1375.

McCaffrey, T.V., Kern, E.B., 1980. Laryngeal regulation of airway resistance. II. Pulmonary receptor reflexes. Ann. Otol. Rhinol. Laryngol. 89, 462–466.

Paintal, A.S., Damodaran, V.N., Guz, A., 1973. Mechanism of excitation of type J receptors. Acta Neurobiol. Exp. (Wars) 33, 15–19.

Paleari, L., Trombino, S., Falugi, C., Gallus, L., Carlone, S., Angelini, C., Sepčić, K., Turk, T., Faimali, M., Noonan, D.M., Albini, A., 2006. Marine sponge-derived polymeric alkylpyridinium salts as a novel tumor chemotherapeutic targeting the cholinergic system in lung tumors. Int. J. Oncol. 29, 1381–1388.

Parham, W.A., Mehdirad, A.A., Biermann, K.M., Fredman, C.S., 2006. Hyperkalemia revisited. Tex. Heart Inst. J. 33, 40–47.

Reed, L.J., Muench, H., 1938. A simple method for estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–496.

Sawyer, N., Mundy, R., 1970. Autonomic responses in the mouse. Pharmacologist 12, 246.

Sepčić, K., 2000. Bioactive alkylpyridinium compounds from marine sponges. J. Toxicol. Toxin Rev. 19, 139–160.

Sepčić, K., Turk, T., 2006. 3-alkylpyridinium compounds as potential non-toxic antifouling agents. In: Fusetani, N., Clare, A.C. (Eds.), Antifouling Compounds, Progress in Molecular and Subcellular Biology, Subseries Marine Molecular Biotechnology. Springer-Verlag, Berlin, Heidelberg, pp. 105–124.

Sepčić, K., Guella, G., Mancini, I., Pietra, F., Dalla Serra, M., Menestrina, G., Tubbs, K., Maček, P., Turk, T., 1997. Characterization of anticholinesterase-active 3-alkylpyridinium polymers from the marine sponge *Reniera sarai* in aqueous solutions. J. Nat. Prod. 60, 991–996.

Šuput, D., Frangež, R., Bunc, M., 2001. Cardiovascular effects of equinatoxin III from the sea anemone *Actinia equina* (L.). Toxicon 9, 1421–1427.

Tucker, S.J., McClelland, D., Jaspars, M., Sepčić, K., MacEwan, D.J., Scott, R.H., 2003. The influence of alkyl pyridinium sponge toxins on membrane properties, cytotoxicity, transfection and protein expression in mammalian cells. Biochim. Biophys. Acta 1614, 171–181.

Turk, T., Frangež, R., Sepčić, K., 2007. Mechanisms of toxicity of 3-alkylpyridinium polymers from marine sponge *Reniera sarai*. Mar. Drugs 5, 157–167.

Turk, T., Sepčić, K., Mancini, I., Guella, G., 2008. 3-alkylpyridinium and 3-alkylpyridine compounds from marine sponges, their synthesis, biological activities and potential use. In: Rahman, Atta-ur- (Ed.), Studies in Natural Product Chemistry; Bioactive Natural Products (Part O). : Studies in Natural Products Chemistry, vol. 35. Elsevier, Amsterdam, pp. 355–397. [etc.].

Van der Meer, C., Valkenburg, P.W., Snijders, P.M., 1986. Death of experimental animals is caused by heart failure due to hyperkalemia. Circ. Shock. 19, 329–345.

Willette, R.N., Barcas, P.P., Krieger, A.J., Sapru, H.N., 1983. Pulmonary resistance and compliance changes evoked by pulmonary opiate receptor stimulation. Eur. J. Pharmacol. 91, 181–188.

Žužek, M.C., Maček, P., Sepčić, K., Cestnik, V., Frangež, R., 2006. Toxic and lethal effects of ostreolysin, a cytolytic protein from edible oyster mushroom (*Pleurotus ostreatus*), in rodents. Toxicon 48, 264–271.

Toxicology 303 (2013) 25–33

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Toxicity of the synthetic polymeric 3-alkylpyridinium salt (APS3) is due to specific block of nicotinic acetylcholine receptors

Marjana Grandič[a], Romulo Aráoz[b], Jordi Molgó[b], Tom Turk[c], Kristina Sepčić[c], Evelyne Benoit[b], Robert Frangež[a,*]

[a] Institute of Physiology, Pharmacology and Toxicology, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia
[b] CNRS, Institut de Neurobiologie Alfred Fessard – FRC 2118, Laboratoire de Neurobiologie et Développement – UPR 3294, 91198 Gif-sur-Yvette Cedex, France
[c] Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 9 August 2012
Received in revised form
28 September 2012
Accepted 1 October 2012
Available online 9 November 2012

Keywords:
APS3
Motor endplate
Nicotinic acetylcholine receptors
Xenopus oocytes
Mouse
Rat

ABSTRACT

The *in vivo* and *in vitro* toxic effects of the synthetic polymeric 3-alkylpyridinium salt (APS3), from the Mediterranean marine sponge *Reniera sarai*, were evaluated on mammals, with emphasis to determine its mode of action. The median lethal doses of APS3 were 7.25 and higher that 20 mg/kg in mouse and rat, respectively. Intravenous administration of 7.25 and 20 mg/kg APS3 to rat caused a significant fall followed by an increase in mean arterial blood pressure accompanied by tachycardia. In addition, cumulative doses of APS3 (up to 60 mg/kg) inhibited rat nerve-evoked skeletal muscle contraction *in vivo*, with a median inhibitory dose (ID_{50}) of 37.25 mg/kg. When administrated locally by intramuscular injection to mouse, APS3 decreased the compound muscle action potential recorded in response to *in vivo* nerve stimulation, with an ID_{50} of 0.5 mg/kg. *In vitro* experiments confirmed the inhibitory effect of APS3 on mouse hemidiaphragm nerve-evoked muscle contraction with a median inhibitory concentration (IC_{50}) of 20.3 μM, without affecting directly elicited muscle contraction. The compound inhibited also miniature endplate potentials and nerve-evoked endplate potentials with an IC_{50} of 7.28 μM in mouse hemidiaphragm. Finally, APS3 efficiently blocked acetylcholine-activated membrane inward currents flowing through *Torpedo* nicotinic acetylcholine receptors (nAChRs) incorporated to *Xenopus* oocytes, with an IC_{50} of 0.19 μM. In conclusion, our results strongly suggest that APS3 blocks muscle-type nAChRs, and show for the first time that *in vivo* toxicity of APS3 is likely to occur through an antagonist action of the compound on these receptors.

1. Introduction

Polymeric 3-alkylpyridinium salts (poly-APS) are one of more than 80 such compounds found in marine sponges of the order Haplosclerida (Almeida and Berlinck, 1997; Sepčić, 2000; Sepčić and Turk, 2006; Turk et al., 2008). Poly-APS were isolated from the Mediterranean marine sponge *Reniera sarai*, and are water soluble with a high degree of polymerization and broad spectrum of biological activities (Sepčić et al., 1997; Turk et al., 2008). They have been reported to be hemolytic, cytotoxic and cytolytic agents, to have antimicrobial and antifouling properties, and also to act as acetylcholinesterase (AChE) inhibitors (reviewed in Turk et al., 2008). Some of these properties may be explained by their capability to induce the formation of transient pores in biological membranes (Tucker et al., 2003). In particular, poly-APS (in the nanomolar range) exert selective cytotoxicity against non-small cell lung cancer (NSCLC) cells, interfering with their cholinergic system (Catassi et al., 2008; Paleari et al., 2006). Up to the dose of 1 mg/kg, poly-APS did not induce any visible effects in treated experimental mice, or produce alterations of their tissues and organs. However, higher doses of poly-APS were found to be lethal for rat, with a median lethal dose (LD_{50}) of 2.7 mg/kg (Bunc et al., 2002; Turk et al., 2007). The effects of natural poly-APS and possible application in medicine and industry led to the synthesis of a series of synthetic analogs with different degree of polymerization and different length of alkyl chains (Houssen et al., 2010; Mancini et al., 2004; Zovko et al., 2012). These compounds were tested for their hemolytic, AChE-inhibitory, antibacterial, antifungal, antifouling, and transfection potential activities (Faimali et al., 2005; Grandič et al., 2012; Zovko et al., 2012). One of the smallest analogs, poly-1,3-butyl pyridinium chloride (APS3), exists as a mixture of two polymers with molecular weight of 1.2 and 3.8 kDa, corresponding to a polymerization grade of 10 and 32 covalently linked N-butyl-3-butyl pyridinium units, in a 9:1 ratio, respectively (Zovko et al., 2012). This compound, in preliminary assays, was found to be non-hemolytic up to

* Corresponding author. Tel.: +386 1 477 91 31.
E-mail address: robert.frangez@vf.uni-lj.si (R. Frangež).

0300-483X/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tox.2012.10.013

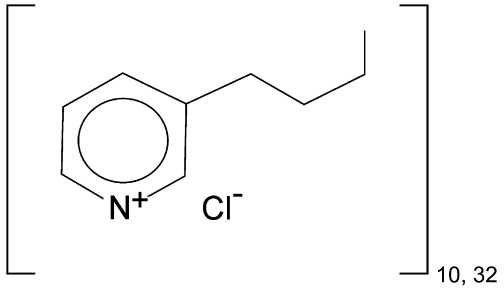


Fig. 1. Chemical structure of APS3 corresponding to a polymerization grade of 10 and 32 covalently linked N-butyl-3-butyl pyridinium units, in a 9:1 ratio, respectively.

the concentration of 50 μg/mL (Zovko and Grandič, unpublished data).

The aim of the present study was to evaluate the *in vivo* and *in vitro* toxic effects of APS3 against rodents, with emphasis to determine its mechanism of action. First and as it is classically done, we examined the mouse toxicity of APS3 and determined the median lethal dose (LD_{50}) as a reference for the following *in vivo* experiments. Second, starting with this reference dose, we studied the *in vivo* effects of the compound on some vital functions (arterial blood pressure, heart rate and breathing) and blood parameters (Na^+, K^+, Ca^{2+} and Cl^- concentrations, hematocrit level) in rats. Third, to evaluate the hypothesized action of the compound as an AChE inhibitor, we looked for APS3-induced *in vivo* modifications of (i) nerve-evoked rat muscle contraction and (ii) mouse neuromuscular excitability. Finally, we undertook *in vitro* experiments on mammalian erythrocytes, mouse neuromuscular junctions and *Xenopus* oocytes having incorporated the *Torpedo* ($\alpha 1_2 \beta 1 \gamma \delta$) nicotinic acetylcholine receptor (nAChR), with emphasis to point out the cellular and molecular mechanism of action of APS3 responsible for its mammalian *in vivo* toxicity.

2. Materials and methods

2.1. Materials

2.1.1. Drugs

The synthetic polymer APS3 (Fig. 1) was synthesized using microwave-assisted polymerization, as previously described (Zovko et al., 2012).

Before use, APS3 was dissolved in a sterile 0.9% saline solution at a stock concentration of 10 mg/mL. Kanamycin, collagenase type II, tricaine, acetylcholine chloride (ACh), 3,4-diaminopyridine (3,4-DAP), neostigmine methyl sulfate (all from Sigma–Aldrich, USA), atropine sulfate (Belupo, Croatia) and μ-conotoxin GIIIB (Bachem, Switzerland) were of the highest grade available. Chanazine [5,6-dihydro-2-(2,6-xylidino)-4H-1,3-thiazin] was purchased from Bayer AG (Leverkusen, Germany), and bioketan (ketamin hydrochloride) from Parke-Davis (Germany).

2.1.2. Experimental animals

Adult male Balb/C mice (24–30 g body weight) were obtained from the animal breeding house of the Faculty of Veterinary, University of Ljubljana. Adult female Swiss-Webster mice (32.7 ± 2.4 g body weight, $n = 9$) were purchased from Janvier Elevage (Le Genest-Saint-Isle, France) and housed at the Gif sur Yvette campus Animal facility. Adult male Wistar albino rats (247–315 g body weight) were obtained from the Medical Faculty, University of Ljubljana. The experimental protocols followed ethical standards, and were approved by the Veterinary administration of the Republic of Slovenia (Permit no. 34401-84/2008/6) and by the French Departmental Direction of Animal Protection (no. A91-453 to E.B.). Adult *Xenopus laevis* female frogs were obtained from the "Centre de Ressources Biologiques Xénopes" (Rennes, France). The *Xenopus* were housed in groups of 8 at the Gif sur Yvette campus Animal facility.

2.2. Methods

2.2.1. In vivo experiments

2.2.1.1. Mouse lethality estimation. Balb/C mice received doses of 4, 6, 7, 7.25, 7.5 and 8 mg/kg by injection of 100 μL saline solution (0.9%) into the right tail vein, as previously described (Grandič et al., 2011). LD_{50} was estimated according to reasonably adopted OECD 425 directive (OECD Guideline 425 "Acute oral toxicity; up and down procedure"), following the 3R principles of Russel and Burch (1959).

2.2.1.2. Cardiorespiratory effects in spontaneously breathing anesthetized rats. The anesthetics chanazine, at a dose of 15 mg/kg of body weight, and bioketan, at a dose of 100 mg/kg of body weight, were simultaneously injected intraperitoneally (*i.p.*) to induce general anesthesia in rats. Body temperature of rats was continuously kept at 37 ± 0.5 °C with the help of heated table. When rats were in deep anesthesia, an incision was made in the ventral cervical region, and cannulae were placed into the prepared jugular vein and carotid artery. Animals used to study the effects of APS3 on muscle contraction, *in vivo*, were artificially ventilated through the aid of a tracheal tube. Mean arterial blood pressure (MAP) was measured as previously described (Grandič et al., 2011). APS3 was administered intravenously (*i.v.*) through a heparinized cannula [50 I.U./mL saline solution (0.9%)] inserted into the left jugular vein. Doses used were 7.25 mg/kg ($n = 6$) and 20 mg/kg ($n = 6$). Solution left in the dead space of cannula was flushed out using 100 μL of 0.9% saline solution. Control rats received the same volume of saline solution ($n = 6$).

2.2.1.3. Rat blood chemistry. Control blood samples (approx. 0.8 mL per animal) were taken from rat left or right sublingual vein under deep general anesthesia (10–15 min after injection of anesthetics). Another set of blood samples was taken directly from the right heart, about 5 min after administration of 0.3 mL of saline solution only, or APS3 at single doses of 7.25 and 20 mg/kg, and at cumulative doses up to 60 (3 + 4 + 6 + 6 + 8 + 5 + 3 + 3 + 5 + 7 + 10) mg/kg (*i.e.* 50–60 min after injection of anesthetics). Blood samples were centrifuged at 1300 × *g* for 10 min to obtain serum samples. Electrolyte (Na^+, K^+, Ca^{2+}, Cl^-) concentrations and hematocrit levels were measured the same day by using techniques previously described (Grandič et al., 2011).

2.2.1.4. In vivo effects on nerve-evoked muscle twitch tension in rat. The rat left sciatic nerve was prepared and embraced with bipolar platinum electrode connected to a stimulator, under deep general anesthesia. The distal tendon of the left *tibialis cranialis* muscle was dissected and attached to an isometric force transducer (Itis, Slovenia). The load applied to transducer was as high as needed to get a maximal contractile response. Supramaximal single nerve pulses (5–10 V intensity and 0.1 ms duration) were applied at a frequency of 0.1 Hz by a S48 stimulator (Grass Instruments, West Warwick, RI, USA). Electrical signals from the force transducer were amplified and digitized at sampling rate of 1 kHz with data acquisition system (Digidata 1440A; Molecular Devices, Union city, CA, USA). Animals received cumulative doses of 3 + 4 + 6 + 6 + 8 + 5 + 3 + 3 + 5 + 7 + 10 mg/kg of APS3 administered *i.v.* with 5 min intervals ($n = 4$). Application of cumulative doses of APS3 was done after artificial ventilation to animals had started, and 15 min after the arterial blood pressure (AP) and heart rate were stabilized. For a given dose of compound, percent of inhibition of twitch tension was calculated at the end of recordings.

2.2.1.5. Local in vivo effects on mouse neuromuscular system. The multimodal excitability properties of the neuromuscular system were studied *in vivo* on Swiss-Webster mouse under isoflurane (AErrane®) anesthesia, by means of minimally invasive electrophysiological methods and using the Qtrac© software, as detailed previously (Boërio et al., 2009). Briefly, electrical stimulations were delivered to the caudal motor nerve by surface electrodes, and the compound muscle action potential (CMAP) was recorded using needle-electrodes inserted into the tail muscle. To study the local action of APS3, intramuscular (*i.m.*) injections of the compound (0.3–3 mg/kg in 100 μL phosphate buffer saline (PBS) solution) were delivered with a 100 μL micro-syringe at the base of mouse tail, between stimulation and ground electrodes. Similar injections were also done with only phosphate buffer saline (PBS) solution. Immediately after a given injection, on-line recordings were initiated to determine the effects of APS3 on selected excitability parameters, such as the excitability threshold and CMAP amplitude recorded continuously as a function of time.

2.2.2. In vitro experiments

2.2.2.1. Determination of hemolytic activity. Rat or bovine erythrocytes were obtained from freshly collected centrifuged citrated blood. The cells were washed twice with excess 0.9% saline solution and once with 140 mM NaCl, 20 mM Tris–HCl buffer (pH 7.4; Grandič et al., 2011). Hemolytic activity was measured with turbidimetric method, as described by Maček and Lebez (1981).

2.2.2.2. Muscle twitch recordings from isolated mouse hemidiaphragms. Balb/C mice were sacrificed by cervical dislocation followed by immediate exsanguination. The diaphragm muscle with the attached phrenic nerves was dissected out, and kept in an oxygenated bath containing Krebs–Ringer solution of the following composition: 154 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES buffer and 11 mM glucose (pH 7.4, room temperature). Each hemidiaphragm preparation was pinned on its lateral side to a silicone-coated glass chamber containing oxygenated Krebs–Ringer solution. The hemidiaphragm medial side with tendon was attached to a silk thread *via* a stainless-steel hook to an isometric mechano-electrical transducer (Itis, Ljubljana, Slovenia). To induce nerve-evoked contraction, the phrenic nerve was stimulated *via* a suction electrode with pulses (10–15 V supramaximal voltage and 0.1 ms duration) at 0.1 Hz frequency, using a square pulse S-48 stimulator (Grass Instruments, West Warwick, RI, USA). For direct muscle stimulation,

pulses (60–100 V amplitude and 0.15 ms duration) were delivered through an electrode assembly placed along the length of the hemidiaphragm and connected to the isolation unit of the S-48 Grass stimulator. To directly or indirectly evoke tetanic muscle contraction, trains of pulses were delivered at 70 Hz for 500 ms duration. For each muscle preparation, the resting tension (typically 1.5–2.5 g) was adjusted to obtain maximal contractile response. Each hemidiaphragm was equilibrated for 20–30 min before starting experiments to obtain maximal contraction and stable resting tension measurements. Muscle twitch tension was measured using an isometric mechano-electrical transducer (Itis, Ljubljana, Slovenia). Electrical signals from the transducer were amplified by a strain gage amplifier (Itis, Ljubljana, Slovenia) and continuously digitized at a sampling rate of 1 kHz, using data acquisition system (Digidata 1440A; Molecular Devices, Sunnyvale, CA, USA). The inhibitory effect of APS3 on nerve-evoked contraction was measured 60–90 min after the onset of APS3 application. Muscle twitch tension blockade produced by APS3 was expressed as percent of the maximal twitch response before exposure to the compound.

2.2.2.3. Membrane potential, miniature endplate potential and endplate potential recordings from isolated mouse hemidiaphragm. All experiments were performed at 20–22 °C on hemidiaphragm preparations equilibrated for 30 min in standard physiological solution. In some experiments, to record full-size endplate potentials (EPPs) without contraction, 2 μM μ-conotoxin GIIIB was added to the physiological solution to block muscle Na^+ channel (Cruz et al., 1985; Hong and Chang, 1989). Membrane potentials, EPPs, and miniature endplate potentials (MEPPs) were recorded from endplate regions using intracellular glass microelectrodes pulled with a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA, USA), in order to have resistances comprised between 10 and 20 MΩ when filled with 3 M KCl. Recordings were performed on each hemidiaphragm preparation before, 45 and 90 min after application of APS3, and 15 min after wash-out of the compound. EPPs were evoked by stimulating the phrenic nerve *via* a suction electrode with supramaximal square pulses of 0.1 ms duration applied at 0.5 Hz. EPPs and MEPPs were digitized using the Digidata 1440A and the pClamp 10 software. Data were analyzed with pClamp-Clampfit 10 (Molecular Devices, Union City, CA, USA). Amplitudes of MEPPs and EPPs were normalized to a membrane potential of −70 mV using the formula $V_c = V_0 \times (-70)/E$, where V_c is corrected amplitude of EPPs or MEPPs, V_0 is the recorded amplitude and E is the resting membrane potential.

2.2.2.4. Membrane preparation enriched in nAChRs from Torpedo marmorata electric tissue. Electrocyte membranes rich in muscle-type ($\alpha 1_2\beta 1\gamma\delta$) nAChR from *T. marmorata* electric tissue were purified according to procedures previously described (Hill et al., 1991) with some modifications as specified here below. The whole membrane purification procedure was performed in a cold room at 4 °C. Freshly dissected electric organ (150–180 g) were sliced with a scalpel and immersed in the *Torpedo* membrane extraction buffer (TMEB) containing 50 mM Tris–HCl, 3 mM EDTA, and 1 mM EGTA supplemented with a cocktail of protease inhibitors (Complete Roche Diagnostics GmbH, Mannheim, Germany), pH = 7.5. The electric tissue was homogenized in 150 mL of TMEB using a Waring blender (Appareils Scientifiques O.S.I., Paris, France) at maximal speed for 1 min (3 times, with 1 min interval). The homogenate was centrifuged at 4000 × *g* for 10 min at 4 °C (Sorvall, Dupont Instruments, Newtown, CT; rotor GSA), and the supernatant (S1) collected and filtered through a gauze of 250 μm pore size. The remaining pellet was re-homogenized and centrifuged, and the resulting supernatant (S2) filtered, as described above. Both supernatants (S1 and S2) were pooled and centrifuged at 25,000 × *g* for 50 min at 4 °C (Sorvall, rotor GSA). The pelleted membranes were resuspended in 100 mL of TMEB, and homogenized using a Potter-Elvehjem (glass/Teflon). Sucrose crystals were added to the crude membrane homogenate to a final concentration of 35% sucrose (w/w). An amount of 16 mL of the 35% sucrose membrane suspension was overlaid on top of 10 mL of 43% sucrose solution in TMEB (w/w) in a 30 mL polycarbonate tube. The samples were centrifuged at 106,000 × *g* during 3 h at 4 °C (Beckman, Palo Alto, CA; rotor 50Ti). The nAChR-rich membranes were collected with a syringe from the interface between the 35% and the 43% sucrose layers. The collected membranes were precipitated by centrifugation (106,000 × *g* for 1 h at 4 °C; Beckman, rotor 50Ti). The precipitated membranes were resuspended in 50 mL of TMEB and centrifuged at 106,000 × *g* for 1 h at 4 °C (Beckman, rotor 50Ti). The membranes were homogenized in 50 mL of 5 mM glycine using a Potter-Elvehjem (glass/Teflon) and centrifuged 106,000 × *g* for 1 h at 4 °C (Beckman, rotor 50Ti). The washing step was repeated once, and the pelleted membranes were resuspended in 5 mL of 5 mM glycine. Protein concentration was determined by the Bradford method (Bio-Rad, Munich, Germany). The protein concentration of the membrane solution was adjusted to 2.5–3.5 mg/mL protein. Aliquots of 1 mL were prepared and stored at −80 °C, until use.

2.2.2.5. Microtransplantation of Torpedo membranes into Xenopus oocytes. X. laevis frogs were anesthetized by immersion for 20 min in a water bath containing 1.5 g/L tricaine. Frogs were operated, and oocytes harvested and prepared as previously described (Krieger et al., 2008). Stages V–VI *Xenopus* oocytes free of follicular cells were kept at 20 °C in Barth's medium containing: 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.5 mM $CaNO_3$, 7.5 mM HEPES (pH 7.6), supplemented with kanamycin (2 mg/mL). For microtransplantation of purified *Torpedo* membranes enriched in $\alpha 1_2\beta 1\gamma\delta$, nAChR, we used microinjection into the oocyte cytoplasm of a membrane suspension (50 nL 2.7 mg/mL protein in 5 mM glycine) with a Nanoliter2000 Micro4 Controller (World Precision Instruments Inc., Stevenage, Herts, UK) mounted on a microscope. Following microinjection, *Xenopus* oocytes were incubated at 18 °C. Incorporation of *Torpedo* electrocyte membranes into the plasma membrane of the oocyte took place 1 day after microinjection.

2.2.2.6. Electrophysiological recordings from Xenopus oocytes having incorporated the Torpedo nAChR. Membrane current recordings on *Xenopus* oocytes were performed at 20 °C using the two-microelectrode voltage-clamp technique and an OC-725B amplifier (Warner Instruments, LLC, Hamden, USA). Oocytes were ready for experiments 1–2 days after microinjection with *Torpedo* membranes. Glass microelectrodes filled with 3 M KCl had resistances of 2–5 MΩ. Each oocyte was placed in the recording chamber (300 μL capacity) and continuously perfused (8–12 mL/min) with a modified Ringer solution containing: 100 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 0.3 mM $BaCl_2$ and 5 mM HEPES. The holding membrane potential was −60 mV. Experimental data were digitized with a Digidata-1322A A/D converter and later analyzed with pCLAMP-9 (Molecular Devices, Union City, CA, USA). Stock solutions of ACh and APS3 were prepared in distilled water. All stock solutions were diluted to the final concentrations in the modified Ringer solution. At the beginning of experiments ACh (50 μM, 2 times the half maximal effective ACh concentration, determined previously) was applied for 15 s twice to each oocyte, to record the amplitude of ACh-induced current under control conditions. After each application of ACh, the oocyte was perfused for 150 s with modified Ringer solution. Then, the oocyte was perfused for 15 s first, with a given concentration of APS3 to test whether the compound had by itself, an agonist effect on nAChRs, and second, immediately after, with a mixture of APS3 and ACh to test the compound effects on the ACh-evoked current. Finally, after 150 s, each oocyte was again exposed twice to ACh (for 15 s) to record the recovery of current amplitude after, thus, 315 s.

2.3. Data analysis and statistics

Data were statistically analyzed using SigmaPlot for Windows 11.0 (Systat Software, Inc., Germany), and results are presented as mean ± SE. The Student two-tailed *t*-test was used for statistical analysis of data, with $P \leq 0.05$ considered statistically significant. Dose–response curves were fitted using the four parameter nonlinear regression model.

3. Results

3.1. Toxicity of APS3 in mouse

The toxicity of APS3 was determined by *i.v.* administration of various doses to mice. At the dose of 4 mg/kg, all animals survived and only one animal showed, 2–4 min after APS3 administration, signs of intoxication characterized by cyanosis, irregular breathing, muscle fasciculation, spasms and movement difficulties. Similar signs of intoxication were observed in all animals injected with doses equal or superior to 6 mg/kg. Moreover, at doses of 6 and 7 mg/kg, 1 out of 3, and at dose of 7.25 mg/kg, 2 out of 4 animals died in less than 5 min after APS3 administration. Surviving animals recovered completely within 30 min after administration of the compound. At doses of 7.5 and 8 mg/kg, all animals died in less than 5 min. Thus, the LD_{50} of APS3 was estimated to 7.25 mg/kg. This value was used as a reference dose for the following *in vivo* rat experiments.

3.2. APS3 on arterial blood pressure, electrocardiogram and respiratory activity in spontaneously breathing anesthetized rat

The effects of APS3 (7.25 and 20 mg/kg *i.v.* administration) were studied *in vivo* on some rat vital functions. At the dose of 7.25 mg/kg, none of the 6 rats died whereas at the dose of 20 mg/kg, 1 out of 6 animals did. These results indicated than the LD_{50} of APS3 in rats was higher than 20 mg/kg. The effects of the compound on MAP were somewhat similar in time-course for the two doses studied although the highest dose was more efficient (Fig. 2A). At the dose of 7.25 mg/kg, the MAP was first significantly decreased from the basal value of 80.38 ± 2.31 mmHg to 67.40 ± 1.86 mmHg within 31.0 ± 1.94 s after APS3 administration, and then significantly increased to 103.55 ± 2.35 mmHg within 152.5 ± 9.61 s, before returning to

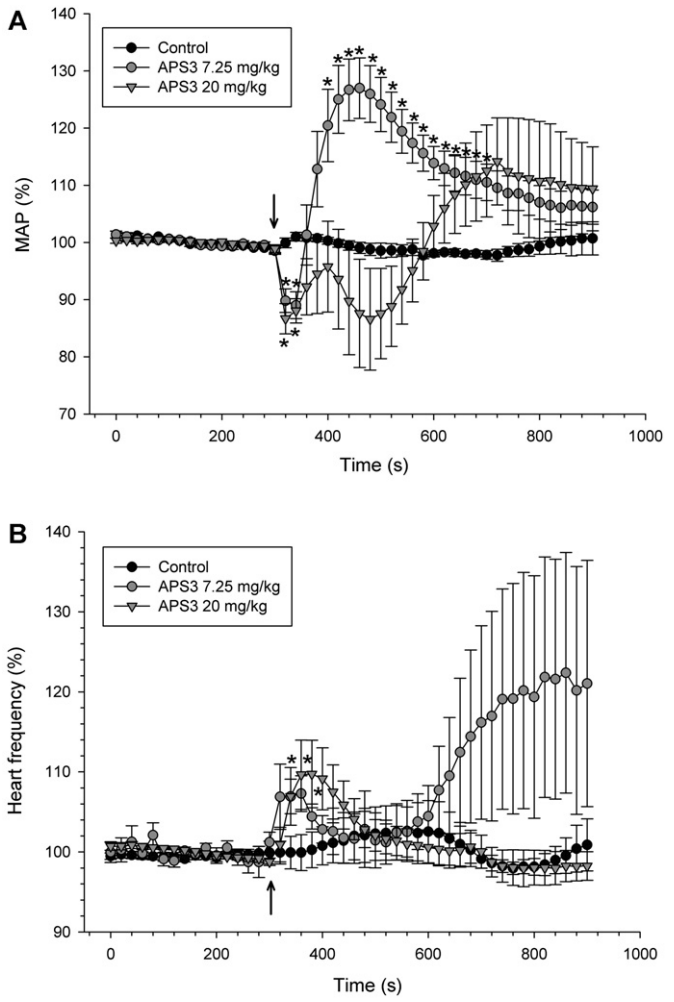


Fig. 2. Effect of APS3 on the mean arterial blood pressure (MAP) (A) and heart frequency (B) in rats. Treated rats ($n = 6$) were *i.v.* administered with 7.25 and 20 mg/kg APS3, and control animals ($n = 5$) with 0.9% saline solution only. Data are expressed as percents of values before administration (arrows). * indicates statistically significant difference ($P \leq 0.05$) between treated and control rats.

basal value. At the dose of 20 mg/kg, the MAP initially significantly fell down from the basal value of 84.26 ± 2.02 mmHg to 67.50 ± 2.40 mmHg within 26.20 ± 1.28 s, and then, after transient fluctuations, was significantly increased to 104.86 ± 4.60 mmHg within 210.80 ± 18.31 s, before returning to basal value.

Following *i.v.* administration of 7.25 and 20 mg/kg APS3, electrocardiogram (ECG) revealed tachycardia (Fig. 2B). At the dose of 7.25 mg/kg, the heart rate was increased, although not significantly, from its basal value of 244.33 ± 11.43 beats per minute (BPM) to 265.17 ± 7.88 BPM within 33.3 ± 6.67 s after APS3 administration, and then, shortly after, returned to basal value before a one more time non-significant increase. At the dose of 20 mg/kg, the heart rate was significantly increased from basal value of 268.00 ± 3.91 BPM to 292.83 ± 8.18 BPM within 56.7 ± 13.08 s, and then gradually fell down toward basal value.

The effects of APS3 on respiratory activity were non-significant since, after *i.v.* administration of 7.25 and 20 mg/kg APS3, it was changed from basal value of $53 \pm 4\ min^{-1}$ to $43 \pm 4\ min^{-1}$ and $53 \pm 7\ min^{-1}$ to $38 \pm 5\ min^{-1}$, respectively (data not shown).

3.3. APS3 on some rat blood parameters

The effects of *i.v.* APS3 administration to rats were studied on serum Na^+, K^+, Ca^{2+} and Cl^- concentrations, as well as on hematocrit blood level, with single doses of 7.25 and 20 mg/kg, and with cumulative doses up to 60 mg/kg (Table 1). The most pronounced effect of the compound was on K^+ serum concentration which was significantly increased with all APS3 doses used. In contrast, serum Na^+ and Ca^{2+} concentrations were not significantly modified at doses of 7.25 and 20 mg/kg and only slightly, but significantly, decreased at cumulative doses. Serum Cl^- concentration and hematocrit level were not significantly changed at all APS3 doses used.

3.4. APS3 on nerve-evoked muscle twitch tension in rats in vivo

To test the eventual AChE inhibitory activity of APS3 *in vivo*, the effects of *i.v.* administration of cumulative doses of the compound (up to 60 mg/kg) were studied on nerve-evoked muscle twitch tension in rats *in vivo* (Fig. 3). The results showed that APS3 induced a dose-dependent inhibition of nerve-evoked skeletal muscle contraction, starting at the cumulative dose of 27 mg/kg and leading to an almost complete block of muscle response at 60 mg/kg of compound (Fig. 3A). The percent of inhibition of twitch tension was calculated as a function of APS3 cumulative doses (19, 27, 32, 35, 38, 43, 50 and 60 mg/kg), leading to a calculated median inhibitory dose (ID_{50}) of 37.25 mg/kg (Fig. 3B).

3.5. APS3 on the multimodal excitability properties of the mouse neuromuscular system in vivo

To further evaluate the hypothesized action of the compound as an AChE inhibitor, the effects of *i.m.* administration of sublethal doses (0.3–3 mg/kg) of APS3 were studied on the multimodal excitability properties of the mouse neuromuscular system. The major effect of the compound was a time- and dose-dependent decrease of CMAP maximal amplitude (Fig. 4), leading to an ID_{50} of 0.5 mg/kg, without significant modification of the other excitability parameters studied. This effect occurred within 15–20 min, depending on APS3 dose injected, and was completely reversed within 2–3 h after compound administration. It is worth noting that the CMAP maximal amplitude remained stable before APS3 injections, or before and after PBS solution injections. This latter result indicates that the injection of the vehicle in which APS3 was dissolved had, by itself, no significant effect on the CMAP maximal amplitude.

3.6. Hemolytic effect of APS3

In order to appraise the cellular mechanism of action of APS3 responsible for its mammal *in vivo* toxicity, the compound was first tested for its hemolytic activity on mammalian cells *in vitro*. The results showed that APS3 did not exhibit any hemolytic activity on rat and bovine erythrocytes, as determined in the range of 3.42 nM to 3.42 μM (data not shown).

3.7. APS3 on mouse muscle contraction in vitro

APS3 was tested *in vitro* for its eventual effects on mouse muscle contraction, since the compound was found to inhibit nerve-evoked rat muscle twitch tension and, more efficiently, mouse CMAP *in vivo* (see Figs. 3 and 4). Micro-molar concentrations of APS3 blocked nerve-evoked single twitch responses (Fig. 5A and B_1) and tetanic contraction (Fig. 5A and B_2) in mouse hemidiaphragm preparations (Fig. 5A), in a concentration-dependent manner (Fig. 5D). The calculated median inhibitory concentration (IC_{50}) for APS3,

Table 1
Effects of APS3 on hematocrit level and serum Na^+, K^+, Ca^{2+} and Cl^- concentrations of rat blood.

	Control rats ($n = 5$)		APS3-treated rats				
			7.25 mg/kg ($n = 6$)		20 mg/kg ($n = 6$)		Up to 60 mg/kg ($n = 4$)
Time after introduction of anesthesia (min)	10–15	50–60	10–15	50–60	10–15	50–60	50–60
K^+ (mM)	4.24 ± 0.22	4.47 ± 0.33	4.48 ± 0.09	5.60 ± 0.35*	4.46 ± 0.08	5.66 ± 0.37*	7.25 ± 0.28*
Na^+ (mM)	149.06 ± 0.82	145.06 ± 0.82	147.54 ± 0.91	145.73 ± 1.09	147.97 ± 1.15	143.16 ± 1.22	138.00 ± 1.19*
Cl^- (mM)	100.72 ± 0.89	100.42 ± 1.29	100.00 ± 1.09	101.60 ± 1.26	100.10 ± 1.50	99.80 ± 1.69	99.23 ± 1.36
Ca^{2+} (mM)	2.71 ± 0.07	2.79 ± 0.04	2.71 ± 0.02	2.76 ± 0.04	2.75 ± 0.03	2.70 ± 0.07	2.58 ± 0.07*
Hematocrit (%)	48.80 ± 0.58	43.40 ± 0.60	50.16 ± 1.37	45.00 ± 0.63	46.75 ± 1.31	47.50 ± 1.99	52.83 ± 3.50

Mean values ± SE of data obtained from control rats, *i.v.* administrated with saline solution only, and from animals treated with 7.25, 20 and up to 60 (cumulative) mg/kg of APS3. Blood samples were collected 10–15 and 50–60 min after the injection of anesthetics (*i.e.* before and about 5 min after saline solution or APS3 administration).
* Statistically significant difference ($P \leq 0.05$) between treated and control rats.

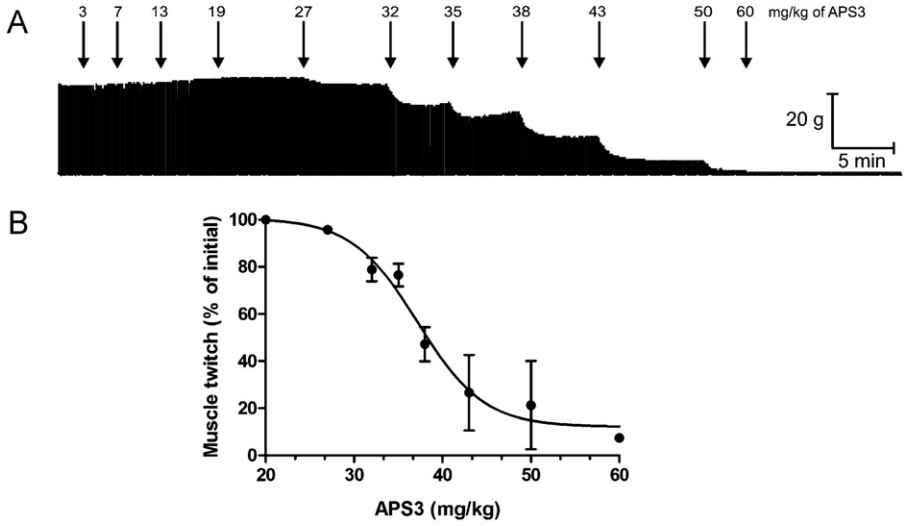


Fig. 3. Effects of APS3 on nerve-evoked twitch tension of the rat tibialis anterior muscle *in vivo*. (A) Representative recording showing the effect of cumulative doses of APS3 on twitch height. Arrows and numbers denote the time of injection of APS3 and the doses used (in mg/kg), respectively. (B) Dose-dependent inhibition of nerve-evoked contraction by APS3 in the rat tibialis anterior muscle *in vivo*. Values are expressed as mean ± SE ($n = 4$).

obtained from curve-fitting analysis using the four parameter non-linear regression model, was 20.3 μM.

Under our experimental conditions, no change in amplitude of twitch contraction was observed during 90 min period of control recordings (data not shown). Nerve-evoked single twitch response and tetanic contraction in the presence of APS3 (0.34–20.55 μM) were almost completely reversed after washing out preparations with a drug-free medium (Fig. 5A). With higher APS3 concentrations (up to 136.98 μM), only partial reversal was observed (data not shown). In all preparations exposed to different APS3 concentrations, we found that single twitch response as well as tetanic contraction of similar amplitude as before the application of APS3 could still be produced by direct muscle stimulation (Fig. 5C_1 and C_2, respectively). The reversible AChE inhibitor neostigmine (1 μM; Alderdice, 1982) was unable to restore single twitch response and tetanic contraction previously inhibited by 20.55 μM APS3 (data not shown). In contrast, 0.3 mM 3,4-DAP, immediately after application, was able to transiently reverse the blocking effect of APS3 on single twitch response and tetanic contraction by about 95% ($n = 3$, data not shown).

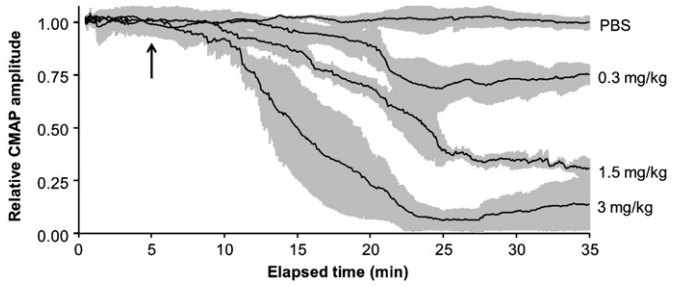


Fig. 4. *In vivo* effects of *i.m.* injections of APS3 and PBS solution on the mouse neuromuscular system. Time-course of the effects of APS3 (0.3, 1.5 and 3 mg/kg) or PBS solution injections on the CMAP maximal amplitude recorded continuously as a function of time. Values are expressed relatively to those before injections, as mean ± SE ($n = 4$). The arrow indicates the time of injections.

3.8. APS3 on resting membrane potential, MEPPs and EPPs of isolated mouse neuromuscular preparations

Since APS3 blocked nerve-evoked muscle contraction in isolated mouse neuromuscular preparations, we tested whether the compound had an action on the resting membrane potential, MEPP and EPP amplitudes using electrophysiological techniques. APS3 (0.07–68.49 μM), *i.e.* in a large range of concentrations that partially or completely blocked nerve-evoked muscle contraction in mouse hemidiaphragms, did not significantly affect the resting membrane potential of muscle fibers recorded in the endplate regions (Fig. 6). The mean resting membrane potential was −64.85 ± 2.29 mV under control conditions, and −65.61 ± 1.96 mV and −69.06 ± 0.47 mV, 45 and 90 min after exposure to 68.49 μM APS3, respectively.

The effects of APS3 (0.07–13.70 μM) on MEPP amplitude and frequency are shown in Fig. 7. APS3, in a concentration-dependent

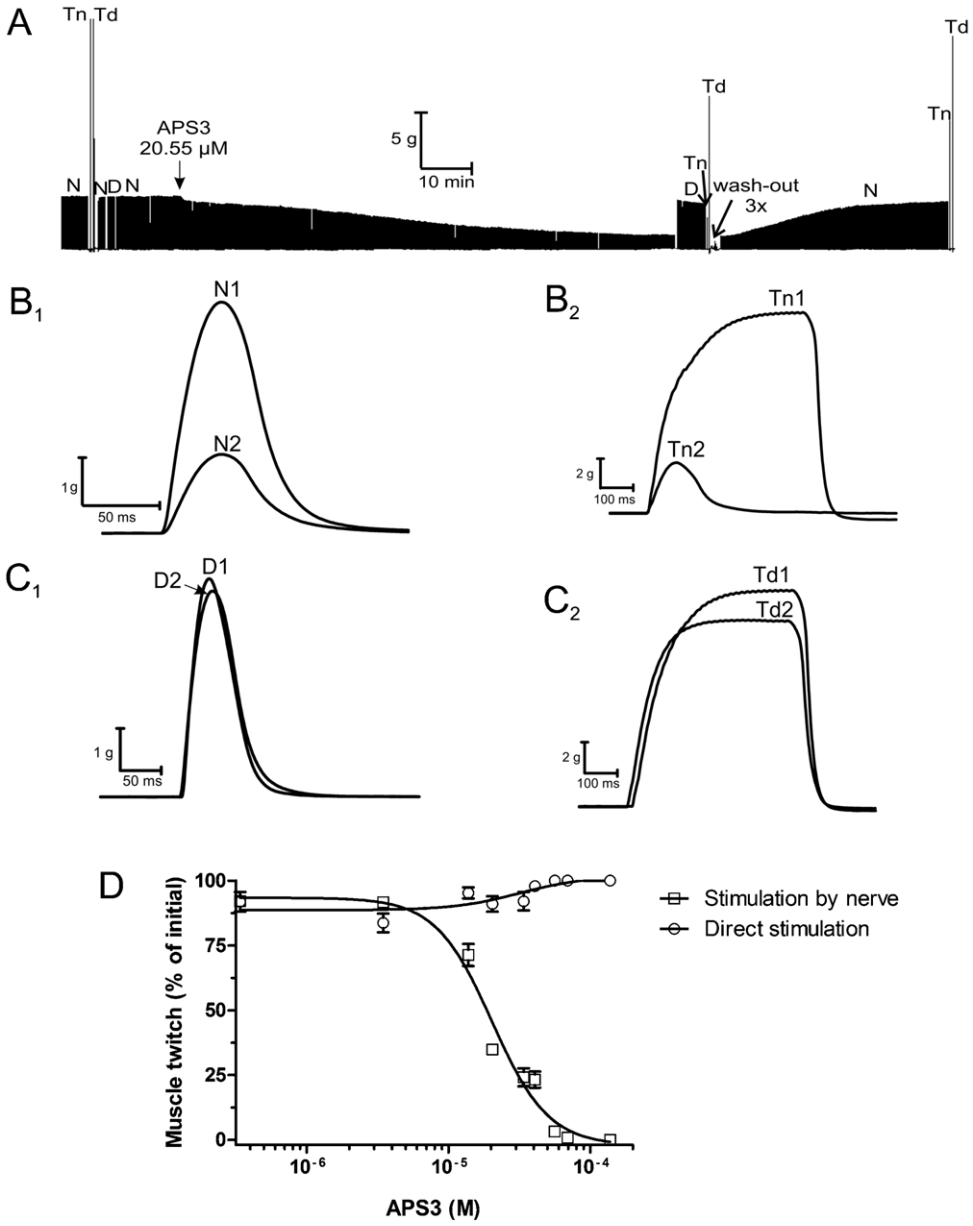


Fig. 5. Effects of APS3 on nerve-evoked and directly elicited single twitch and tetanic muscle contraction in isolated mouse hemidiaphragm neuromuscular preparations. (A) Representative muscle contraction recording showing the partial block of nerve-evoked muscle twitch and tetanic contraction by 20.55 μM APS3. N – nerve-evoked muscle contraction, D – directly elicited muscle contraction, Tn – nerve-evoked tetanic contraction, Td – directly elicited tetanic contraction. (B_1) Representative traces of nerve-evoked single twitch before (N1) and 90 min after (N2) application of 20.55 μM APS3. Data recorded from the same muscle showing the blocking effects of APS3. (B_2) Representative traces of tetanic contraction (70 Hz) before (Tn1) and 90 min after (Tn2) application of 20.55 μM APS3. Data recorded from the same muscle showing the blocking effects of the compound. (C_1) Representative traces of single muscle twitch elicited by direct muscle stimulation before (D1) and 90 min after (D2) application of APS3. Data from the same mouse hemidiaphragm preparation showing the absence of effect of 20.55 μM APS3. (C_2) Representative traces of tetanic contraction evoked by direct muscle stimulation before (Td1) and 90 min after (Td2) application of 20.55 μM APS3, showing no marked effect of the compound. (D) Concentration-dependent inhibition curve of APS3 on mouse hemidiaphragm twitch expressed as percent of the maximal response. Values are expressed as mean ± SE ($n = 5$).

manner, significantly reduced the amplitude of MEPPs recorded in superficial fibers of the mouse hemidiaphragm (Fig. 7A and B), without significant change in their frequency (Fig. 7C). APS3 concentrations higher than 6.85 μM completely blocked MEPPs.

Intracellular recordings in endplates of the mouse hemidiaphragm revealed that exposure to APS3 (0.07–68.49 μM) for 45 and 90 min significantly reduced the amplitude of full sized EPPs in a dose-dependent manner (Fig. 8), without significant change in their half-decay time course (Fig. 8A). It is worth noting that, at APS3 concentrations of 13.70–34.25 μM which completely blocked MEPPs, the nerve stimulation still evoked EPPs, although of lower amplitude compared to control. The calculated IC_{50} for APS3-induced decrease of EPP amplitude was 7.28 μM (Fig. 8B).

3.9. APS3 on Torpedo $\alpha 1_2\beta 1\gamma\delta$ nAChRs incorporated to Xenopus oocytes

Finally, we tested APS3 for its activity on *Torpedo* ($\alpha 1_2\beta 1\gamma\delta$) nAChRs incorporated to *Xenopus* oocytes, to appraise the molecular mechanism of action of the compound. APS3, alone and at all concentrations studied (6.8 pM to 6.84 μM), did not change the oocyte resting membrane conductance and potential (data not shown).

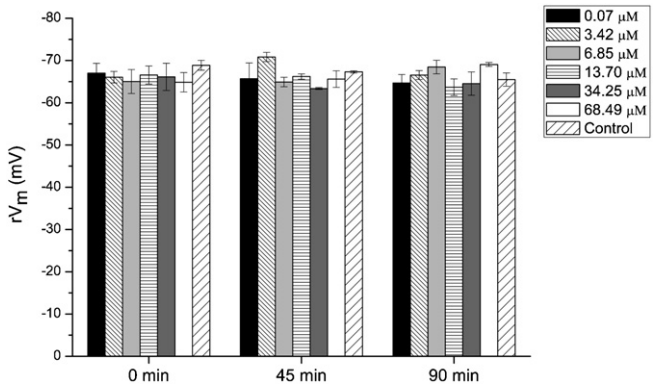


Fig. 6. Concentration- and time-dependent lack of effect of APS3 on the resting membrane potential of isolated mouse muscle fibers. Recordings were performed at the endplate region of the mouse hemidiaphragm. Muscles were exposed to 0.07–68.49 μM APS3, before (time 0) and after 45 and 90 min of APS3 action. Each value represents the mean ± SE of resting membrane potential recorded from 3 hemidiaphragms (n = 6–8 fibers from each).

In contrast, perfusing the oocyte with various concentrations of APS3 together with 50 μM ACh for 15 s resulted in a concentration-dependent reduction of ACh-evoked currents with an IC_{50} of 0.19 μM (Fig. 9). The Hill coefficient of the concentration-response curve was 1.65, indicating a stoichiometry of 2:1 interaction between nAChRs and APS3, respectively. It is worth noting that the APS3-induced inhibition of ACh-evoked currents showed no voltage dependency (data not shown), and was reversible after 315 s wash out of the compound (Fig. 9, *inset*).

4. Discussion

The present study evaluated the *in vivo* and *in vitro* toxic effects of APS3 in mammals, with emphasis to determine its cellular and molecular mechanism of action.

The results obtained from the toxicity evaluation of APS3 *in vivo* can be summarized as follow: (i) *i.v.* administration of the compound was toxic to both mouse and rat with an estimated LD_{50} of 7.25 and higher that 20 mg/kg, respectively; (ii) at 7.25 and 20 mg/kg, the compound mainly decreased and then increased MAP in rats, accompanied by tachycardia, while respiratory activity was not altered; (iii) at 7.25, 20 and cumulative doses up to 60 mg/kg, it increased the K^+ serum concentration of rat blood samples, while Na^+, Ca^{2+} and Cl^- concentrations and the hematocrit level were not markedly modified, except a slight decrease of Na^+ and Ca^{2+} concentrations at high doses; and (iv) it inhibited the nerve-evoked rat muscle contraction and mouse CMAP with ID_{50} of 37.25 and 0.5 mg/kg, respectively. The results obtained from *in vitro* evaluation of the toxicity of APS3 can be summarized as follow: (i) the compound had no hemolytic activity up to 3.42 μM; (ii) it inhibited mouse hemidiaphragm nerve-evoked muscle contraction with an IC_{50} of 20.3 μM, without affecting directly elicited muscle contraction; (iii) it blocked MEPPs and nerve-evoked EPPs in mouse hemidiaphragm, with an IC_{50} of 7.28 μM, without modifying the resting membrane potential of muscle fibers; and (iv) it inhibited ACh-activated membrane inward currents flowing through *Torpedo* nAChRs incorporated to *Xenopus* oocytes, with an IC_{50} of 0.19 μM.

The toxicity of APS3 to rat was found to be about 3-times lower than that to mouse (*e.g.* present results), and more than 7-times lower than that of natural poly-APS in rat (*i.e.* ~2.7 mg/kg; Bunc et al., 2002). From this point of view, APS3 is similar to the related compound APS12-2 (Grandič et al., 2011). However, in contrast to other synthetic APS-based compounds including APS12-2 and natural poly-APS (Grandič et al., 2011; Houssen et al., 2010; Turk et al., 2008), APS3 (up to 3.42 μM) did not exhibit hemolytic activity. On one hand, as a consequence of their hemolytic activity, *i.v.* injections of APS12-2 and natural poly-APS to rat have been reported to produce hyperkalemia, leading to arrhythmias and cardiorespiratory arrest, and thus to rapid death of animals (Bunc et al., 2002; Grandič et al., 2011). On the other hand, in relation with its non-hemolytic activity, we did not observe any APS3-induced arrhythmias and

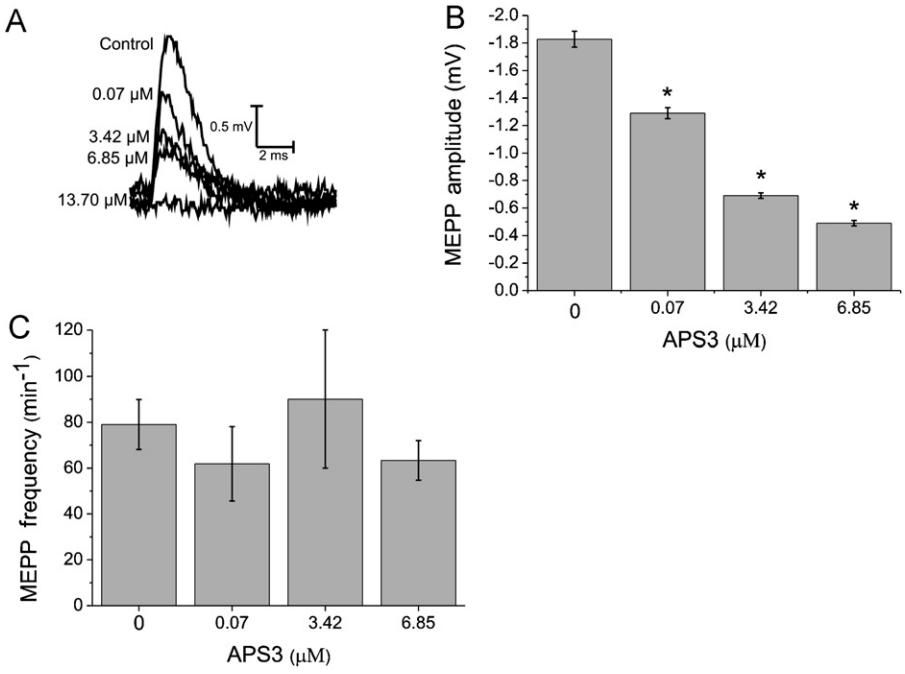


Fig. 7. Effects of APS3 on MEPP amplitude and frequency in isolated mouse hemidiaphragm. (A) Examples of MEPP traces recorded under control conditions and after the indicated concentrations of APS3 applied for 90 min. At concentrations higher than 6.85 μM, the compound completely blocked MEPPs. (B) MEPP amplitude in muscles exposed to different APS3 concentrations. (C) Effects of APS3 on MEPP frequency in the same preparations. Each bar represents mean value ± SE recorded from 3 hemidiaphragms (n = 6–8 fibers from each). * indicates statistically significant difference ($P \leq 0.05$).

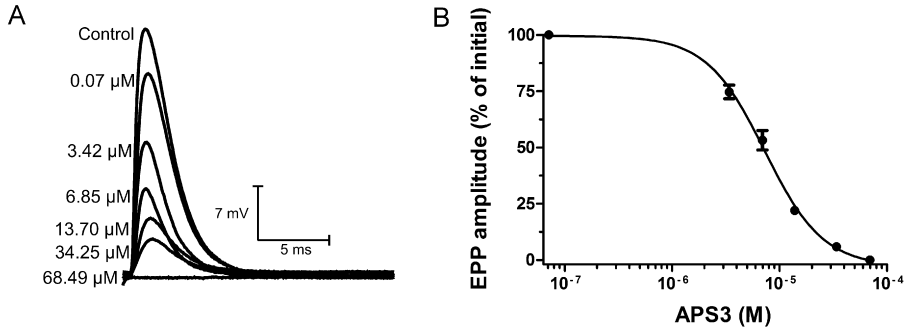


Fig. 8. APS3 blocks, in a concentration-dependent manner, full size endplate potentials in isolated mouse hemidiaphragm. (A) EPP traces recorded under control conditions and 90 min after exposure of muscle to the APS3 concentrations indicated in front of the respective traces. Note that the decrease of EPP amplitudes occurred without change in the resting membrane potential of muscle fibers. (B) Concentration-dependent inhibition curve of APS3 on EPP amplitude expressed as percent of the maximal amplitude. Each value represents the mean ± SE of EPPs recorded from 3 preparations.

failure of vital functions in rat, despite an increase in serum K^+ concentration. However, this increase, although significant, was small compared to that produced by APS12-2 (Grandič et al., 2011). In contrast, APS3 produced mainly a decrease followed by an increase in the mean arterial blood pressure of rat, accompanied by tachycardia, while respiratory activity was not altered.

The APS3-induced initial significant drop of MAP is not the result of a direct decreased heart frequency since the rate was even increased by the compound. A possible explanation of the APS3-induced initial hypotension would be that the compound, as do natural poly-APS (Turk et al., 2008), acts as a competitive AChE inhibitor since bradycardia and hypotension are frequent signs of poisoning with AChE inhibitors (Dube et al., 1993; Kassa and Fusek, 1998; Kojima et al., 1992). According to this hypothesis, an increased release of norepinephrine from postganglionic sympathetic neurons, as the result of excessive cholinergic activity at sympathetic ganglia due to an indirect nicotinic effect of AChE inhibitors, may lead to normal or even increased heart frequency (Bao et al., 1997). In this case, the increase in heart frequency would be a compensatory response to hypotension following APS3 administration. In support to this hypothesis, APS3 has been shown to be a potent competitive AChE inhibitor (with an inhibitory constant of 125 ng/mL), as revealed by *in vitro* measurements on electric eel AChE (Zovko and Turk, unpublished data). However, under our experimental conditions, we did not register any effect of APS3 related to AChE inhibition, such as muscle fasciculation and increased amplitude of muscle contraction while studying the *in vivo* effects of AP3 on nerve-evoked rat muscle contraction, or an increased duration of EPP decay recorded *in vitro*. Another possibility would be that APS3, which is structurally related to quaternary ammonium compounds (such as atracurium, mivacurium, and pancuronium), produces, as do quaternary ammonium compounds (Fisher, 1999), serious arterial hypotension and tachycardia related to histamine release. Although this latter possibility remains to be further investigated, one cannot exclude that APS3-induced increased heart frequency is related to an antagonistic action of the compound on ganglionic $(\alpha3)_2(\beta4)_3$ nAChRs.

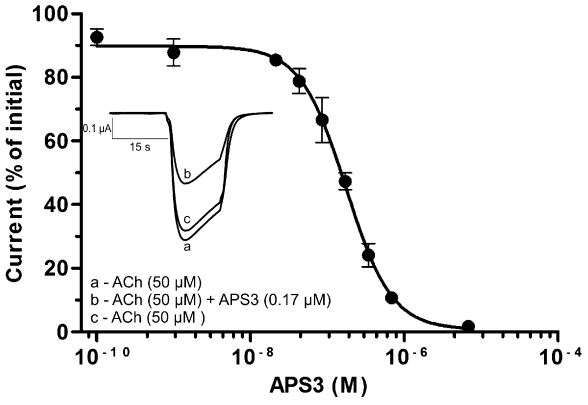


Fig. 9. Concentration-dependent antagonistic effects of APS3 on ACh-evoked currents in *Torpedo marmorata* muscle type nAChRs incorporated to *Xenopus* oocyte membrane. The inward currents were evoked by 15 s application of ACh (50 μM) to oocytes without (control) and with APS3 (0.07–68.49 μM). The current amplitude recorded in response to a given APS3 concentration was normalized to the maximum amplitude of current evoked by control ACh. *Inset*: ACh-evoked currents before (a), during (b) and 315 s after (c) 0.17 μM APS3.

The fact that APS3 exerts an antagonist action on nAChRs is in agreement with the observation that the compound inhibited, *in vivo*, nerve-evoked rat muscle contraction and mouse CMAP, and is further supported by the results obtained on mouse isolated neuromuscular preparations. These results reveal that APS3 blocked, in a concentration-dependent manner, nerve-evoked single twitch response and tetanic contraction. The neuromuscular blocking potency in mouse hemidiaphragm produced by APS3 was approximately 15-times lower when compared to the conventional competitive muscle-type nAChRs inhibitor D-tubocurarine (IC_{50} = 20.30 μM for APS3 *vs.* 1.42 μM for D-tubocurarine; Nguyen-Huu et al., 2005; Ridtitid et al., 1998). APS3, in a concentration-dependent manner (0.07–6.85 μM), reduced MEPP amplitude without significantly affecting their frequency, nor the resting membrane potential of muscle fibers. These results strongly suggest that the compound exerted, at the neuromuscular junction, a postsynaptic action on nAChRs. All together, our results indicate that APS3 reduced the activity of nAChR-channels without altering either muscle fiber passive membrane properties or the ACh release process. Exposure of muscles treated with APS3 to the reversible AChE inhibitor neostigmine had no effect on the muscle response. Although the low concentration of neostigmine (1 μM) used did not inhibit completely AChE activity, an inhibitory action of APS3 on endplate acetylcholinesterases would be expected to prevent further neostigmine action. Furthermore, no evidence was obtained in the present study of an eventual anticholinesterase effect of APS3. This eventual effect would have resulted in an enhancement of twitch amplitude and a prolongation of the decay phase of synaptic potentials (MEPPs and full-size EPPs) since the lack of AChE activity allows ACh to persist in the synaptic cleft and to activate repetitively nAChRs of the endplate (Van der Kloot et al., 1994), unless such an action was masked by the block of nAChRs by APS3, which is expected to reduce the number of receptors available for repetitive binding. In contrast, the APS3-induced blockade of nerve-evoked muscle contraction was antagonized by treatment with the potassium channel blocker 3,4-DAP which increases ACh release from motor terminals (Hong

and Chang, 1990; Molgó et al., 1980) and prolongs the duration of action potentials in skeletal muscle and thereby greatly improves skeletal muscle contraction performance (Khan and Lemeignan, 1983; Van Lunteren et al., 2008). In the range of concentrations studied, APS3 had little or no effect on muscle excitability and on excitation-contraction coupling processes in muscle, as determined by analyzing the contractile responses upon single and tetanic direct muscle stimulation.

Finally, the hypothesis suggesting that APS3 exerted an antagonist action on nAChRs was confirmed by experiments performed on *Xenopus* oocytes microtransplanted with muscle-type *Torpedo* nAChRs. Electrophysiological recordings demonstrated that APS3, with high potency, inhibited ACh-evoked nicotinic currents flowing through muscle-type nAChRs incorporated to the membrane of *Xenopus* oocytes. The antagonistic mechanism on nAChRs may involve binding of the drug molecule to the open channel leading to reduced current flow, competitive antagonism or allosteric modulation of nAChRs (Goodman Gilman, 2001). However, we observed that the APS3-induced inhibition of ACh-evoked currents was not voltage dependent, which strongly suggests that APS3 did not bind preferentially to open nAChR-channels but to both closed and open channels. Moreover, the Hill coefficient obtained from four parameter fit to the concentration-response curve for APS3, calculated for oocytes having incorporated the *Torpedo* ($\alpha 1_2 \beta 1 \gamma \delta$) nAChR, was 1.65. This value, lower than 2, may indicate that the compound interacted with nAChRs in a molecular ratio of 2:1 and exerted allosteric modulation of these receptors, probably by binding externally to their non-conducting part.

In conclusion, our results show for the first time that *in vivo* toxicity of APS3 is likely to occur through a reversible antagonistic action of the compound on nAChRs in motor endplates.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors gratefully acknowledge the Slovenian Research Agency (grants P4-0053-0406-04, BI-FR/11-12-PROTEUS-002 and J1-4044), and the European 7th Frame Program (grant 2009-1/117 PHARMATLANTIC) for financial support. We thank Boštjan Drolc, Katarina Babnik, and Patricia Villeneuve for excellent technical support.

References

Alderdice, M.T., 1982. Further comparison of the effects of physostigmine and neostigmine on frog neuromuscular transmission. Clin. Exp. Pharmacol. Physiol. 9, 35–43.

Almeida, A.M.P., Berlinck, R.G.S., 1997. Alcalóides alquilpiridínicos de esponjas marinhas. Quim. Nova 20, 170–185.

Bao, G., Randhawa, P.M., Fletcher, E.C., 1997. Acute blood pressure elevation during repetitive hypocapnic and eucapnic hypoxia in rats. J. Appl. Physiol. 82, 1071–1078.

Boërio, D., Greensmith, L., Bostock, H., 2009. Excitability properties of motor axons in the maturing mouse. J. Peripher. Nerv. Syst. 14, 45–53.

Bunc, M., Strupi Šuput, J., Vodovnik, A., Šuput, D., 2002. Toxic effects of head-to-tail 3-alkylpiridinium polymers isolated from the marine sponge *Raniera sarai* in rat. Toxicon 40, 843–849.

Catassi, A., Servent, D., Paleari, L., Cesario, A., Russo, P., 2008. Multiple roles of nicotine on cell proliferation and inhibition of apoptosis: implication on lung carcinogenesis. Mutat. Res. 659, 221–231.

Cruz, L.J., Gray, W.R., Olivera, B.M., Zeikus, R.D., Kerr, L., Yoshikami, D., Moczydlowski, E., 1985. *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. J. Biol. Chem. 260, 9280–9288.

Dube, S.N., Kumar, P., Kumar, D., Das Gupta, S., 1993. Route-specific cardiorespiratory and neuromuscular changes following organophosphorous poisoning in rats. Arch. Int. Pharmacodyn. Ther. 321, 112–122.

Faimali, M., Garaventa, F., Mancini, I., Sicurelli, A., Guella, G., Piazza, V., Greco, G., 2005. Antisettlement activity of synthetic analogues of polymeric 3-alkylpyridinium salts isolated from the sponge *Reniera sarai*. Biofouling 21, 49–57.

Fisher, D.M., 1999. Clinical pharmacology of neuromuscular blocking agents. Am. J. Health Syst. Pharm. 56 (Suppl 1), S4–S9.

Goodman Gilman, A., 2001. Goodman and Gilman's the Pharmacological Basis of Therapeutics, 11th ed. McGraw Hill, New York, pp. 193–206, 645–658.

Grandič, M., Sepčić, K., Turk, T., Juntes, P., Frangež, R., 2011. In vivo toxic and lethal cardiovascular effects of a synthetic polymeric 1,3-dodecylpyridinium salt in rodents. Toxicol. Appl. Pharmacol. 255, 86–93.

Grandič, M., Zovko, A., Frangež, R., Turk, T., Sepčić, K., 2012. Binding and permeabilization of lipid bilayers by natural and synthetic 3-alkylpyridinium polymers. Bioorg. Med. Chem. 20, 1659–1664.

Hill Jr., J.A., Nghiêm, H.O., Changeux, J.P., 1991. Serine-specific phosphorylation of nicotinic receptor associated 43K protein. Biochemistry 30, 5579–5585.

Hong, S.J., Chang, C.C., 1989. Use of geographutoxin II (mu-conotoxin) for the study of neuromuscular transmission in mouse. Br. J. Pharmacol. 97, 934–940.

Hong, S.J., Chang, C.C., 1990. Facilitation by 3,4-diaminopyridine of regenerative acetylcholine release from mouse motor nerve. Br. J. Pharmacol. 101, 793–798.

Houssen, W.E., Lu, Z., Edrada-Ebel, R., Chatzi, C., Tucker, S., Sepčić, K., Turk, T., Zovko, A., Shen, S., Mancini, I., Scott, R.H., Jaspars, M., 2010. Chemical synthesis and biological activities of 3-alkyl pyridinium polymeric analogues of marine toxins. J. Chem. Biol. 3, 113–125.

Khan, R.A., Lemeignan, M., 1983. Effects of 3,4-diaminopyridine on mechanical and electrical responses of frog single muscle fibres. Acta Pharmacol. Toxicol. (Copenh.) 52, 181–187.

Kassa, J., Fusek, J., 1998. The positive influence of a cholinergic–anticholinergic pretreatment and antidotal treatment on rats poisoned with supralethal doses of soman. Toxicology 128, 1–7.

Kojima, T., Tsuda, S., Shirasu, Y., 1992. Non-cholinergic mechanisms underlying the acute lethal effects of P=S type organophosphorus insecticides in rats. J. Vet. Med. Sci. 54, 529–533.

Krieger, F., Mourot, A., Araoz, R., Kotzyba-Hibert, F., Molgó, J., Bamberg, E., Goeldner, M., 2008. Fluorescent agonists for the Torpedo nicotinic acetylcholine receptor. Chembiochem 9, 1146–1153.

Maček, P., Lebez, D., 1981. Kinetics of hemolysis induced by equinatoxin, a cytolytic toxin from the sea anemone *Actinia equina*. Effects of some ions and pH. Toxicon 19, 233–244.

Mancini, I., Sicurelli, A., Guella, G., Turk, T., Maček, P., Sepčić, K., 2004. Synthesis and bioactivity of linear oligomers related to polymeric alkylpyridinium metabolites from the Mediterranean sponge *Reniera sarai*. Org. Biomol. Chem. 2, 1368–1375.

Molgó, J., Lundh, H., Thesleff, S., 1980. Potency of 3,4-diaminopyridine and 4-aminopyridine on mammalian neuromuscular transmission and the effect of pH changes. Eur. J. Pharmacol. 61, 25–34.

Nguyen-Huu, T., Dobbertin, A., Barbier, J., Minic, J., Krejci, E., Duvaldestin, P., Molgó, J., 2005. Cholinesterases and the resistance of the mouse diaphragm to the effect of tubocurarine. Anesthesiology 103, 788–795.

Paleari, L., Trombino, S., Falugi, C., Gallus, L., Carlone, S., Angelini, C., Sepčić, K., Turk, T., Faimali, M., Noonan, D.M., Albini, A., 2006. Marine sponge-derived polymeric alkylpyridinium salts as a novel tumor chemotherapeutic targeting the cholinergic system in lung tumors. Int. J. Oncol. 29, 1381–1388.

Ridtitid, W., Rattanaprom, W., Thaina, P., Chittrakarn, S., Sunbhanich, M., 1998. Neuromuscular blocking activity of methanolic extract of *Piper sarmentosum* leaves in the rat phrenic nerve-hemidiaphragm preparation. J. Ethnopharmacol. 6, 135–142.

Sepčić, K., Guella, G., Mancini, I., Pietra, F., Dalla Serra, M., Menestrina, G., Tubbs, K., Maček, P., Turk, T., 1997. Characterization of anticholinesterase-active 3-alkylpyridinium polymers from the marine sponge *Reniera sarai* in aqueous solutions. J. Nat. Prod. 60, 991–996.

Sepčić, K., 2000. Bioactive alkylpyridinium compounds from marine sponges. J. Toxicol. Toxin Rev. 19, 139–160.

Sepčić, K., Turk, T., 2006. 3-Alkylpyridinium compounds as potential non-toxic antifouling agents. In: Fusetani, N., Clare, A.C. (Eds.), Antifouling Compounds, Progress in Molecular and Subcellular Biology, Subseries Marine Molecular Biotechnology. Springer-Verlag, Berlin/Heidelberg, pp. 105–124.

Tucker, S.J., McClelland, D., Jaspars, M., Sepčić, K., MacEwan, D.J., Scott, R.H., 2003. The influence of alkyl pyridinium sponge toxins on membrane properties, cytotoxicity, transfection and protein expression in mammalian cells. Biochim. Biophys. Acta 1614, 171–181.

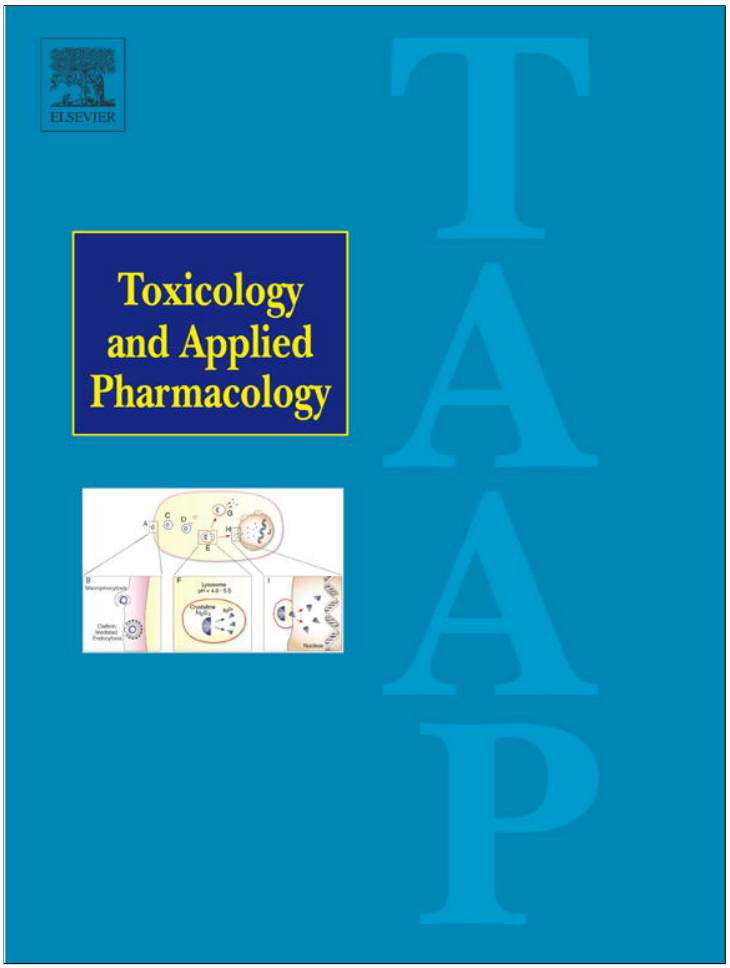
Turk, T., Frangež, R., Sepčić, K., 2007. Mechanisms of toxicity of 3-alkylpyridinium polymers from marine sponge *Reniera sarai*. Mar. Drugs 5, 157–167.

Turk, T., Sepčić, K., Mancini, I., Guella, G., 2008. 3-Alkylpyridinium and 3-alkylpyridine compounds from marine sponges, their synthesis, biological activities and potential use. In: Atta-ur-Rahman (Ed.), Studies in Natural Product Chemistry; Bioactive natural products (Part O), Studies in Natural Products Chemistry, vol. 35. Elsevier, Amsterdam, pp. 355–397.

Van der Kloot, W., Balezina, O.P., Molgó, J., Naves, L.A., 1994. 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. Pflügers Arch. 428, 114–126.

Van Lunteren, E., Moyer, M., Pollarine, J., 2008. Long-lasting in vivo inotropic effects of the K^+ channel blocker 3,4-diaminopyridine (DAP) during fatigue-inducing stimulation. Muscle Nerve 38, 1616–1622.

Zovko, A., Vaukner, M., Sepčić, K., Pohleven, F., Jaklič, D., Gunde-Cimerman, N., Turk, T., 2012. Antifungal and antibacterial activity of 3-alkylpyridinium polymeric analogs of marine toxins. Int. Biodeterior. Biodegr. 68, 71–77.



(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

Toxicology and Applied Pharmacology 265 (2012) 221–228

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

The non-competitive acetylcholinesterase inhibitor APS12-2 is a potent antagonist of skeletal muscle nicotinic acetylcholine receptors

Marjana Grandič [a], Romulo Aráoz [b], Jordi Molgó [b], Tom Turk [c], Kristina Sepčić [c], Evelyne Benoit [b], Robert Frangež [a,*]

[a] Institute of Physiology, Pharmacology and Toxicology, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, SI-1000 Ljubljana, Slovenia
[b] CNRS, Institut de Neurobiologie Alfred Fessard, FRC 2118, Laboratoire de Neurobiologie et Développement, UPR 3294, F-91198 Gif-sur-Yvette Cedex, France
[c] Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 4 July 2012
Revised 28 September 2012
Accepted 30 September 2012
Available online 6 October 2012

Keywords:
APS12-2
Neuromuscular junction
Nicotinic acetylcholine receptors
Xenopus oocytes
AChE inhibitors
Torpedo marmorata
Motor endplate

ABSTRACT

APS12-2, a non-competitive acetylcholinesterase inhibitor, is one of the synthetic analogs of polymeric alkylpyridinium salts (poly-APS) isolated from the marine sponge *Reniera sarai*. In the present work the effects of APS12-2 were studied on isolated mouse phrenic nerve–hemidiaphragm muscle preparations, using twitch tension measurements and electrophysiological recordings. APS12-2 in a concentration-dependent manner blocked nerve-evoked isometric muscle contraction (IC_{50} = 0.74 μM), without affecting directly-elicited twitch tension up to 2.72 μM. The compound (0.007–3.40 μM) decreased the amplitude of miniature endplate potentials until a complete block by concentrations higher than 0.68 μM, without affecting their frequency. Full size endplate potentials, recorded after blocking voltage-gated muscle sodium channels, were inhibited by APS12-2 in a concentration-dependent manner (IC_{50} = 0.36 μM) without significant change in the resting membrane potential of the muscle fibers up to 3.40 μM. The compound also blocked acetylcholine-evoked inward currents in *Xenopus* oocytes in which *Torpedo* ($\alpha1_2\beta1\gamma\delta$) muscle-type nicotinic acetylcholine receptors (nAChRs) have been incorporated (IC_{50} = 0.0005 μM), indicating a higher affinity of the compound for *Torpedo* ($\alpha1_2\beta1\gamma\delta$) than for the mouse ($\alpha1_2\beta1\gamma\varepsilon$) nAChR. Our data show for the first time that APS12-2 blocks neuromuscular transmission by a non-depolarizing mechanism through an action on postsynaptic nAChRs of the skeletal neuromuscular junction.

Introduction

APS12-2 is a synthetic polymeric 1,3-dodecylpyridinium salt with 14.7 kDa of molecular mass structurally related to natural polymeric alkylpyridinium salts (poly-APS) from the marine sponge *Reniera sarai* (Sepčić et al., 1997) that shows a broad spectrum of biological activities (Turk et al., 2008). APS12-2 was shown to act as a potent non-competitive acetylcholinesterase (AChE) inhibitor with an inhibitory constant (K_i) of 0.034×10^{-3} μM (Houssen et al., 2010). It was also proved to exert hemolytic, antibacterial and antifungal actions, and to induce stable transfection of nucleated mammalian cells with heterologous DNA (Houssen et al., 2010; Zovko et al., 2012). Recent research revealed its selective cytotoxicity towards the cells of non-small cell lung cancer (NSCLC), which is the most common form of lung cancer (Zovko and Turk, unpublished results). NSCLC cells express cholinergic signaling including α7-nicotinic receptors (Catassi et al., 2008; Egleton et al., 2008; Paleari et al., 2006). In previous work the *in vivo* effects of APS12-2 were studied by injecting the substance intravenously. It was shown that the *in vivo* toxicity of APS12-2 is low and, due to its high hemolytic potential, hyperkalemia is mainly responsible for its cardiotoxicity (Grandič et al., 2011). APS12-2 is structurally related to quaternary ammonium compounds, some of which can block muscle nAChRs. Some AChE inhibitors in fact can have dual effects; besides inhibiting AChE, they can also modulate muscle-type nAChR conductance. One example is the reversible AChE inhibitor physostigmine that binds to *Torpedo* nAChRs (Sherby et al., 1985) which can either activate nAChRs in micromolar concentration, or directly block nAChRs in millimolar concentration, depending on the concentration (Okonjo et al., 1991). Another AChE inhibitor, bis(7)-tacrine, also reversibly inhibits *Torpedo* nAChRs (IC_{50} = 0.162 μM). The inhibition is not voltage-dependent, and bis(7)-tacrine enhances desensitization of nAChRs (Ros et al., 2001). Also the selective AChE inhibitor BW284c51 blocks ACh-activating current in *Torpedo* nAChRs (IC_{50} = 0.2–0.5 μM), and enhances receptor desensitization (Olivera-Bravo et al., 2005).

Since in the case of APS12-2 it was not possible to detect any effect on nerve-evoked muscle contraction *in vivo* in rats, due to the prevailing effects deriving from its potent hemolytic potential, we performed *in vitro* studies on isolated mouse neuromuscular preparations and on *Xenopus* oocytes microtransplanted with *Torpedo* ($\alpha1_2\beta1\gamma\delta$) nAChRs in order to reveal the toxin mode and sites of

* Corresponding author.
E-mail address: robert.frangez@vf.uni-lj.si (R. Frangež).

0041-008X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2012.09.024

action on the tissue and at the cellular level. APS12-2 and structurally related compounds are promising new chemotherapeutic agents that exhibit very low *in vivo* toxicity in experimental animals (Grandič et al., 2011). In view of their putative use to treat lung cancer, it is very important to evaluate other possible adverse effects of these compounds. In particular, due to the structural similarity between APS12-2 and quaternary ammonium compounds that have been reported to block muscle nAChRs, the aim of this study was to investigate the effects and the underlying mechanisms of APS12-2 on neuromuscular transmission.

Materials and methods

Materials

Drugs and solutions. Tricaine, kanamycin, collagenase type II, acetylcholine chloride, neostigmine methyl sulfate and 3,4-diaminopyridine were from Sigma–Aldrich (Saint Quentin Fallavier, France). Atropine sulfate was purchased from Belupo, Croatia and μ-conotoxin GIIIB was from Bachem, Switzerland. All chemicals were of the highest grade available.

APS12-2 was synthesized using a microwave-assisted polymerization procedure (Houssen et al., 2010). In addition, it was structurally characterized by nuclear magnetic resonance (NMR), and its molecular weight determined by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and electrospray ionization mass spectrometry (ESI-MS). APS12-2 was found to be a 14.7 kDa polymer composed of sixty 1,3-dodecylpyridinium monomer units. Before use, the compound was dissolved in distilled water.

Experimental animals. Adult male Balb/C mice (24–28 g body weight) were purchased from the animal breeding house in Veterinary Faculty, University of Ljubljana. The experiments followed ethical standards, and were approved by Veterinary Administration of the Republic of Slovenia (permit no. 34401-20/2009/30). Adult female Swiss-Webster mice (31.6 ± 2.2 g body weight, $n = 6$) were purchased from Janvier Elevage (Le Genest-Saint-Isle, France) and housed at the Gif-sur-Yvette campus animal facility. Adult *Xenopus laevis* female frogs were obtained from the Centre de Ressources Biologiques Xenopes (Rennes, France). The *Xenopus* were housed in groups of 8 at the Gif-sur-Yvette campus animal facility.

Methods

Muscle twitch recordings from isolated mouse hemidiaphragms. Adult male Balb/C mice were euthanized by cervical dislocation followed by immediate exsanguination. The diaphragm muscle with corresponding phrenic nerves was isolated and mounted in a 3 mL Rhodorsil-lined organ bath containing the following oxygenated physiological solution composed of (in mM): 154 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES and 11 D-glucose, pH 7.4, at 22–24 °C. The lateral side of hemidiaphragm preparation was tightly pinned to the Rhodorsil-lined bath. The medial side with tendon was attached with a silk thread *via* a stainless steel hook to an isometric mechano-electrical transducer (Itis, Ljubljana, Slovenia). The motor nerve of isolated neuromuscular preparations was stimulated *via* a suction electrode with pulses of 0.1 ms duration, 0.1 Hz stimulation rate and supramaximal voltage of 5–10 V, using a square pulse S-48 stimulator (Grass Instruments, West Warwick, RI, USA). For direct muscle stimulation electrical field stimulation was carried out (pulses of 60–100 V, 150 μs in duration) by means of a platinum electrode assembly placed along the length of the hemidiaphragm and connected to the isolation unit of the S-48 Grass stimulator. In order to directly or indirectly evoke tetanic muscle contraction, trains of pulses (500 ms duration at 70 Hz) were used. The resting tension for each muscle preparation (typically 1.5–2.5 g) was adjusted in order to achieve maximal contractile response upon nerve-evoked muscle stimulation. Each hemidiaphragm preparation was allowed to equilibrate for 20–30 min before starting experiments to achieve maximal contractile response and stable resting tension. Muscle twitch tension was measured using a Grass FT03 force transducer. Electrical signals were amplified by P122 strain gage amplifier (Grass Instruments, West Warwick, RI, USA), and continuously digitized at a sampling rate of 1 kHz using a data acquisition system (Digidata 1440A; Molecular Devices, Sunnyvale, CA, USA). The inhibitory effect of APS12-2 on nerve-evoked contraction was measured 60–90 min after APS12-2 application. Muscle twitch tension blockade produced by APS12-2 was expressed as percentage of the maximal twitch response before exposure to APS12-2. Concentration–response curves of APS12-2 were plotted 60–90 min after drug perfusion.

Membrane potential, miniature endplate potential and endplate potential recordings from mouse hemidiaphragm. Resting membrane potential, miniature endplate potentials (MEPPs) and endplate potentials (EPPs) were obtained from superficial muscle fibers using intracellular borosilicate glass microelectrodes with an Axoclamp 900A microelectrode amplifier (Molecular Devices, Sunnyvale, CA, USA). Microelectrodes were pulled with a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA, USA) and filled with 3 M KCl. Only microelectrodes with 10–20 MΩ resistance were used. EPPs were recorded in the endplate region, indicated by the presence of MEPPs. EPPs were evoked by stimulating the phrenic nerve *via* a bipolar suction electrode with supramaximal square pulses of 0.1 ms duration at 0.5 Hz using an S-48 stimulator (Grass Instruments, West Warwick, RI, USA). EPPs and MEPPs were digitized (Digidata 1440A and pCLAMP 10, Axon Instruments) at 25 kHz and stored for later analysis using pCLAMP-Clampfit 10 software (Axon Instruments). All experiments were performed at 22–24 °C. Muscles were pretreated for 30 min with 2 μM μ-conotoxin GIIIB (Chevessier et al., 2008). Thereafter, EPPs were measured in the presence of 2 μM μ-conotoxin GIIIB which preferentially blocks muscle type voltage-gated sodium channels and allows measuring the full-size EPP amplitude (Cruz et al., 1985; Hong and Chang, 1989). Amplitudes of MEPPs and EPPs were normalized to a membrane potential of −70 mV using the formula $V_c = V_0 \times (-70)/E$, where V_c is corrected amplitude of EPPs or MEPPs, V_0 is recorded amplitude and E is resting membrane potential (Pardo et al., 2006).

Electrophysiological recordings from Xenopus oocytes having incorporated the Torpedo nAChR. The nAChRs expressed in the electric organ of *Torpedo marmorata* fish are similar in subunit composition and functional properties to those of fetal vertebrate neuromuscular junctions (reviewed by Sine, 2012). Therefore, in order to have additional information on the effects of APS12-2 on this muscle-type nAChR we performed micro-transplantation of electrocyte purified *Torpedo* membranes to *Xenopus* oocytes to study the $\alpha 1_2\beta 1\gamma\delta$ nAChR incorporated to the oocyte membrane. *T. marmorata* electrocyte membranes enriched with $\alpha 1_2\beta 1\gamma\delta$ nAChR were purified, as previously described (Krieger et al., 2008) and aliquots (2.7 mg/mL total protein) in 5 mM glycine were kept at −80 °C until use.

Adult *X. laevis* frogs were anesthetized by immersion in a water solution containing 1.5 g/L tricaine for 20 min. Frogs were operated and oocytes were prepared as described (Krieger et al., 2008). Oocytes were kept at 18 °C in Barth's medium containing: 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.5 mM $CaNO_3$, 7.5 mM HEPES, and pH 7.6, supplemented with kanamycin (2 mg/mL) to prevent bacterial contamination.

Stage V and VI oocytes were microinjected each with 50 nL of purified *Torpedo* membranes using a Nanoliter 2000 Micro4 Controller (World Precision Instruments Inc., Stevenage, Herts, U.K.) and incubated at 18 °C. Oocytes were used 1–2 days after microinjection with *Torpedo* membranes. Viability of oocytes was checked, and Barth's medium was replaced daily. Membrane current recordings were performed at 20 °C using the two-microelectrode voltage-

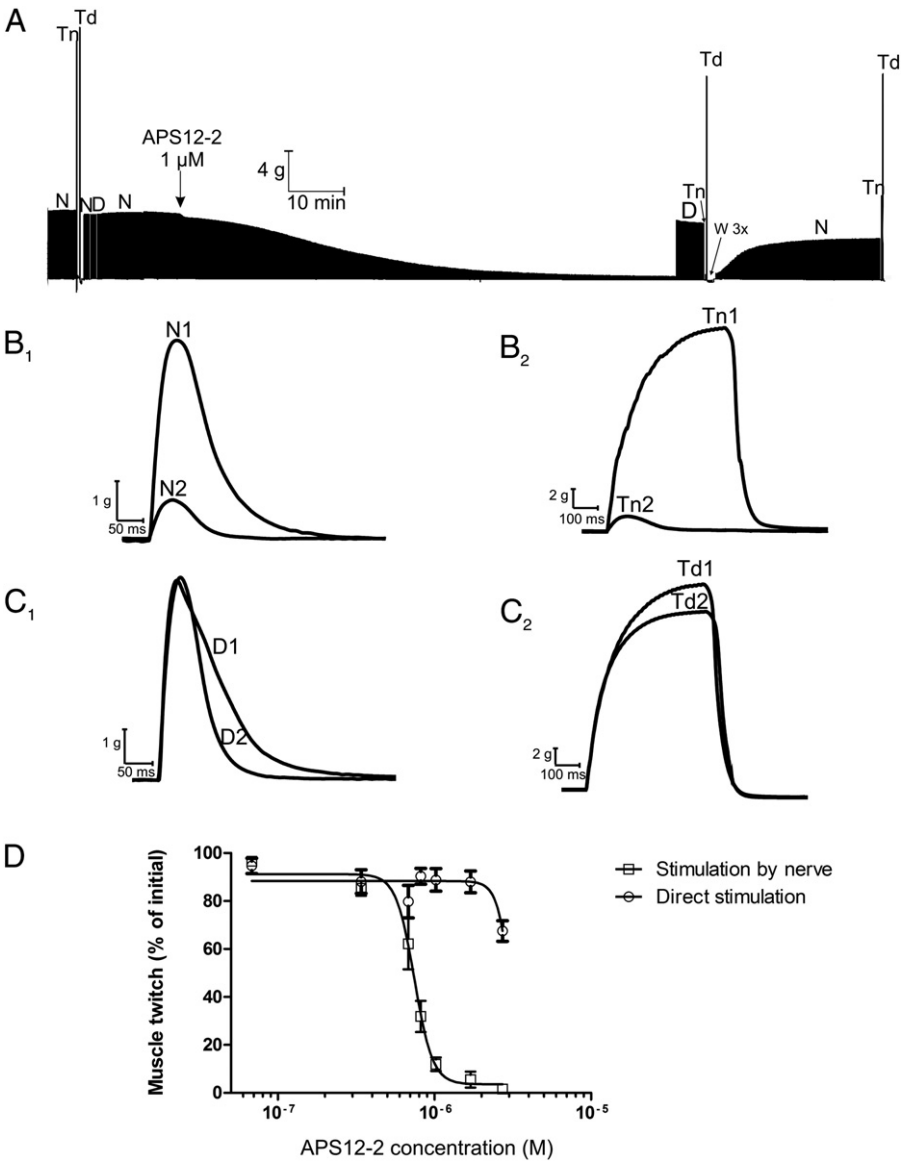


Fig. 1. Effects of APS12-2 on both nerve-evoked or directly-elicited single twitch and tetanic contractions in isolated mouse hemidiaphragm neuromuscular preparations. (A) Representative muscle contraction recordings showing the partial block of nerve-evoked muscle twitch and tetanic contraction by APS12-2 at a concentration of 1.02 μM. N — denotes nerve-evoked muscle contraction; D — denotes directly-elicited muscle contraction; Tn — denotes nerve-evoked tetanic contraction; Td — denotes directly-elicited tetanic contraction; W — wash-out. (B_1) Representative recordings of nerve-evoked single twitch before (N1), and 90 min after application of APS12-2 (N2), data recorded from the same muscle showing the blocking effects of APS12-2 (1.02 μM). (B_2) Representative recordings of tetanic contraction (at 70 Hz) before (Tn1), and 90 min after application of APS12-2 (Tn2), data recorded from the same muscle showing its blocking effects (1.02 μM). (C_1) Representative recordings showing that APS12-2 (1.02 μM) has little effect on the peak amplitude but prolongs the time course of the twitch of single muscle twitch elicited by direct muscle stimulation before (D1), and 90 min after application of APS12-2 (D2). Data are from the same mouse hemidiaphragm. (C_2) Representative recordings showing tetanic contractions elicited by direct muscle stimulation before (Td1), and 90 min after application of APS12-2 (Td2). (D) Concentration-dependent inhibition curve for directly-elicited and nerve-evoked contraction for APS12-2 on mouse hemidiaphragms, expressed as percent of the maximal twitch response. Values are expressed as the mean ± SE ($n = 5$ different muscles).

clamp technique and an OC-725B amplifier (Warner Instruments, LLC, Hamden, USA). Intracellular borosilicate glass microelectrodes were filled with 3 M KCl and had resistances of 2–5 MΩ. Each oocyte was placed in the recording chamber (300 μL capacity), equilibrated for about 5 min and then continuously superperfused (8–12 mL/min) with a modified Ringer's solution containing (mM): 100 NaCl, 2.8 KCl, 1 $MgCl_2$, 0.3 $BaCl_2$ and 5 HEPES, where $BaCl_2$ substitution to $CaCl_2$ prevents secondary activation of a Ca^{2+}-dependent Cl-current (Sands et al., 1993).

The holding membrane potential was maintained at −60 mV. Experimental data were digitized with a Digidata-1322A A/D converter, and later analyzed with pCLAMP-9 software (Molecular Devices, Union City, CA, USA). Stock solutions of ACh and APS12-2 were prepared in distilled water. All stock solutions were diluted to the final concentrations in the modified Ringer solution.

The half maximal effective ACh concentration (EC_{50}) was 25 μM, as determined by a concentration–response curve in oocytes having incorporated the *Torpedo* muscle-type nAChR and voltage-clamped at −60 mV. We used 50 μM ACh (2 times the EC_{50}) which activated about 80% of the nAChR. ACh (50 μM) was usually applied twice in order to record control current amplitudes. ACh applications were followed by APS12-2 perfusion which was immediately followed by perfusion with the mixture of APS12-2 and ACh. Finally, each oocyte was washed-out and again exposed twice to ACh to record the

recovery of current amplitudes. A computer-controlled solution-exchange system (VC-6, Warner Instruments) allowed limiting the duration of each drug exposure to 15 s. Recovery time between each application was 150 s. Amplitudes of the currents recorded in response to each drug concentration were normalized to the maximum amplitude of the current evoked by the control ACh.

Local in vivo effects on mouse neuromuscular system. The multimodal excitability properties of the mouse neuromuscular system were studied *in vivo* on adult mice, by means of minimally-invasive electrophysiological methods, using the Qtrac© software written by Prof. Hugh Bostock (Institute of Neurology, London, England). The experiments were performed in accordance with the guidelines established by the French Council on animal care "Guide for the Care and Use of Laboratory Animals": EEC86/609 Council Directive-Decree 2001-131, on mice under isoflurane (AErrane®) anesthesia. The experimental protocols were approved by the French Departmental Direction of Animal Protection (no. A91-453 to E.B.).

Briefly, electrical stimulations were delivered to the caudal motor nerve by means of surface electrodes, and the compound muscle action potential (CMAP) was recorded using needle-electrodes inserted into the tail muscle. To study the local action of APS12-2, intramuscular injections of the compound [1.1–2.2 μmol/kg mouse in 100 μL phosphate buffer saline (PBS) solution] were delivered with a 100 μL micro-syringe at the base of the mouse tail, between stimulation and ground electrodes. Similar injections were also done with only PBS solution. Immediately after a given injection, on-line recordings were initiated to determine the effects of APS12-2 on selected excitability parameters, such as the excitability threshold and CMAP amplitude recorded continuously as a function of time.

Data analysis and statistics

The data were statistically analyzed using Sigma Plot for Windows version 11.0 (Systat Software Inc., USA). The Student two-tailed test was used for statistical analysis of data. The results are presented as mean ± S.E. The statistical significances were set at the P value ≤ 0.05. Concentration–response curves were fitted using the four parameter nonlinear regression model.

Results

APS12-2 blocks nerve-evoked muscle contraction in vitro

One of the goals of this study was first to determine the effects of the non-competitive AChE inhibitor APS12-2 on directly- and indirectly-evoked isometric muscle twitch and tetanic contraction in the mouse hemidiaphragm. The following APS12-2 concentrations were used: 0.07, 0.34, 0.68, 0.82, 1.02, 1.70 and 2.72 μM. An example of the time-course of APS12-2 effects on muscle contraction is shown in Fig. 1A. APS12-2 blocked nerve-evoked single twitch (Fig. 1B_1) and tetanic contraction (Fig. 1B_2) in a concentration-dependent manner (Fig. 1D). From the concentration–response curve it was calculated an IC_{50} = 0.74 μM for APS12-2.

No drop in the amplitude of muscle twitch was observed in control experiments during 90 min (record and data not shown). After concentration-dependent blockade of indirectly-evoked muscle twitch, thorough washing of the preparation with standard physiological solution partially restored indirectly-evoked muscle twitch and tetanic contractions (Fig. 1A). In all preparations exposed to different APS12-2 concentrations we found that single twitch, as well as tetanic contraction of about the same amplitude as before the application of APS12-2, could still be produced by direct electrical muscle stimulation (Figs. 1C_1 and C_2, respectively). With most APS12-2 concentrations used, the drug did not reduce the amplitude of directly-elicited muscle contraction except at 2.72 μM which was the highest concentration used (Fig. 1D). During the development of the contraction block, obtained 30 min after application of 0.82 μM APS12-2, the reversible AChE inhibitor neostigmine (1 μM; Alderdice, 1982; Goodman Gilman, 2001) did not augment single twitch and tetanic muscle contraction (not shown). In contrast, 30 min after application of APS12-2, when twitch responses were reduced to about 30%, 300 μM 3,4-diaminopyridine transiently reversed the blocking effect of APS12-2 on single twitch and tetanic contraction by about 86% (n = 3; not shown).

Effects of APS12-2 on the resting membrane potential of muscle fibers

At the concentration of 3.40 μM, APS12-2 completely blocked nerve-evoked muscle contraction. APS12-2 in all the concentrations

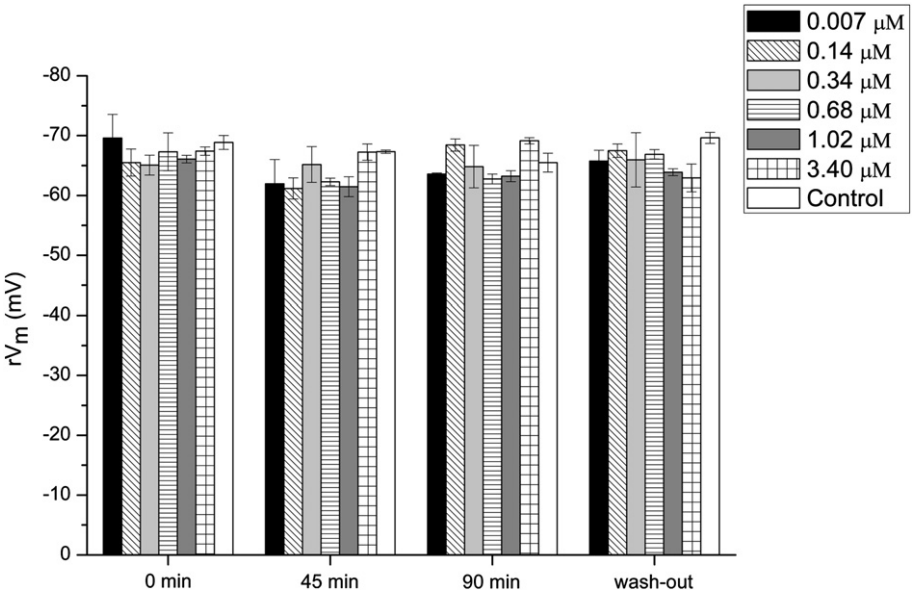


Fig. 2. Concentration- and time-dependent lack of effect of APS12-2 on the resting membrane potential (rV_m) of muscle fibers. Recordings were performed at the end-plate region of muscle fibers in mouse hemidiaphragms. Muscles were exposed to 0.007, 0.14, 0.34, 0.68, 1.02 and 3.40 μM APS12-2, before (time 0), after 45 and 90 min of APS12-2 exposure, and after wash-out of the drug from the medium for 15 min. Each column represents the mean ± SE of rV_m recorded from 3 different left hemidiaphragms (by sampling 6–8 fibers from each muscle).

used (0.007, 0.14, 0.34, 0.68, 1.02 and 3.40 μM) did not significantly affect the resting membrane potential recorded in the endplate region of muscle fibers ($P>0.05$; Fig. 2). The average membrane potentials measured from 6 to 8 fibers were -67.40 ± 0.70 mV, -67.24 ± 1.35 mV and -69.14 ± 0.52 mV before application, 45 min and 90 min after exposure to the highest concentration of APS12-2 (3.40 μM), respectively (Fig. 2).

Effects of APS12-2 on MEPP and EPP amplitude

The effects of APS12-2 on MEPP amplitude and frequency are shown in Fig. 3. At concentrations of 0.007, 0.14 and 0.34 μM, APS12-2 significantly ($P\leq0.05$) reduced the amplitude of MEPPs measured in superficial muscle fibers of the mouse hemidiaphragm (Figs. 3A and B). The mean MEPP frequency was not significantly ($P>0.05$) affected by APS12-2 at concentrations that affected MEPP amplitude (Fig. 3C). Above 0.68 μM, APS12-2 completely blocked MEPP amplitudes. However, when MEPP amplitude was completely blocked, nerve stimulation still evoked EPPs of low amplitude (Fig. 4A).

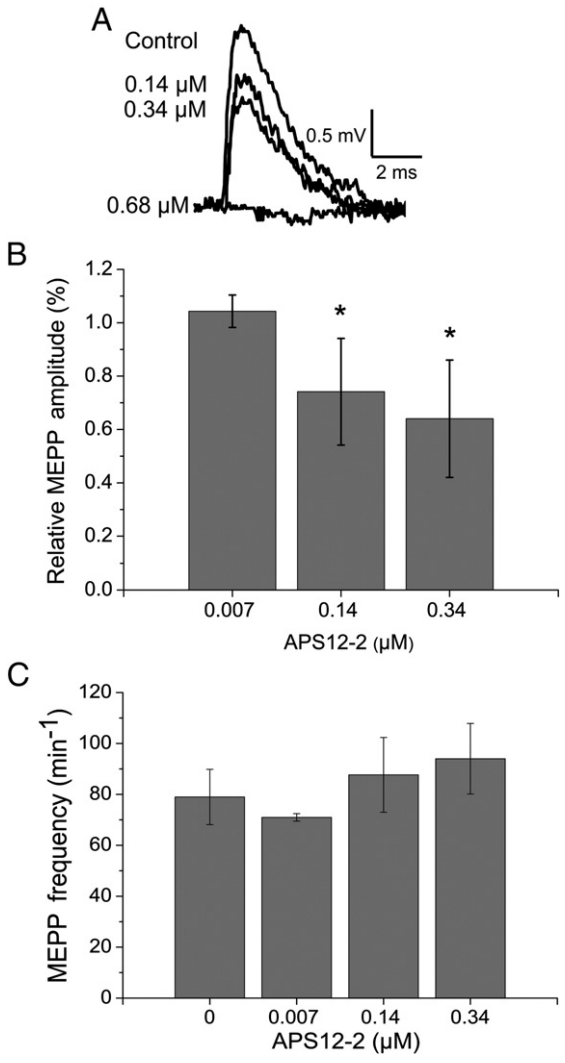


Fig. 3. Effects of APS12-2 on MEPP amplitude and frequency. (A) Representative traces of MEPPs recorded from the same mouse hemidiaphragm under control conditions and after indicated concentrations of APS12-2 applied for 90 min. (B) MEPP amplitude recorded from mouse hemidiaphragms exposed to different APS12-2 concentrations, and normalized to that obtained before APS12-2 application. At concentrations higher than 0.68 μM, APS12-2 completely blocked MEPP amplitudes (not shown). (C) Effects of APS12-2 on MEPP frequency in the same preparation. Each bar represents the mean value ± SE obtained from 6 different nerve–muscle preparations. * indicates statistically significant difference ($P\leq0.05$).

Electrophysiological recordings from endplates of the mouse hemidiaphragm, exposed to APS12-2 (from 0.007 to 3.40 μM) for 45 and 90 min (Fig. 4A), revealed that APS12-2 significantly reduced the amplitude of full size EPPs in a concentration-dependent manner (Fig. 4B) with a calculated IC_{50} of 0.36 μM. No significant changes in the EPP half-decay time ($P>0.05$) were observed with APS12-2 in the range of concentration studied (from 0.007 to 3.40 μM). The effects of APS12-2 on MEPPs and EPPs were fully reversible at the concentration of 0.007 μM, and partially reversible at higher drug concentrations (not shown).

APS12-2 blocks Torpedo $\alpha1_2\beta1\gamma\delta$ nAChRs incorporated to Xenopus oocytes

APS12-2 alone at all concentrations used (0.001 to 2.5 nM) did not change the oocyte resting membrane conductance and membrane potential. When oocytes were exposed to ACh (50 μM) and a series of concentrations of APS12-2, a blockade of ACh-induced current was evident. ACh-evoked currents, recorded at a holding potential of −60 mV were inhibited by APS12-2 (0.001 to 2.5 nM) in a concentration-dependent manner as shown in Fig. 5A, and reversibly. Examples of the ACh-evoked currents are shown in Fig. 5A, either in the presence of ACh before, and after APS12-2 was applied together with ACh. APS12-2-induced ACh current inhibition on nAChRs expressed in *Xenopus* oocytes, showed no voltage-dependence (data not shown). The normalized concentration-dependent data were fitted by the four parameter non-linear ordinary fit method producing an IC_{50} of 0.55 nM (Fig. 5B). The Hill coefficient of the dose–response curve was 3.82, indicating stoichiometry of 4:1 interaction between *T. marmorata* nAChRs and APS12-2.

APS12-2 blocks in vivo the compound muscle action potential in mice

The major effect of an intramuscular injection of APS12-2 on the multimodal excitability properties of the mouse neuromuscular system was a time- and dose-dependent decrease of CMAP maximal amplitude, as exemplified in Fig. 6 for 2.2 μmol/kg mouse of compound injected, without significant modification of the other excitability parameters studied. This effect occurred within 15–30 min, depending on the APS12-2 dose injected, and was completely reversed within 2–3 h after compound injections. It is worth noting that the CMAP maximal amplitude remained stable before APS12-2 injections (Fig. 6B), or before and after PBS solution injections (Figs. 6A and B). This latter result indicates that the injection of the vehicle in which APS12-2 was dissolved had, by itself, no significant effect on the CMAP maximal amplitude.

Discussion

In a previous study we reported that APS12-2 toxicity to mice was low ($LD_{50}=11.5$ mg/kg). The rapid death of experimental animals, following intravenous injection, is the result of cardiorespiratory arrest due to APS12-2 hemolytic activity (Grandič et al., 2011). The data obtained in the present work on isolated neuromuscular preparations revealed that APS12-2 causes concentration-dependent block of nerve-evoked twitches and tetanic contractions. The neuromuscular block produced by the APS12-2 in the mouse hemidiaphragm occurs at lower equimolar concentrations than that reported for D-tubocurarine (DTC), a non-depolarizing antagonist of muscle type nAChRs ($IC_{50}=0.74$ μM *vs.* DTC 1.1 or 1.42 μM, respectively; Nguyen-Huu et al., 2005; Ridtitid et al., 1998). APS12-2 in low concentrations, without affecting frequency, decreased MEPP amplitude measured in superficial muscle fibers without a significant decrease of muscle fiber resting membrane potential indicating the postsynaptic activity of the compound. The neuromuscular block was not antagonized by the low concentration of neostigmine (1 μM) used, which does not inhibit completely AChE activity. If APS12-2 would exert

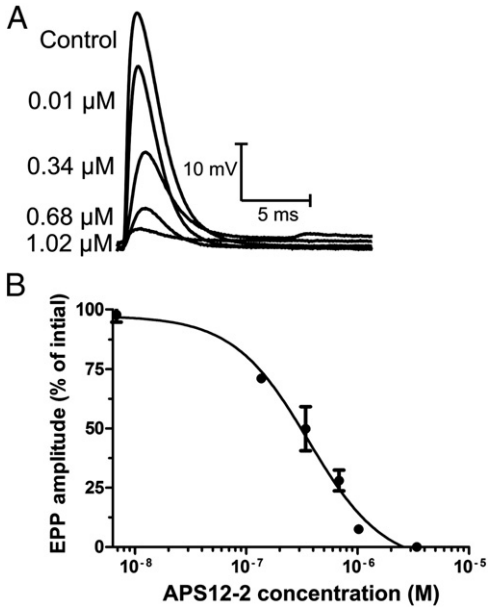


Fig. 4. APS12-2 blocks in a concentration-dependent manner full size endplate potentials recorded after blocking voltage-dependent sodium channels with 2 μM μ-conotoxin GIIIB. (A) Examples of EPP tracings recorded under control conditions (control), and 90 min after exposure of nerve–muscle preparation to APS12-2 (concentrations are indicated in front of the respective tracings). Note that the decrease of EPP amplitudes occurred without changes in the resting membrane potential of muscle fibers. (B) Block of full-size EPPs as a function of APS12-2 concentration on isolated mouse hemidiaphragm. Each point represents the mean value ± SE obtained from 3 different nerve–muscle preparations.

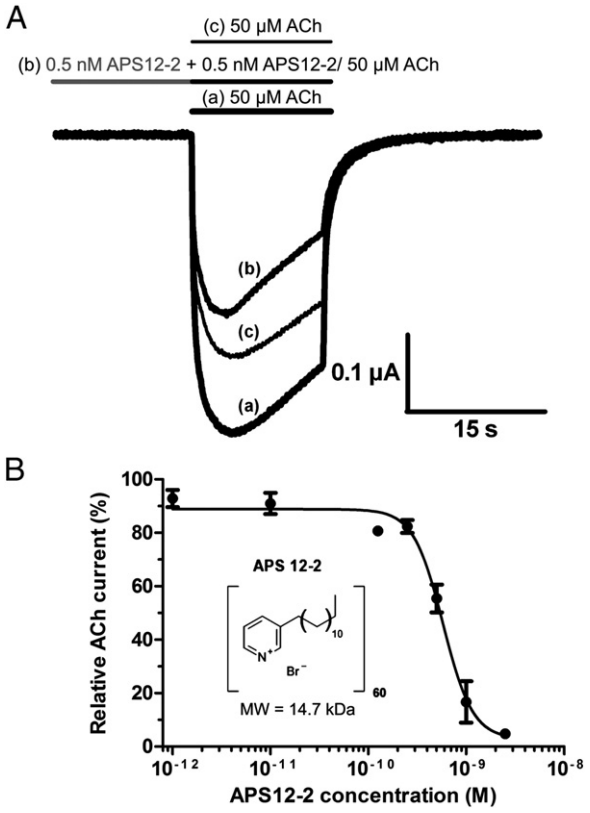


Fig. 5. Antagonistic effects of APS12-2 in *Xenopus* oocytes having incorporated into their membranes *Torpedo* muscle-type $\alpha 1_2\beta 1\gamma\delta$ nAChR. (A) Examples of ACh-evoked currents recorded before (50 μM ACh; trace a), during the action of 0.5 nM APS12-2 with 50 μM ACh (trace b), and after 150 s washout of APS12-2 molecules from the medium with 50 μM ACh (trace c). (B) Concentration-dependent inhibition of ACh-evoked currents by APS12-2 in oocytes having incorporated the *Torpedo* muscle-type nAChR. Amplitudes of the ACh current peak, recorded at a holding membrane potential of −60 mV in the presence of APS12-2 were normalized to control currents and fitted to the Hill equation. Each point represents the mean value ± SE of data obtained from 4 to 5 different oocytes.

an inhibitory action on endplate acetylcholinesterases it would be expected to prevent further neostigmine action. However, no evidence was obtained in the present study of an eventual anticholinesterase effect of APS12-2 which would result in an enhancement of twitch amplitude, and a prolongation of the decay phase of synaptic potentials (MEPPs and full-size EPPs), since the lack of AChE activity allows ACh to persist in the synaptic cleft and to activate repetitively nAChRs of the endplate (Van der Kloot et al., 1994), unless such an action is masked by the APS12-2-induced block of nAChRs which reduces availability of receptors for repetitive binding. The absence of evidence of an APS12-2-induced AChE inhibition, in contrast to previous studies (Houssen et al., 2010), may be due to the polycationic nature of APS12-2 leading to limited capacity of the compound to reach its targets. The muscle twitch blockade produced by APS12-2 was almost completely reversed (86%) by the potassium channel blocker 3,4-diaminopyridine, which increases ACh release from motor terminals (Hong and Chang, 1990; Molgó et al., 1980) and prolongs the duration of action potentials in skeletal muscle, thereby greatly improving skeletal muscle contractile performance (Khan and Lemeignan, 1983; Van Lunteren et al., 2008).

In order to determine possible direct effects of APS12-2 on sarcolemma or muscle excitation–contraction coupling processes, single twitch and tetanic responses of muscle with direct electrical stimulation were performed. After partial or complete block of nerve-evoked muscle contraction produced by APS12-2, muscle contraction of the same amplitude as before APS12-2 application could be evoked by direct electrical stimulation of muscle fibers. A direct effect of APS12-2 on muscle membrane excitability is not likely, since direct electrical stimulation produced muscle twitch responses similar in amplitude to those obtained before the application of APS12-2 in the same preparations. These results clearly indicate that APS12-2 has no direct effect on muscle fibers. Therefore, based on the described results from direct muscle stimulation experiments, a direct effect of APS12-2 on the sarcolemma or physiological processes (excitation–contraction coupling) leading to the block of contraction is unlikely. The muscle response to the direct electrical stimulation was reduced only at the highest concentration of APS12-2 used (2.72 μM; Fig. 1D). We anticipated that above 2.72 μM, APS12-2 could depolarize and inactivate voltage-dependent sodium channels in the muscle fibers, since it was shown that APS12-2 may permeabilize natural and artificial membranes in nanomolar concentrations (Grandič et al., 2012). This assumption was rejected in this study by the intracellular recordings of membrane potential in the muscle preparations exposed to different concentrations of APS12-2 and different times of exposition to the compound, clearly showing that APS12-2 has no effect on the resting membrane potential at all the concentrations used. In the following experiments, we showed that APS12-2 reduces MEPP and full-size EPP amplitudes, indicating a postsynaptic nAChR effect, since changes of postsynaptic potentials are proportional to the number of nAChR open channels. In concentrations insufficient to block neuromuscular transmission (Fig. 4A), APS12-2 significantly decreased MEPP amplitude (Fig. 3B) without affecting the resting membrane potential (Fig. 2), the rate of MEPP frequency (Fig. 3C) and their decay suggesting that this compound reduces the number of open nAChRs not by altering either muscle fiber passive membrane properties or neurotransmitter ACh release. Subsequent electrophysiological recordings demonstrated the high potency of APS12-2 to inhibit functional responses (nicotinic current) of muscle-type *Torpedo* nAChRs incorporated to *Xenopus* oocytes (Fig. 5). Differences in the calculated IC_{50} concentrations of APS12-2 on muscle contraction (IC_{50} = 0.74 μM) and full-sized EPPs (IC_{50} = 0.36 μM) are expected and may be related to the safety margin of neuromuscular transmission

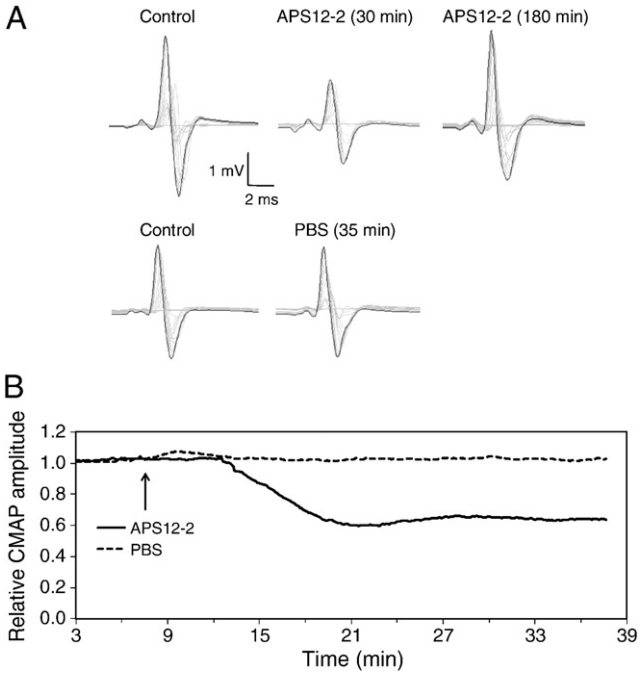


Fig. 6. *In vivo* effects of intramuscular injections of APS12-2 (2.2 μmol/kg mouse) and PBS solution on the multimodal excitability properties of mouse neuromuscular system. (A) Superimposed traces of the CMAP recorded from tail muscle following increasing intensities of caudal motor nerve stimulation, before and after injections of APS12-2 or PBS solution. (B) Time-course of the effects of APS12-2 or PBS solution injections on the CMAP maximal amplitude recorded continuously as a function of time. Values are expressed relatively to those before injections. The arrow indicates the time of injections.

(Wood and Slater, 2001). The fact that APS12-2 had an IC_{50} for ACh-induced current in *Xenopus* oocytes, lower than that obtained from measuring full size EPP amplitude in the mouse hemidiaphragm, may indicate a higher affinity of the compound for *Torpedo* ($\alpha1_2\beta1\gamma\delta$) than for the mouse ($\alpha1_2\beta1\gamma\varepsilon$) nAChR.

A number of blocking mechanisms have been proposed for the nAChRs. The simplest of them involves binding the drug molecule to the open channel leading to plugging and reduced current flow (Adams, 1976; Pascual and Karlin, 1998; Rozman et al., 2010). Another possible mechanism involves binding of competitive antagonists with the nAChRs at the end-plate and thereby competitive-blockade of the ACh binding, as it was shown for the DTC in low concentrations (Bowman, 2006). At higher concentrations, DTC antagonize the channel directly in a noncompetitive fashion (Colquhoun et al., 1979). The Hill coefficient derived from four parameter fits to concentration–response curves for APS12-2 calculated for oocytes expressing $\alpha1_2\beta1\gamma\delta$ nAChRs was 3.82, indicating that APS12-2 binds to the nAChRs in a respective molecular ratio of 4:1. A high Hill coefficient could also indicate possible allosteric effects of APS12-2 by interacting with regions other than the ACh binding site of nAChRs. Conductivity affected by APS12-2 was not voltage-sensitive, indicating that APS did not interact preferably with the open-state of nAChRs.

In conclusion, our results show that APS12-2 in the sub-micromolar range reduces indirectly evoked muscle contraction, MEPP and EPP amplitudes, and blocks native nAChRs. Our results also suggest that APS12-2 might act as a slowly dissociating antagonist of the nAChR. These inhibitory effects of APS12-2 on nerve-evoked diaphragm contraction may contribute to the respiratory arrest caused by the compound. Furthermore, when APS12-2 is locally applied *in vivo* to mice (by intramuscular injection), it is able to produce a marked and reversible inhibition of the CMAP recorded from the tail muscle in response to nerve stimulation, without significant modification of other excitability parameters, as expected if the compound blocks the muscle-type of nAChRs. It is worth noting that all the above effects may limit the use of APS12-2 as an anticancer drug since these effects and the antiproliferative action of the compound towards NSCLC cells occurred in the same concentration range.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors gratefully acknowledge the Slovenian Research Agency (grants P4-0053-0406-04, BI-FR/11-12-PROTEUS-002 and J1-4044), and the European 7th Framework Program (grant 2009-1/117 PHARMATLANTIC) for financial support. We thank Boštjan Drolc, Katarina Babnik, and Patricia Villeneuve for excellent technical support.

References

Adams, P.R., 1976. Drug blockade of open end-plate channels. J. Physiol. 260, 531–552.
Alderdice, M.T., 1982. Further comparison of the effects of physostigmine and neostigmine on frog neuromuscular transmission. Clin. Exp. Pharmacol. Physiol. 9, 35–43.
Bowman, W.C., 2006. Neuromuscular block. Br. J. Pharmacol. 147, 277–286.
Catassi, A., Servent, D., Paleari, L., Cesario, A., Russo, P., 2008. Multiple roles of nicotine on cell proliferation and inhibition of apoptosis: implication on lung carcinogenesis. Mutat. Res. 659, 221–231.
Chevessier, F., Girard, E., Molgó, J., Bartling, S., Koenig, J., Hantaï, D., Witzemann, V., 2008. A mouse model for congenital myasthenic syndrome due to MuSK mutations reveals defects in structure and function of neuromuscular junctions. Hum. Mol. Genet. 17, 3577–3595.
Colquhoun, D., Dreyer, F., Sheridan, R.E., 1979. The actions of tubocurarine at the frog neuromuscular junction. J. Physiol. 293, 247–284.
Cruz, L.J., Gray, W.R., Olivera, B.M., Zeikus, R.D., Kerr, L., Yoshikami, D., Moczydlowski, E., 1985. *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. J. Biol. Chem. 260, 9280–9288.
Egleton, R.D., Brown, K.C., Dasgupta, P., 2008. Nicotinic acetylcholine receptors in cancer: multiple roles in proliferation and inhibition of apoptosis. Trends Pharmacol. Sci. 29, 151–158.
Goodman Gilman, A., 2001. Goodman and Gilman's the Pharmacological Basis of Therapeutics, 11th ed. McGraw Hill, New York, pp. 193–206. 645–658.
Grandič, M., Sepčić, K., Turk, T., Juntes, P., Frangež, R., 2011. In vivo toxic and lethal cardiovascular effects of a synthetic polymeric 1,3-dodecylpyridinium salt in rodents. Toxicol. Appl. Pharmacol. 255, 86–93.
Grandič, M., Zovko, A., Frangež, R., Turk, T., Sepčić, K., 2012. Binding and permeabilization of lipid bilayers by natural and synthetic 3-alkylpyridinium polymers. Bioorg. Med. Chem. 20, 1659–1664.
Hong, S.J., Chang, C.C., 1989. Use of geographutoxin II (mu-conotoxin) for the study of neuromuscular transmission in mouse. Br. J. Pharmacol. 97, 934–940.
Hong, S.J., Chang, C.C., 1990. Facilitation by 3,4-diaminopyridine of regenerative acetylcholine release from mouse motor nerve. Br. J. Pharmacol. 101, 793–798.
Houssen, W.E., Lu, Z., Edrada-Ebel, R., Chatzi, C., Tucker, S., Sepčić, K., Turk, T., Zovko, A., Shen, S., Mancini, I., Scott, R.H., Jaspars, M., 2010. Chemical synthesis and biological activities of 3-alkyl pyridinium polymeric analogues of marine toxins. J. Chem. Biol. 3, 113–125.
Khan, R.A., Lemeignan, M., 1983. Effects of 3,4-diaminopyridine on mechanical and electrical responses of frog single muscle fibres. Acta Pharmacol. Toxicol. (Copenh) 52, 181–187.
Krieger, F., Mourot, A., Araoz, R., Kotzyba-Hibert, F., Molgó, J., Bamberg, E., Goeldner, M., 2008. Fluorescent agonists for the *Torpedo* nicotinic acetylcholine receptor. Chembiochem 9, 1146–1153.
Molgó, J., Lundh, H., Thesleff, S., 1980. Potency of 3,4-diaminopyridine and 4-aminopyridine on mammalian neuromuscular transmission and the effect of pH changes. Eur. J. Pharmacol. 61, 25–34.
Nguyen-Huu, T., Dobbertin, A., Barbier, J., Minic, J., Krejci, E., Duvaldestin, P., Molgó, J., 2005. Cholinesterases and the resistance of the mouse diaphragm to the effect of tubocurarine. Anesthesiology 103, 788–795.
Okonjo, K.O., Kuhlmann, J., Maelicke, A., 1991. A second pathway of activation of the *Torpedo* acetylcholine receptor channel. Eur. J. Biochem. 200, 671–677.
Olivera-Bravo, S., Ivorra, I., Morales, A., 2005. The acetylcholinesterase inhibitor BW284c51 is a potent blocker of *Torpedo* nicotinic AchR incorporated into the *Xenopus* oocyte membrane. Br. J. Pharmacol. 144, 88–97.
Paleari, L., Trombino, S., Falugi, C., Gallus, L., Carlone, S., Angelini, C., Sepčić, K., Turk, T., Faimali, M., Noonan, D.M., Albini, A., 2006. Marine sponge-derived polymeric alkylpyridinium salts as a novel tumor chemotherapeutic targeting the cholinergic system in lung tumors. Int. J. Oncol. 29, 1381–1388.
Pardo, N.E., Hajela, R.K., Atchison, W.D., 2006. Acetylcholine release at neuromuscular junctions of adult tottering mice is controlled by N-(Cav2.2) and R-type (Cav2.3) but not L-type (cav1.2) Ca^{2+} channels. J. Pharmacol. Exp. Ther. 319, 1009–1020.

Pascual, J.M., Karlin, A., 1998. Delimiting the binding site for quaternary ammonium lidocaine derivatives in the acetylcholine receptor channel. J. Gen. Physiol. 112, 611–621.

Ridtitid, W., Rattanaprom, W., Thaina, P., Chittrakarn, S., Sunbhanich, M., 1998. Neuromuscular blocking activity of methanolic extract of *Piper sarmentosum* leaves in the rat phrenic nerve–hemidiaphragm preparation. J. Ethnopharmacol. 61, 135–142.

Ros, E., Aleu, J., Gomez de Aranda, I., Cantí, C., Pang, Y.P., Marsal, J., Solsona, C., 2001. Effects of bis(7)-tacrine on spontaneous synaptic activity and on the nicotinic ACh receptor of *Torpedo* electric organ. J. Neurophysiol. 86, 183–189.

Rozman, K.B., Araoz, R., Sepčić, K., Molgo, J., Šuput, D., 2010. Parazoanthoxanthin A blocks *Torpedo* nicotinic acetylcholine receptors. Chem. Biol. Interact. 187, 384–387.

Sands, S.B., Costa, A.C., Patrick, J.W., 1993. Barium permeability of neuronal nicotinic receptor α7 expressed in *Xenopus* oocytes. Biophys. J. 65, 2614–2621.

Sepčić, K., Guella, G., Mancini, I., Pietra, F., Dalla Serra, M., Menestrina, G., Tubbs, K., Maček, P., Turk, T., 1997. Characterization of anticholinesterase-active 3-alkylpyridinium polymers from the marine sponge *Reniera sarai* in aqueous solutions. J. Nat. Prod. 60, 991–996.

Sherby, S.M., Eldefrawi, A.T., Albuquerque, E.X., Eldefrawi, M.E., 1985. Comparison of the actions of carbamate anticholinesterases on the nicotinic acetylcholine receptor. Mol. Pharmacol. 27, 343–348.

Sine, S.M., 2012. End-plate acetylcholine receptor: structure, mechanism, pharmacology, and disease. Physiol. Rev. 92, 1189–1234.

Turk, T., Sepčić, K., Mancini, I., Guella, G., 2008. 3-Alkylpyridinium and 3-alkylpyridine compounds from marine sponges, their synthesis, biological activities and potential use. In: Rahman, Atta-ur- (Ed.), Studies in Natural Product Chemistry; Bioactive Natural Products (Part O). Studies in Natural Products Chemistry, vol. 35. Elsevier, Amsterdam, pp. 355–397. [etc.].

Van der Kloot, W., Balezina, O.P., Molgó, J., Naves, L.A., 1994. 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, 114–126.

Van Lunteren, E., Moyer, M., Pollarine, J., 2008. Long-lasting in vivo inotropic effects of the K^+ channel blocker 3,4-diaminopyridine (DAP) during fatigue-inducing stimulation. Muscle Nerve 38, 1616–1622.

Wood, S.J., Slater, C.R., 2001. Safety factor at the neuromuscular junction. Prog. Neurobiol. 64, 393–429.

Zovko, A., Vaukner Gabrič, M., Sepčić, K., Pohleven, F., Jaklič, D., Gunde-Cimerman, N., Lu, Z., Edrada-Ebel, R., Houssen, W., Mancini, I., Defant, A., Jaspars, M., Turk, T., 2012. Antifungal and antibacterial activity of 3-alkylpyridinium polymeric analogs of marine toxins. Int. Biodeterior. Biodegrad. 68, 71–77.

Bioorganic & Medicinal Chemistry xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Binding and permeabilization of lipid bilayers by natural and synthetic 3-alkylpyridinium polymers

Marjana Grandič [a], Ana Zovko [b], Robert Frangež [a], Tom Turk [b], Kristina Sepčić [b,*]

[a] Institute of Physiology, Pharmacology and Toxicology, 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

ARTICLE INFO

Article history:
Received 7 November 2011
Revised 12 January 2012
Accepted 16 January 2012
Available online xxxx

Keywords:
3-Alkylpyridinium compounds
Hemolysis
Membrane permeabilization
Anticancer drugs
Transfection

ABSTRACT

Naturally occurring 3-alkylpyridinium polymers from the marine sponge *Reniera sarai* are membrane-active compounds exerting a selective cytotoxicity towards non small cell lung cancer cells, and stable transfection of nucleated mammalian cells. In view of their possible use as chemotherapeutics and/or transfection tools, three poly-APS based synthetic compounds were tested on their activity using natural and artificial lipid membranes. The tested compounds were found to be very stable over a wide range of temperature, ionic strength, and pH, and to prefer the solid-ordered membrane state. Their membrane-damaging activity increases with the length of their alkyl chains and the degree of polymerization.

1. Introduction

Water-soluble polymeric 3-alkylpyridinium salts (poly-APS), isolated from crude extracts of the Mediterranean marine sponge *Reniera sarai*, belong to the large group of about 80 different biologically active 3-alkylpyridinium and 3-alkylpyridine compounds found in several sponges of the order Haplosclerida.[1–4] Poly-APS are polymers with the molecular weight of 5520 Da, corresponding to 29 *N*-butyl-3-butyl pyridinium units, in which the 3-alkyl chain is bound head-to-tail to the nitrogen of the adjacent subunit.[5] They exert a very broad spectrum of biological activities, which is not surprising given that the activities of 3-alkylpyridinium compounds, as well as their potency, increase with the degree of polymerization. At concentrations above 0.23 mg/mL, poly-APS form large supramolecular aggregates with an average hydrodynamic radius of 23 ± 2 nm.[5] This behavior resembles that of other structurally related cationic detergents.[6]

At concentrations below 0.5 μg/mL, poly-APS have been described as being hemolytic, selectively cytotoxic against non small cell lung cancer cells, and to inhibit the enzyme acetylcholinesterase (AChE), the growth of marine bacteria, and the settlement of marine zoo- and phytoplankton on submerged surfaces (reviewed in[4]). Among the biological effects of poly-APS, their action on biological membranes is intriguing, as it can result in the formation of transient pores and stable transfection of nucleated mammalian cells with heterologous DNA, finding a possible use in gene therapy.[7] At higher concentrations, however (above 1 mg/mL) poly-APS can be toxic and lethal to rodents on intravenous application.[8]

In view of the possible use of poly-APS based synthetic compounds as transfection or chemotherapeutic agents, or as additives to environmental-friendly antifouling paints, several 3-alkylpyridinium oligomers and polymers (3-APS) have recently been synthesized, and their hemolytic, AChE-inhibitory, toxic, antibacterial, antifouling, antifungal, and transfection potential tested.[9–13] Among them APS12 and APS12-2, two large polymeric 1,3-dodecylpyridinium salts of 12.5 and 14.7 kDa molecular mass, were shown to be particularly interesting, as they are more potent and less toxic transfecting agents than the natural poly-APS.[11]

Previous studies have shown that the ability of poly-APS to form lesions in biological membranes can be explained, at least partially, by their surfactant-like characteristics and behavior in aqueous solutions. The hemolytic activity of poly-APS was analyzed and compared to that of the structurally-related monomeric cationic surfactants, cetylpyridinium chloride and cetyltrimethylammonium bromide. It was found that poly-APS induce the formation

Abbreviations: AChE, acetylcholinesterase; 3-APS, 3-alkylpyridinium salt; CH, cholesterol; CMC, critical micelle concentration; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; NSCLC, non small cell lung cancer cells; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPC+, 1-hexadecanoyl-2-(9Z-octadecanoyl)-*sn*-glycero-3-ethylphosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; SV, sonicated vesicles.

* Corresponding author. Tel.: +386 1 320 33 00; fax: +386 1 257 33 90.
E-mail addresses: marjana.grandic@vf.uni-lj.si (M. Grandič), ana.zovko@bf.uni-lj.si (A. Zovko), robert.frangez@vf.uni-lj.si (R. Frangež), tom.turk@bf.uni-lj.si (T. Turk), kristina.sepcic@bf.uni-lj.si (K. Sepčić).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2012.01.027

of discrete lesions (5.8 nm in diameter) in erythrocyte membranes by a colloid-osmotic type of lysis. Hemolysis was attenuated or prevented by various divalent cations and phosphatidic acid.[6] Recent studies of poly-APS induced transfection showed that this process was, surprisingly, more effective at lower temperatures (7–12 °C).[14] All these results suggest that the structure and physical properties of the membrane could greatly influence the poly-APS membrane activity. In this work, we have further elucidated these interactions, as well as the effects of different conditions on the stability of natural and synthetic 3-APS. The influence of the 3-APS structure (the compounds differ in the length of their alkyl chains and degree of polymerization) on their membrane activity was also studied. The results reported in this study are of interest in view of possible use of these compounds in medicine and/or industry.

2. Materials and methods

2.1. Materials

Natural polymeric alkylpyridinium salts (poly-APS) were purified from the marine sponge *Reniera sarai*,[5] and freeze-dried. Before use, they were dissolved in 140 mM NaCl, 20 mM Tris·HCl, pH 7.4 (erythrocyte buffer) or 140 mM NaCl, 20 mM Tris·HCl, 1 mM EDTA, pH 8.0 (vesicle buffer). 3-APS compounds (APS7, APS8 and APS12-2) were synthesized using a microwave-assisted polymerization procedure.[11,13] All the compounds were structurally characterized by NMR, and their molecular weights determined by the use of MALDI-TOF or ESIMS. Deconvoluted data of the high resolution weight spectral data of APS7 indicated a polymerization grade of m = 8 and 24 at a ratio of 2:1 for the monomer $C_{12}H_{18}N^+$.[13] APS8 and APS12-2 were proven to be of 11.9 and 14.7 kDa, respectivelly, which is consistent with 63 and 60 monomer units, respectively.[11] The structures of the used compounds are shown in Fig. 1. Wool grease cholesterol (CH), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) and 1-hexadecanoyl-2-(9Z-octadecanoyl)-*sn*-glycero-3-ethylphosphocholine ($POPC^+$) were from Avanti Polar Lipids (Alabaster, USA). All lipids were dissolved in chloroform or other organic solvents according to the manufacturer's instructions.

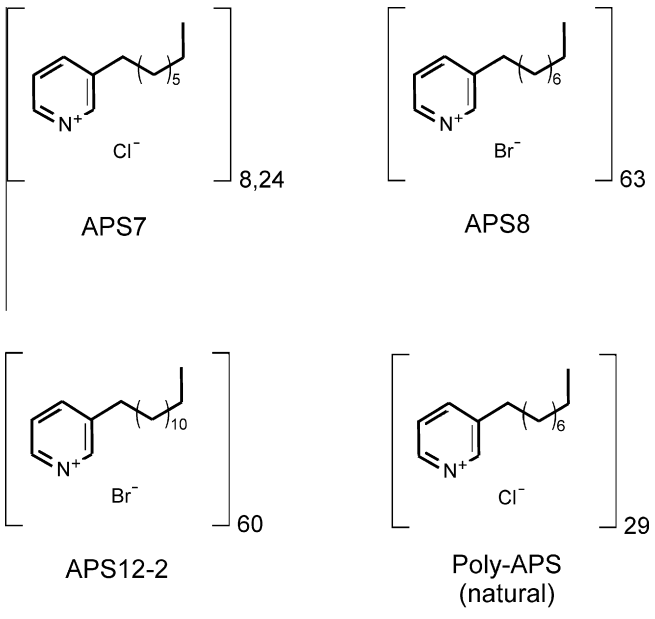


Figure 1. Chemical structures of 3-alkylpyridinium compounds used in this study.

Triton X-100, rhodamine 6G, and calcein were from Sigma. All chemicals were of the highest grade available.

2.2. Methods

2.2.1. Determination of critical micelle concentration

Critical micelle concentrations (CMCs), that is, the aggregation points of different 3-APS were determined using the fluorescent probe rhodamine 6G which was added, to give a final concentration of 10 μM, to vials containing 3-APS ranging from 0 to 1 mg/mL in water. The solutions were mixed and left in the dark for 48 h at, variously, room temperature, 4, 15, 25, or 40 °C. Samples were excited at 480 nm and their fluorescence emission recorded at 550 nm. Light scattering from similar samples was recorded at the excitation and emission wavelengths of 400 nm. In both experiments, a Jasco FP-750 spectrofluorometer (JASCO Ltd., Essex, UK), equipped with a water-thermostated cell holder and a 1 cm path length, magnetically stirred (830 rpm) quartz cuvette, was used. Slit widths with a nominal band-pass of 5 nm were used for both excitation and emission.

2.2.2. Preparation of sonicated vesicles (SV)

Lipid films were formed by removing the organic solvent from a lipid solution by rotary evaporation and vacuum drying. Lipids, at a final concentration of 10 or 5 mg/mL, were swollen in vesicle buffer or in 80 mM calcein, respectively, and vortexed vigorously to give multilamellar vesicles which were further exposed to 8 cycles of freezing and thawing and sonicated using a 750 Watt Ultrasonic Processor (Cole Parmer, USA) as described.[15] After centrifugation (20 min, 16,000*g*, 25 °C), the vesicles were incubated for 45 min at 40 °C. They were kept at 4 °C prior to use for no longer than 5 days. In the case of calcein-containing vesicles, extra-vesicular calcein was removed by gel filtration on a Sephadex G-50 column.

2.2.3. Hemolytic activity

Hemolytic activity was measured on rat erythrocytes by a turbidimetric method.[15] Rat erythrocytes were obtained by centrifugation of freshly collected citrated blood and washed 3–5 times with excess 0.9% saline and once with 140 mM NaCl, 20 mM Tris·HCl buffer, pH 7.4. Typically, 100 μL of 3-APS solution in erythrocyte buffer was added to 100 μL of rat erythrocyte suspension in the same buffer. The initial turbidity of the mixture at 630 nm was 0.5. The decrease of turbidity was recorded for 30 min using a kinetic microplate reader (Dynex Technologies, USA) to determine the time required for 50% hemolysis, t_{50}. The 3-APS concentration causing 50% lysis (e.g., the drop of the turbidity at 630 nm to 0.25) in 2 min was termed $HC_{0.5}$ (μg/mL). Unless otherwise stated, all the experiments were performed at 25 °C. For pH values ranging from 6.0 to 9.5, the erythrocyte buffer was adjusted to the required pH. Both erythrocytes and 3-APS were dissolved in the same buffer 5 min before the experiment, and the final concentrations of 3-APS in the reaction mixture were calculated to give the $HC_{0.5}$. The same strategy was used for monitoring the effect of ionic strength, where erythrocyte buffers containing different concentrations of NaCl (100, 140, 180, 220, 260, 300, 340, or 380 mM) were used. To assess the effect of sonication on different 3-APS, 2 mL of 3-APS solutions were sonicated using a 750 Watt Ultrasonic Processor (Cole Parmer, USA), output scale 4 and 50% duty cycle (room temperature) for 0, 15, 30, 45, 60, 90 or 120 min, and the hemolytic activity at their $HC_{0.5}$ concentrations assessed immediately and after 24 h. Temperature stability of 3-APS was determined by exposing 2 mL of 3-APS solutions to −196, 25, 50, 75, and 100 °C for 30 min and then the hemolytic activity was assessed. The effect of temperature on hemolytic activity was monitored by following the whole process of measuring hemolytic activity at 4, 10, 25, and 37 °C. In both cases, 3-APS were used at their $HC_{0.5}$ concentrations.

Percent of hemolysis was expressed as 100 − % of inhibition of hemolysis (= $At_{12}/(A_{max} - A_{min})$), where At_{12} is the turbidity at 12 min, A_{max} is maximal turbidity (initial turbidity of the lysing mixture at 630 nm), and A_{min} is the turbidity after total lysis of the erythrocytes resulting from the addition of Triton X-100 to a final concentration of 1 mM.

2.2.4. Inhibition of 3-APS induced hemolysis

Binding of 3-APS to SV with different lipid compositions (DOPC, POPC, DPPC, and POPC:cholesterol, POPC:POPS, and POPC:$POPC^+$ in 1:1 molar ratios) was estimated by measuring the residual hemolytic activity of unbound 3-APS, using a kinetic microplate reader (Dynex Technologies, USA). Typically, 100 μL of SV at various lipid concentrations (0–2.5 mg/mL) in erythrocyte buffer were pipetted onto a multiwell-plate. 50 μL of 3-APS (in final $HC_{0.5}$ concentrations) was added and the plate incubated for 30 min at 25 °C to allow binding of the 3-APS to SV. Hemolysis was then assayed by adding 100 μL of rat erythrocyte suspension in vesicle buffer. The decrease in turbidity at 630 nm was recorded for 30 min to determine the time necessary for 50% hemolysis ($t_{0.5}$). The lysing mixture had an initial turbidity of 0.5 at 630 nm.

2.2.5. Permeabilization of SV

Vesicle permeabilization was assayed using a Jasco FP-750 spectrofluorometer (JASCO Ltd., Essex, UK) equipped with a water-thermostated cell holder, and using 1 cm path length, magnetically stirred (830 rpm) quartz cuvette. Slit widths with a nominal band-pass of 5 nm were used for both excitation and emission. The excitation and emission wavelengths were set to 485 and 535 nm. 5–10 μL of calcein-loaded SV with different lipid compositions (DOPC, POPC, DPPC, or POPC:cholesterol, POPC:POPS, and POPC:$POPC^+$ in 1:1 molar ratios) were added to the cuvette containing 1 mL of filtered vesicle buffer, followed by an appropriate amount of 3-APS. The release of calcein was then recorded for 10 min at 25 °C. Maximal (100%) calcein release was obtained by solubilization of SV with Triton X-100, 1 mM final concentration, and the percentage of calcein release then calculated for each well.[16] To assess the influence of different parameters (temperature, ionic strength, pH, sonication) on 3-APS induced membrane permeabilization, POPC vesicles, and 3-APS, in concentrations inducing 50% calcein release from these vesicles under the above-mentioned conditions, were used. For pH values ranging from 6.0 to 9.5, we used the vesicle buffer adjusted to a specific pH. The effect of ionic strength was assessed using vesicle buffers with different final concentrations of NaCl (100, 140, 180, 220, 260, 300, 340, or 380 mM). To assess the effect of sonication on 3-APS, 2 mL of 3-APS solution were sonicated as described in the Section 2.2.3 for 0, 15, 30, 45, 60, 90, and 120 minutes, and their permeabilization potential assessed immediately, and after 24 h. Temperature stability of 3-APS was studied by exposing 2 mL of different 3-APS solutions to −196, 25, 50, 75, and 100 °C for 30 min, while the effect of temperature on vesicle permeabilization was monitored by following calcein release at 4, 25, and 37 °C.

3. Results and discussion

3.1. Determination of critical micelle concentration (CMC)

To assess their critical micelle concentrations (CMCs), 3-APS were tested in the concentration range of 0–1 mg/mL, and their CMCs determined by the use of rhodamine 6G (Fig. 2A), and light scattering (Fig. 2B), as described in the Section 2. The CMC of poly-APS at room temperature was found to be 0.33 mg/mL, slightly higher than that determined previously.[5] It increased with increasing temperature (Fig. 2B). The CMCs of synthetic 3-APS could not be determined (Fig. 2A), which suggests that their CMCs are higher than 1 mg/mL. Thus all the tested compounds exert their biological activities in their monomeric states.

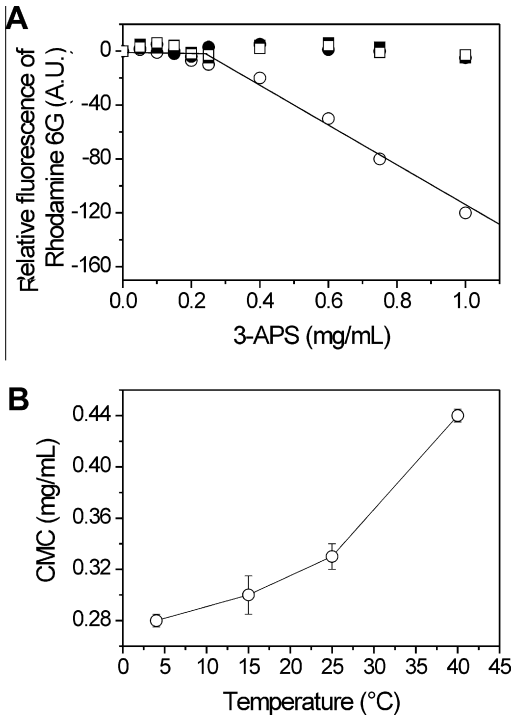


Figure 2. A: Determination of critical micelle concentration (CMC) for poly-APS (○), APS7 (●), APS8 (□) and APS12-2 (■) at 25 °C. 3-APS were tested in the concentration range of 0–1 mg/mL, and CMC was determined by using Rhodamine 6G as described in Section 2.2.1. B: Temperature-dependence of poly-APS CMC. The CMC was determined by recording the light scattering at excitation and emission wavelengths of 400 nm.

3.2. Influence of 3-APS structure on permeabilization of natural and artificial lipid membranes

Three synthetic 3-APS, differing in the length of their alkyl chains (7–12 C-atoms), degree of polymerization (8 to 63 monomeric subunits), and the nature of their counterions (chloride or bromide), were assayed for their ability to induce the formation of lesions in natural and artificial lipid membranes. Their activities were compared to that of the natural polymeric alkylpyridinium compound, poly-APS (Fig. 3A). Hemolysis rates of 1 s^{-1} for APS12-2, APS8, and APS7 were measured at 0.5, 1.2, and 7 μg/mL, respectively, indicating that the hemolytic activity increases with the increasing alkyl chain length. The membrane activity of these synthetic 3-APS also correlates with their degree of polymerization. However, all the three tested compounds were far more active than the natural compound, poly-APS.

A similar trend was observed when monitoring the membrane-permeabilizing activity on artificial lipid vesicles composed of POPC (Fig. 3B). All the compounds showed membrane-permeabilizing activities in a concentration range similar to that in the case of erythrocyte membranes, however the sequence of the compounds according to their permeabilization potential was slightly different. 50% calcein release was achieved by 0.35, 1, 1.2, and 10 μg/mL of APS8, APS12-2, APS7, and poly-APS, respectively. APS8 also proved to be the most potent membrane-permeabilizing agent in the case of artificial vesicles made of other pure phospholipids (DOPC, DPPC, Fig. 5) or POPC:POPS equimolar mixtures (not shown). However, when phospholipids (POPC, DPPC) were combined with an equimolar content of cholesterol, the permeabilizing

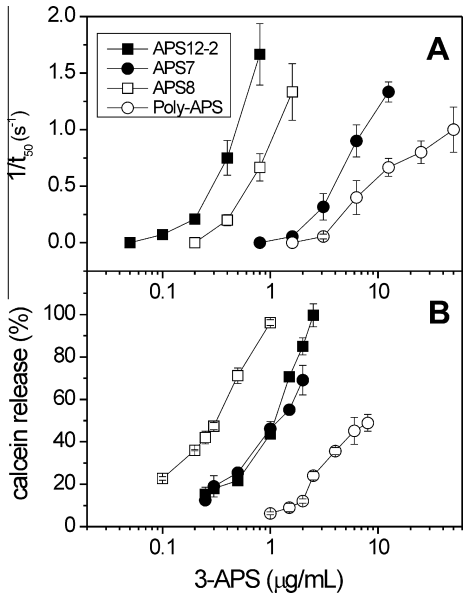


Figure 3. Hemolytic activity on rat erythrocytes (A) and permeabilization of calcein-loaded POPC vesicles (B) induced by different concentrations of APS12-2 (■), APS8 (□), APS7 (●), and poly-APS (○) at 25 °C. The experiments were performed as described in the Sections 2.2.3 and 2.2.5. $1/t_{50}$ = reciprocal half-time of hemolysis, i.e., time necessary to induce the lysis of 50% of the erythrocytes. Each point is the mean of three independent experiments ± SE.

ability of all the compounds was considerably lower, and APS12-2 was always the most active (Fig. 5). Overall, these results corroborate those obtained by other authors, showing that the membrane-disrupting potential of amphiphilic compounds correlates with their alkyl chain length and the number of positive charges.[17–19] The higher hemolytic potential of APS12-2 and APS8 could also be explained by the nature of their counterions, since the pyridinium salts containing Br^- were found to be more membrane-damaging than those containing chloride or other counterions.[20,21] Hydrated bromide ions, having the greater mobility and the smaller radii, could be more effective in modifying the membrane surface potential, thus enhancing the interaction between the pyridinium compound and the membrane.[20,21]

3.3. Influence of a lipid membrane phase on 3-APS activity

The lysis of red blood cells induced by poly-APS (Fig. 4) is temperature-dependent, with the lowest value observed at 4 °C, the maximum at 10 °C, and slightly decreasing hemolysis at 25 and 37 °C. Similar results, with an even more pronounced maximum at 10 °C, were obtained with bovine erythrocytes (not shown).

The same temperature trend was observed with all the three tested synthetic 3-APS. These results are in accordance with those of McLaggan et al.,[14] which showed more effective transfection of poly-APS at lower temperatures (7–12 °C), and also with the results obtained by other authors[21–24] on temperature-dependence of hemolytic activity of ionic detergents. The phase transition in erythrocyte membranes occurs between 18 °C and 25 °C,[25] indicating that at lower temperatures the erythrocyte membranes exist in a solid-ordered state that obviously facilitates 3-APS membrane activity. On the other hand, at higher temperatures (>37 °C), local rearrangements deriving from the formation of a multibilayer state, and subsequent exposition of lipid-free areas of the erythrocyte surface, facilitate a spontaneous membrane lysis.[26] The requirement of a solid ordered membrane phase for 3-APS activity is further confirmed by the results obtained with lipid vesicles, where all the tested 3-APS showed the highest activity with DPPC vesicles, followed by those composed from POPC and DOPC (Fig. 5). The fact that these lipids show phase transitions at 41 °C,[27] −3 °C,[28] and −19°C[29] respectively, indicates that only the DPPC ones exist in the solid ordered phase at room temperature. The 3-APS lytic potential is further reduced by the introduction of 50 mol % of cholesterol (Fig. 5). The addition of this lipid broadens the phase transition and leads to the formation of the so-called liquid ordered phase, in which membrane lipids are tightly packed, but still exhibit rapid lateral mobility.[30] At 25 °C, the POPC:cholesterol (1:1, mol:mol) vesicles exist in this state,[31] and are clearly the most resistant to the action of all studied 3-APS (Fig. 5). The liquid ordered phase is believed to be the physical state of membrane rafts—transient, dynamic and unstable cell membrane entities involved in several important biological functions, such as exocytosis and endocytosis, signal transduction, pathogen entry, and attachment of various ligands.[32–34] Our combined results suggest that 3-APS are excluded from lipid rafts when interacting with cell membranes.

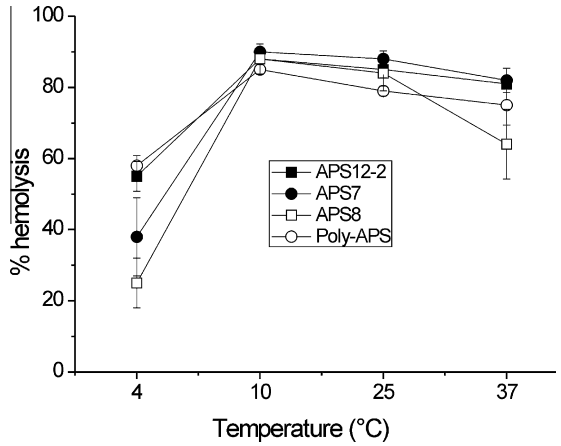


Figure 4. Temperature-dependence of 3-APS induced hemolysis. A suspension of rat erythrocytes was incubated for 2 h at each temperature, then APS12-2 (■), APS8 (□), APS7 (●), and poly-APS (○) at their final $HC_{0.5}$ concentration were added and hemolysis measured as described in the Section 2.2.3. Each point is the mean of three independent experiments ± SE.

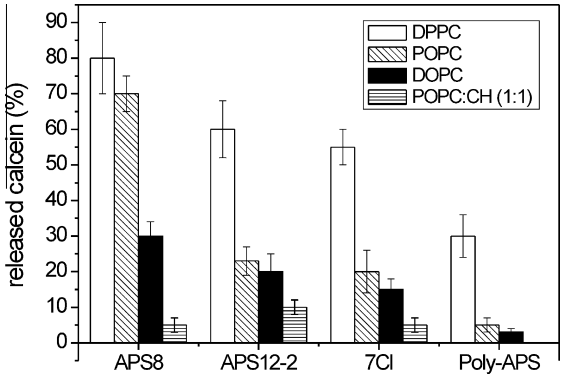


Figure 5. Effect of lipid composition on 3-APS induced permeabilization of calcein-loaded vesicles composed of phosphatidylcholines with different degrees of saturation of fatty acid chains (DOPC, POPC, DPPC), and equimolar mixture of POPC and cholesterol at 25 °C. The 3-APS concentration in all the experiments was 0.5 μg/mL. Each bar is the mean of three independent experiments ± SE. DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; CH, cholesterol.

3.4. Influence of membrane surface charge, ionic strength, and pH on 3-APS activity

The effect of the membrane surface charge on 3-APS binding was determined using the hemolysis-inhibition assay, by pre-incubating 3-APS with different concentrations of lipid vesicles. The results clearly indicate the importance of the charge for the initial membrane—3-APS interaction (Fig. 6A). The highest degree of poly-APS induced inhibition was exhibited by vesicles composed of POPC in combination with negatively charged phosphatidylserine (50% inhibition at 3 μg/mL SV), followed by those of pure POPC (50% inhibition at 30 μg/mL SV), and finally vesicles composed of POPC supplemented with a positively charged phosphatidylcholine, where no inhibition of hemolysis was observed up to 3 mg/mL of lipid vesicles. As discussed previously, addition of cholesterol significantly decreased the 3-APS binding step, and 50% inhibition of poly-APS induced hemolysis could be observed only at 350 μg/mL of POPC:cholesterol (1:1, mol:mol) SV. The above-described trends were similar with all other tested 3-APS (not shown). The hemolytic activity of poly-APS is abolished in the presence of negatively charged phosphatidic acid.[6] These combined results indicate that the preference of poly-APS for negatively charged membrane lipids results from their polycationic structure. This preference was clearly observed also in the further steps of 3-APS-membrane interactions, that is, in the process of membrane permeabilization, as shown for poly-APS in Fig. 6B.

Both 3-APS induced hemolysis (Fig. 7) and permeabilization of POPC vesicles (not shown) are slightly inhibited on increasing the ionic strength (in this case the NaCl concentration) of the buffer. This phenomenon strongly suggests charge shielding of the negatively charged groups at the membrane by Na^+, preventing the access of 3-APS to binding sites on the membrane, as already described for the inhibitory action by some divalent cations.[6,35]

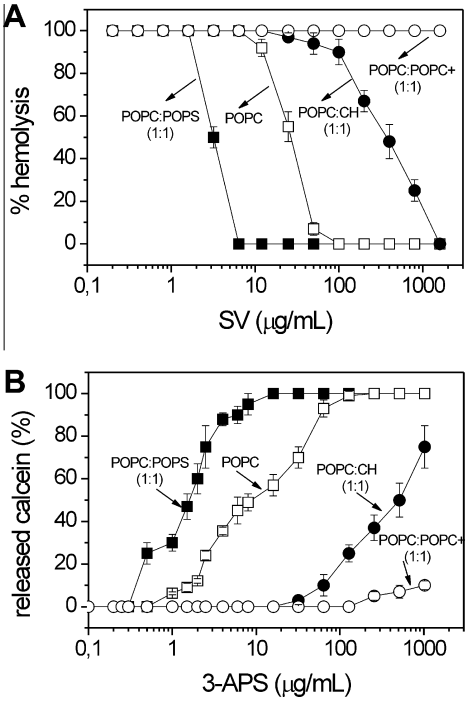


Figure 6. Inhibition of poly-APS induced hemolysis of rat erythrocytes by sonicated lipid vesicles (A), and poly-APS induced permeabilization of calcein-loaded sonicated lipid vesicles (B). The lipid vesicles were composed of POPC:POPS (1:1, mol:mol, ■), POPC (□), POPC : cholesterol (1:1, mol:mol, ●), and POPC:$POPC^+$ (1:1, mol:mol, ○). The experiments were performed at 25 °C, as described in the Sections 2.2.4 and 2.2.5. Each point is the mean of three independent experiments ± SE. POPS = 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine, $POPC^+$ = 1-hexadecanoyl-2-(9Z-octadecanoyl)-*sn*-glycero-3-ethylphosphocholine. Other abbreviations are as given in Fig. 5.

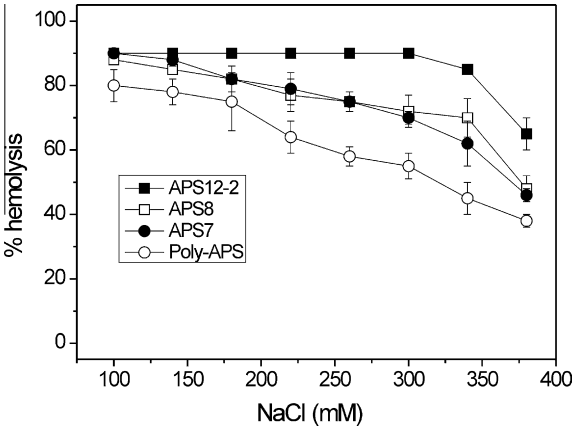


Figure 7. Effect of NaCl concentration on 3-APS induced hemolysis of rat erythrocytes. Erythrocyte buffer with different NaCl concentrations was used in the test. The concentrations of 3-APS used were 2 μg/mL (APS12-2, ■), 3.5 μg/mL (APS8, □), 8 μg/mL (APS7, ●), and 10 μg/mL (poly-APS, ○). Each point is the mean of three independent experiments ± SE.

On the other hand, the rates of hemolysis and vesicle permeabilization showed a steady, slow increase with increasing pH, as shown in Fig. 8 for hemolysis in the pH-range from 6 to 9.5. This could reflect stronger interactions of 3-APS with negatively charged groups at the membrane surface, exposed after their deprotonation at higher pH.

3.5. Influence of temperature and physical stress on 3-APS stability

Aqueous solutions of poly-APS are stable for months.[36] Synthetic 3-APS are also stable; their membrane-damaging activity was unaltered even after 1-hour exposure to 100 °C, or after five cycles of freezing–thawing (not shown). In contrast, sonication had a pronounced negative effect on their hemolytic and vesicle-permeabilizing activities, as shown in Fig. 9 for permeabilization of calcein-loaded POPC lipid vesicles. This effect was especially pronounced on poly-APS, which lost about 78% of its membrane activity after a 15-min sonication. However, this is unlikely to be due to sonication-induced disruption of the supramolecular

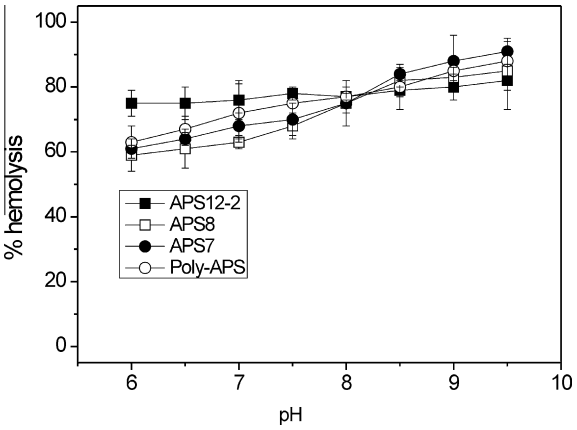


Figure 8. Effect of pH on 3-APS induced hemolysis of rat erythrocytes. Erythrocyte buffer with different pH values was used in the experiment. The concentrations of 3-APS used were 1.5 μg/mL (APS12-2, ■), 2.25 μg/mL (APS8, □), 6.3 μg/mL (APS7, ●), and 7 μg/mL (poly-APS, ○). Each point is the mean of two independent experiments ± SE.

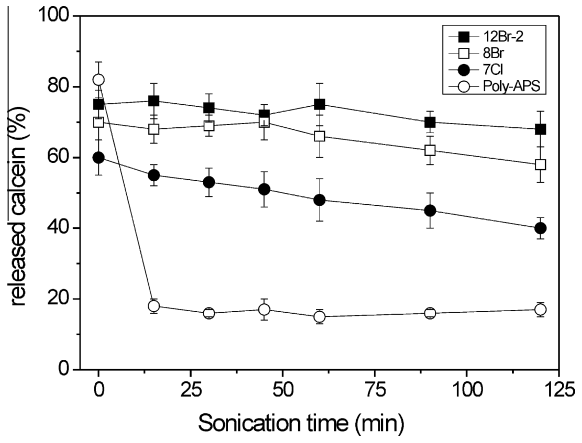


Figure 9. Effect of time of sonication on 3-APS induced permeabilization of sonicated vesicles composed of POPC. The concentrations of different 3-APS used in the test were 0.7 μg/mL (APS12-2, ■), 0.2 μg/mL (APS8, □), 0.7 μg/mL (APS7, ●), and 3 μg/mL (poly-APS, ○). Each point is the mean of three independent experiments ± SE.

structures that can be formed by these natural alkylpyridinium salts, since (i) the concentration of poly-APS used was far below their CMC (Fig. 2,[5]), and (ii) the loss of membrane-damaging potential was irreversible, that is, it did not recover in the next 24 h (not shown). Synthetic 3-APS were far more resistant, even to longer sonication times (Fig. 9).

4. Conclusions

The three tested synthetic 3-APS analogues are shown to be very stable over a wide range of temperature, ionic strength, and pH. Their membrane-damaging activity is greatly influenced by their structure, and increases with the length of their alkyl chains and the degree of polymerization. When interacting with artificial and natural lipid membranes, these compounds appear to prefer the solid-ordered membrane state. This could be the reason for their relatively low cytotoxicity to non-target cell membranes that, according to the current model of cell membrane structure, consist of co-existing liquid-disordered and liquid-ordered domains.[37] Recently, it was found that poly-APS exert a selective cytotoxicity towards non small cell lung cancer (NSCLC) cells, which are the most common form of lung cancer and are also linked to tobacco use.[38] These cells express molecules belonging to the cholinergic system, such as cholinoacetyltransferase, vesicular acetylcholine transporter and acetylcholinesterase, so the selective cytotoxicity of poly-APS probably derives from the interruption of the NSCLC cells' cholinergic system through acetylcholinesterase inhibition, resulting in their apoptosis. The cytotoxic concentrations of poly-APS against NSLC cells are hence significantly lower than those inducing lysis of other cell types. The results obtained in this study, combined with preliminary encouraging results on NSCLC cytotoxicity and the antifouling activity of synthetic 3-APS (to be published elsewhere), support these compounds as promising candidates for use in non-toxic antifouling paints, and/or in medicine as selective chemotherapeutics.

Acknowledgments

This work was supported by the Slovenian Research Agency (ARRS). The authors wish to thank Mrs. Irena Pavešič for excellent technical assistance, and Professor Roger Pain for critical reading of the manuscript.

References and notes

1. Almeida, A. M. P.; Berlinck, R. G. S. *Quim. Nova* **1997**, *20*, 170.
2. Sepčić, K. J. *Toxicol. Toxin Rev.* **2000**, *19*, 139.
3. Sepčić, K.; Turk, T. In *Antifouling Compounds, Progress in Molecular and Subcellular Biology, Subseries Marine Molecular Biotechnology*; Fusetani, N., Clare, A. C., Eds.; Springer: Berlin, Heidelberg, 2006; pp 105–124.
4. Turk, T.; Sepčić, K.; Mancini, I.; Guella, G. In *Studies in Natural Product Chemistry; Bioactive natural products (Part O), Studies in natural products chemistry*; Rahman, Atta-ur, Eds.; Elsevier: Amsterdam, 2008; Vol. 35, pp 355–397.
5. Sepčić, K.; Guella, G.; Mancini, I.; Pietra, F.; Dalla Serra, M.; Menestrina, G.; Tubbs, K.; Maček, P.; Turk, T. *J. Nat. Prod.* **1997**, *60*, 991.
6. Malovrh, P.; Sepčić, K.; Turk, T.; Maček, P. *Comp. Biochem. Physiol. C* **1999**, *124*, 221.
7. Tucker, S. J.; McClelland, D.; Jaspars, M.; Sepčić, K.; MacEwan, D. J.; Scott, R. H. *Biochim. Biophys. Acta* **2003**, *1614*, 171.
8. Turk, T.; Frangež, R.; Sepčić, K. *Mar. Drugs* **2007**, *5*, 157.
9. Mancini, I.; Sicurelli, A.; Guella, G.; Turk, T.; Maček, P.; Sepčić, K. *Org. Biomol. Chem* **2004**, *2*, 1368.
10. Faimali, M.; Garaventa, F.; Mancini, I.; Sicurelli, A.; Guella, G.; Piazza, V.; Greco, G. *Biofouling* **2005**, *21*, 49.
11. Houssen, W. E.; Lu, Z.; Edrada-Ebel, R.; Chatzi, C.; Tucker, S.; Sepčić, K.; Turk, T.; Zovko, A.; Shen, S.; Mancini, I.; Scott, R. H.; Jaspars, M. J. *Chem. Biol.* **2010**, *3*, 113.
12. Grandič, M.; Sepčić, K.; Turk, T.; Juntes, P.; Frangež, R. *Toxicol. Appl. Pharm.* **2011**, *255*, 86.
13. Zovko, A.; Vaukner Gabrič, M.; Sepčić, K.; Pohleven, F.; Jaklič, D.; Gunde-Cimerman, N.; Lu, Z.; Edrada-Ebel, R.; Houssen, W.; Mancini, I.; Defant, A.; 440 Jaspars, M.; Turk, T. *Int. Biodeter. Biodegrad.* **2012**, *68*, 71.
14. McLaggan, D.; Adjimatera, N.; Sepčić, K.; Jaspars, M.; MacEwan, D. J.; Blagbrough, I. S.; Scott, R. H. *BMC Biotechnol.* **2006**, *6*, 6.
15. Sepčić, K.; Berne, S.; Potrich, C.; Turk, T.; Maček, P.; Menestrina, G. *Eur. J. Biochem.* **2008**, *270*, 1199.
16. Menestrina, G. *FEBS Lett.* **1988**, *232*, 217.
17. Kleszczyńska, H.; Bielecki, K.; Sarapuk, J.; Bonarska-Kujawa, D.; Pruchnik, H.; Trela, Z.; Łuczyński, J. *Z. Naturforsch., C* **2009**, *61*, 715.
18. Zarif, L.; Riess, J. G.; Pucci, B.; Pavia, A. A. *Biomater. Artif. Cells Immobil. Biotechnol.* **1993**, *21*, 597.
19. Kuroda, K.; DeGrado, W. F. *J. Am. Chem. Soc* **2005**, *127*, 4128.
20. Sarapuk, J.; Kleszczyńska, H.; Rózycka-Roszak, B. *Biochem. Mol. Biol. Int.* **1998**, *44*, 1105.
21. Sarapuk, J.; Kleszczyńska, H.; Pernak, J.; Kalewska, J.; Rózycka-Roszak, B. *Z. Naturforsch., C* **1999**, *54*, 952.
22. Chernitsky, E. A.; Senkovich, O. A. *Membr. Cell. Biol.* **1997**, *11*, 475.
23. Hoyrup, P.; Davidsen, J.; Jorgensen, K. *J. Phys. Chem.* **2001**, *105*, 2649.
24. Davidsen, J.; Mouritsen, O. G.; Jorgensen, K. *Biochim. Biophys. Acta* **2002**, *1964*, 156.
25. Moore, D. J.; Sills, R. H.; Patel, N.; Mendelsohn, R. *Biochemistry* **1996**, *35*, 229.
26. Gershfeld, N. L.; Murayama, M. *J. Membr. Biol.* **1988**, *101*, 67.
27. Crow, J. H.; Tablin, F.; Tsvetkova, N.; Oliver, A. E.; Walker, N.; Crowe, L. M. *Cryobiology* **1999**, *38*, 180.
28. Filippov, A.; Orädd, G.; Lindblom, G. *Biophys. J* **2003**, *84*, 3079.
29. Munford, M. L.; Lima, V. R.; Vieira, T. O.; Heinzelmann, G.; Creczynski-Pasa, T. B.; Pasa, A. A. *Microsc. Microanal.* **2005**, *11*, 90.
30. Lichtenberg, D.; Goni, F. M.; Heerklotz, H. *Trends Biochem. Sci.* **2005**, *30*, 430.
31. de Almeida, R. F. M.; Fedorov, A.; Prieto, M. *Biophys. J.* **2003**, *85*, 2406.
32. Edidin, M. *Annu. Rev. Biophys. Biomol. Struct.* **2003**, *32*, 257.
33. London, E. *Curr. Opin. Struct. Biol.* **2002**, *12*, 480.
34. Simons, K.; Ikonen, E. *Nature* **1997**, *387*, 569.
35. McClelland, R. M.; Evans, I.; Abidin, I.; Sharma, S.; Choudhry, F. Z.; Jaspars, M.; Sepčić, K.; Scott, R. H. *Br. J. Pharm.* **2003**, *139*, 1399.
36. Eleršek, T.; Kosi, G.; Turk, T.; Pohleven, F.; Sepčić, K. *Biofouling* **2008**, *24*, 137.
37. McConnell, H. M.; Vrljic, M. *Annu. Rev. Biophys. Biomol. Struct.* **2003**, *32*, 469.
38. Paleari, L.; Trombino, S.; Falugi, C.; Gallus, L.; Carlone, S.; Angelini, C.; Sepčić, K.; Turk, T.; Faimali, M.; Noonan, D. M.; Albini, A. *Int. J. Oncol.* **2006**, *29*, 1381.

Toxicology in Vitro 27 (2013) 627–631

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Effects of synthetic analogues of poly-APS on contractile response of porcine coronary arteries

Marjana Grandič [a], Blanka Premrov Bajuk [a], Kristina Sepčić [b], Marinka Drobnič Košorok [a], Robert Frangež [a,*]

[a] Institute of Physiology, Pharmacology and Toxicology, 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

ARTICLE INFO

Article history:
Received 4 September 2012
Accepted 11 November 2012
Available online 20 November 2012

Keywords:
APS12-2
APS3
Porcine
Coronary
Cardiotoxicity

ABSTRACT

APS12-2 and APS3 are synthetic analogues of polymeric alkylpyridinium salts (poly-APS) isolated from the marine sponge *Reniera sarai*. The aim of the present study was to determine the possible direct contractile effects of these two synthetic molecules on coronary arteries, in order partly to explain hemodynamic and cardiotoxic effects of APS12-2 previously observed in *in vivo* studies and to reveal possible adverse effects on the organism in the case of their clinical use. In contrast to APS3, APS12-2 caused a concentration-dependent vascular smooth muscle contraction of isolated porcine coronary ring preparations in a concentration-range from 1.36 to 13.60 μM. Lanthanum chloride (5 mM) and verapamil (10 μM) completely abolished the APS12-2 evoked contraction of the coronary rings. Pre-incubation with indomethacin (10 μM) had no effect on the contractile responses of coronary ring preparations. These results indicate that APS12-2 contracts vascular smooth muscle in a concentration-dependent manner, due to an increase of Ca^{2+} influx through the voltage-gated Ca^{2+} channels. Our data show for the first time that APS12-2 induces concentration-dependent contraction of coronary ring preparations, which may contribute to the cardiotoxic effects of APS12-2, in addition to hyperkalemia.

1. Introduction

Poly-APS, water-soluble polymeric alkylpyridinium salts, which were originally purified from the marine sponge *Reniera sarai* (Sepčić et al., 1997), belong to a larger group of 3-alkylpyridine and 3-alkylpyridinium alkaloids that have been intensively investigated during the last 20 years. These compounds have been found to possess a variety of biological actions and to have possible applications in medicine and technologies of antifouling coatings (reviewed in Turk et al., 2008). In particular, the ability of nanomolar concentrations of poly-APS selectively to inhibit the proliferation of non-small cell lung cancer (NSCLC) cells (Paleari et al., 2006) has opened the possibility of their potential therapeutic use. Following research on the biological activity of the natural poly-APS compound, a series of synthetic oligomeric and polymeric alkylpyridinium salts (sAPS) was synthesized (Houssen et al., 2010; Mancini et al., 2004; Zovko et al., 2012) and their biological potential assessed, with several of them being found to be very promising for putative use in medicine. In particular, compounds with a higher degree of polymerization, such as 1,3-dodecylpyridinium bromide (APS12-2), were shown to be promising tools for stable transfection of nucleated mammalian cells with heterologous DNA (Houssen et al., 2010), and for inhibiting the growth of NSCLC cells (to be published elsewhere). In view of the possible use of APS12-2 and other sAPS as transfecting and/or anticancer agents, it is extremely important to evaluate their possible adverse effects on the cells, tissues and organs of the treated animals, and to detect their possible toxic and lethal effects. In fact, the natural poly-APS compound was found to be lethal for mice by causing cardiorespiratory arrest with a half lethal dose of 2.7 mg/kg (Bunc et al., 2002; Turk et al., 2007). It was recently shown that poly-APS diminished endothelium-dependent relaxation of isolated rat thoracic aorta in a 1 μM concentration and significantly decreased coronary flow in the heart (Lunder et al., 2012). In previous studies, we also showed that APS12-2 and APS3 increase arterial blood pressure and produce ECG signs related to myocardial hypoxia and arrhythmias. However, their toxicity in mice was found to be lower than that of the natural poly-APS, with respective LD_{50} values of 7.25 mg/kg and 11.5 mg/kg for APS3 (Grandič et al., 2012b) and APS12-2 (Grandič et al., 2011). Cardiotoxic action, especially in the case of APS12-2, could be related to increased coronary resistance associated with a drop of coronary blood flow and myocardial hypoxia, as previously shown for poly-APS (Lunder et al., 2012). The aim of the present study was therefore to evaluate the direct toxic effects *in vitro* of APS12-2 and APS3 on coronary arteries, and to determine

* Corresponding author.
E-mail address: robert.frangez@vf.uni-lj.si (R. Frangež).

0887-2333/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tiv.2012.11.012

their mechanism of action. The concentration-dependent contractile effects of APS3 and APS12-2 on coronary artery were first examined. Subsequently, starting with a concentration that produced a significant contractile response (6.8 μM), the pathways involved in the physiological contractile response were studied. Pharmacological blocking was used in order to highlight the cellular and molecular mechanism(s) of action of APS12-2 and APS3 responsible for their *in vivo* toxicity to mammals.

2. Materials and methods

2.1. Drugs and reagents

Substance P was dissolved in distilled water. Indomethacin was dissolved in absolute ethanol at a concentration of 20 mM. The volume of ethanol used in the experiments never exceeded 0.1% v/v and alone had no effect on the preparation response. Verapamil and lanthanum chloride were dissolved directly in Krebs–Henseleit solution. All chemicals used were purchased from Sigma Aldrich (Saint Luis, USA) and were of the highest grade available. APS12-2 and APS3 (Fig. 1) were synthesized using a microwave-assisted polymerization procedure (Houssen et al., 2010; Zovko et al., 2012).

Both compounds were structurally characterized by NMR and their molecular weights determined by the use of MALDI-TOF or ESIMS (Houssen et al., 2010; Zovko et al., 2012). APS12-2 is a 14.7 kDa polymer composed of sixty 1,3-dodecylpyridinium monomer units. APS3 is a mixture of two polymers with molecular weights of 1.2 and 3.8 kDa, corresponding to a polymerization grade of 10 and 32 covalently linked N-butyl-3-butyl pyridinium units in a 9:1 ratio, respectively. Both substances were dissolved in distilled water at stock solutions of 10 mg/mL and kept at 4 °C prior to use.

2.2. Preparation of coronary arterial rings

Hearts from adult pigs were collected daily at a local slaughter house and immersed in ice-cooled Krebs–Henseleit solution immediately after they had been removed. The hearts were transported to the laboratory within 30 min, where the left descending coronary artery was dissected 2–3 cm from its origin. Fat and connective tissue were removed and the artery was cut into 4 mm long rings. Rings were mounted between two stainless steel hooks connected to a mechano-electrical transducer (Itis, Slovenia). Electrical signals were amplified by P122 strain gage amplifier (Grass Instruments, West Warwick, RI, USA) and continuously digitized at a sampling rate of 200 Hz, using a data acquisition system (Digidata 1440A; Molecular Devices, Sunnyvale, CA, USA). Rings were immersed in a 10 mL organ bath filled with Krebs–Henseleit solution of the following composition (mM): 118 NaCl; 4.7 KCl; 2.5 $CaCl_2$; 1.6 $MgSO_4$; 1.2 KH_2PO_4; 24 $NaHCO_3$ and 10 D-glucose, pH 7.4, constantly oxygenized with 95% O_2 and 5% CO_2. Experiments were performed at 37 ± 0.1 °C. The arterial rings were equilibrated for 60 min, with the resting tension being set to 4 g (Kužner et al., 2004; Rattmann et al., 2012).

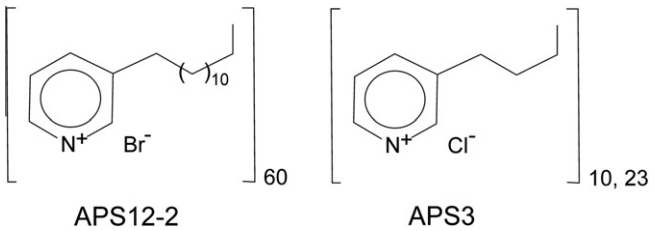


Fig. 1. Chemical structures of APS3 and ASP12-2.

2.3. Vascular reactivity studies

Initially, a set of experiments with increasing concentrations of potassium chloride (10, 20, 30, 40, 50, 60 and 70 mM) was performed and a concentration-response curve was obtained ($n = 8$ different preparations for each concentration of KCl used). The maximum increase in contractile response was elicited with 70 mM KCl. The subsequent experiments were performed with 40 mM KCl, which provoked 95% of the maximum contractile response. In each experiment, 40 mM KCl was first applied three times to produce stable contractions (Fig. 2). The last contraction was taken as a reference value (100%) and was used to calculate the amplitude of the sAPS-induced contraction. The presence of intact endothelium was assessed using 60 nM of substance P applied on rings pre-contracted with KCl. Substance P is a neuropeptide producing endothelium-dependent vasodilatation of pig coronary arteries due to activation of different K_{Ca} currents and membrane hyperpolarization (Frieden et al., 1999), and also induces relaxation in smooth muscle *via* the NK1 receptor (Cathieni et al., 1999). The concentration-dependent responses of arterial rings to APS12-2 and APS3 were assessed in the second set of experiments. APS12-2 was used at concentrations of 1.4, 2.7, 4.1, 6.8 and 13.6 μM ($n = 4–8$ different preparations for each concentration of APS12-2 used), while APS3 was used at concentrations of 41.1, 68.5 and 137 μM ($n = 4–8$ different preparations for each concentration of APS3 used). Selected dose range corresponds to the sub-

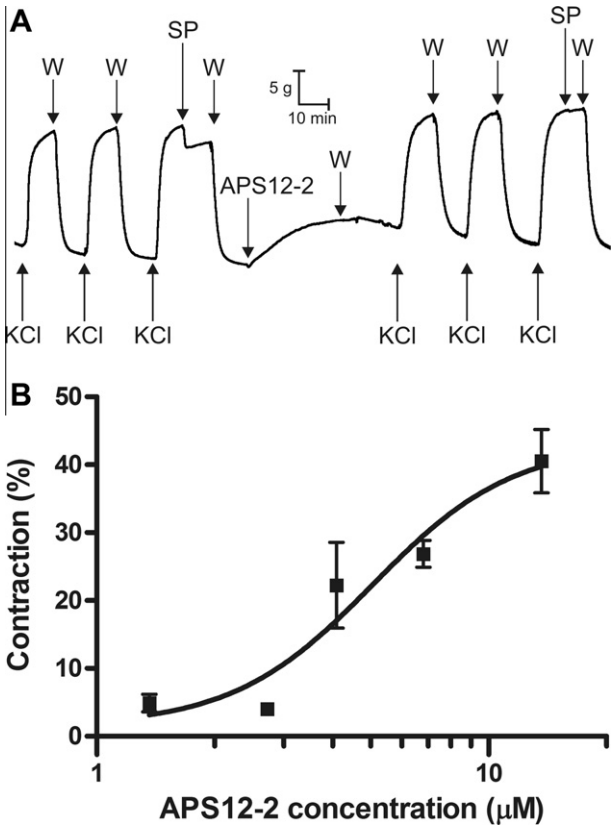


Fig. 2. Effects of APS12-2 on isolated coronary ring tension. (A) Representative isometric contractile response of porcine coronary ring preparations to high external K^+ (40 mM), APS12-2 (13.60 μM), and relaxation response of high potassium-pre-contracted coronary ring to SP (60 nM). (B) Concentration-dependent contractile response curve for coronary ring contraction elicited with APS12-2 expressed as a percent of the maximum twitch response to 40 mM KCl ($n = 32$). Values are expressed as the mean ± SE ($n = 4–8$ different preparations for each concentration of APS12-2 used). Abbreviations used: KCl – potassium chloride, W – washout, and SP – substance P.

lethal and lethal concentrations of APS12-2 and APS3 in blood and extracellular fluid calculated from *in vivo* experiments in rats injected with different doses of substances tested (Grandič et al., 2011, 2012b). After application of sAPS and wash out, potassium chloride (3×) and substance P (1×) were applied again to check the contractility response and preservation of the intact endothelium (Fig. 2). In the third set of experiments, three different inhibitors were used to determine the mechanisms of APS12-2–induced contraction of the arterial rings. Before treatment with APS12-2, the arterial rings were incubated for 15 min with the following inhibitors: verapamil (10 μM; $n = 6$), lanthanum chloride (5 mM; $n = 6$) and indomethacin (10 μM; $n = 8$).

2.4. Data analysis and statistics

Data were statistically analyzed using Sigma Plot for Windows, version 11.0 (Systat Software Inc., USA). The percentage of contraction produced by sAPS was calculated based on the maximum contractile response obtained with 40 mM KCl. One-way analysis of variance (ANOVA) was performed with the Bonferroni test to assign differences between group comparisons when an overall significance ($P < 0.05$) was attained. Data are presented as mean values ± SE.

3. Results

3.1. Effects of sAPS on the tension of coronary arterial rings with preserved endothelium

No spontaneous contractile responses of coronary ring preparations were observed under control conditions (not shown). Fig. 2A shows representative recordings of changes in tension induced by 40 mM KCl and 13.6 μM APS12-2. Exposure of the coronary rings to 40 mM KCl produced a rapid increase in ring tension followed by a plateau phase, which is assumed to represent a 100% contractile response. Expressed as a percentage of the maximum porcine coronary ring tension following depolarization evoked by 40 mM potassium, APS12-2 provoked a response of 22.2 ± 6.3% at 4.1 μM, and reached a maximum contractile response of 40.5 ± 4.6% at 13.6 μM (Fig. 2B). APS3 in a concentration up to 137 μM had no effect on resting coronary muscle tension (not shown). In contrast to APS3, APS12-2 induced a concentration-dependent increase in coronary ring tension (Fig. 2B). After washout of the preparation, the tension returned smoothly to the basal level (Fig. 2A). Contractile responses to 40 mM KCl were 8.4 ± 2 g, and to the highest APS12-2 concentration tested (i.e., 13.6 μM) were 3.7 ± 0.6 g. A noticeable effect was the ability of APS12-2 to diminish the endothelium-mediated relaxation of coronary ring preparations (pre-contracted with 40 mM KCl) with substance P (Fig. 2A).

Since the smooth muscle contraction is mostly dependent on the influx of Ca^{2+} from the extracellular space, a series of coronary arterial ring preparations were pretreated with lanthanum chloride ($LaCl_3$), a non-selective blocker of cationic channels ($P < 0.05$, $n = 6$). $LaCl_3$ (5 mM) completely abolished the APS12-2-induced contractile response (Fig 3). In order to assess the role of voltage-dependent Ca^{2+} channels and to estimate their role in APS12-2–induced smooth muscle contraction, vascular preparations were exposed to 10 μM verapamil, which selectively blocks L-type voltage dependent calcium channels (VDCC; $P < 0.05$, $n = 6$). It can clearly be seen in Fig. 3 that the contraction of coronary vessel rings pretreated with verapamil was also completely prevented when 6.80 μM APS12-2 was applied. The role of cyclooxygenase (COX) derived prostanoids in vasoconstrictor responses of APS12-2 using the non-selective COX inhibitor indomethacin in a concentration 10 μM was also examined ($P < 0.05$, $n = 8$). Indomethacin had no significant effect on APS12-2–evoked vascular muscle contractions (Fig. 3). The effects of indomethacin, verapamil and $LaCl_3$ on APS12-2–induced smooth muscle contractions are summarized in Fig. 3.

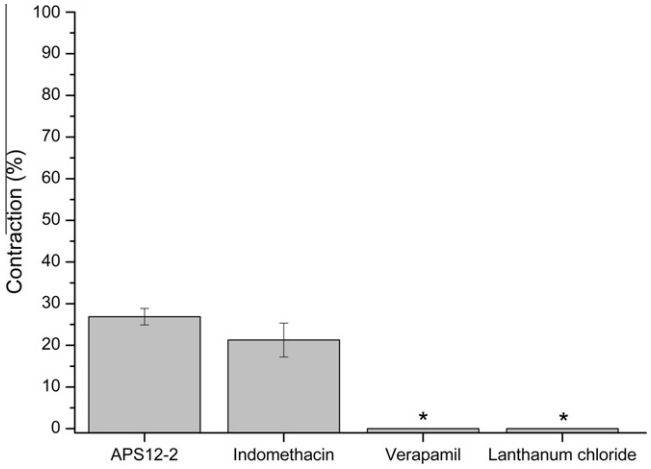


Fig. 3. Effect of different substances on APS12-2-induced (6.8 μM) contraction of porcine coronary artery. Ring tension induced by 6.80 μM APS12-2 is expressed as % of the maximum artery tension evoked by 40 mM KCl. Coronary rings were incubated for 30 min before the addition of inhibitors in the following final concentrations: verapamil, 10 μM ($n = 6$); lanthanum chloride 5 mM ($n = 6$) and indomethacin 10 μM ($n = 8$). In the control experiments (APS12-2), the contractility of arterial rings was determined in the absence of inhibitors. Results are expressed as mean ± SE. Statistically significant differences ($P \leqslant 0.05$) from the control value are indicated by asterisks.

4. Discussion

APS12-2 administered to rats at sub-lethal doses (4 and 5.5 mg/kg body weight) caused a decrease of arterial blood pressure followed by a persistent increase above the basal values. In a lethal dose (11.5 mg/kg), APS12-2 produced a progressive, irreversible drop of arterial blood pressure, accompanied by signs of myocardial hypoxia and arrhythmias (Grandič et al., 2011). In contrast to APS12-2, APS3 produced a reversible transient drop of arterial blood pressure without arrhythmias and exerted no cardiotoxic action at concentrations up to 20 mg/kg in rats (Grandič et al., 2012b). In order to study the effects of APS12-2 and APS3, better to reveal the mechanisms of APS12-2 in cardiovascular effects and to provide more data on mechanistic specificity, *in vitro* experiments were performed on isolated porcine coronary vessels. APS12-2 induced concentration-dependent, sustained contraction of isolated porcine coronary artery rings, which *in vivo* may significantly contribute to its cardiotoxic effect. Since concentrations between 4.1 and 13.6 μM in the muscle tension study produced a maximum contractile response of vascular smooth muscle (Fig. 2B), it can be concluded that APS12-2 may contribute to hemodynamic changes or even fatal cardiorespiratory arrest in rats. However, the interpretation of these results needs some caution in view of the use of porcine coronary ring preparations as a model for APS12-2 testing. On the other hand, APS3 in a single dose of 20 mg/kg, or in cumulative doses up to 60 mg/kg, produced only small and transient, although significant changes of arterial blood pressure (Grandič et al., 2012b). In this study, we used high concentrations of APS3 (from 41.1 to 137 μM). It can therefore be concluded that the intrinsic capacities of each sAPS molecule to permeabilize lipid vesicles and natural lipid membranes play an important role, as has already been shown (Grandič et al., 2012a). In addition, the biological actions of alkylpyridinium compounds are greatly influenced by their structure. Most of these

actions, including the effect on lipid vesicles and natural membranes, increase with the length of the compound's alkyl chains, with the degree of polymerization and with the presence of a bromine counterion (Grandič et al., 2012a). On the basis of these observations, it is not surprising that APS12-2, a bromide containing twelve carbon atoms in the alkyl chains, exerted a significantly more pronounced biological effect than APS3, which is a chloride with a three-carbon alkyl tail.

Since we did not obtain any contractile or relaxing response of vascular smooth muscle in concentrations of APS3 up to 137 μM, only APS12-2 was further used for evaluation of the mechanism of action.

The main trigger in vascular smooth muscle contraction is the increase of cytosolic Ca^{2+} activity due to an influx of Ca^{2+} from the extracellular space and from intercellular stores (Elmoselhi and Grover, 1997; Kuriyama et al., 1998). The vascular muscle contraction observed in the presence of APS12-2 may be due to Ca^{2+} influx through existing membrane conductance or through the formation of large transient membrane pores produced by APS12-2 (Houssen et al., 2010; McClelland et al., 2003). In order to test these hypotheses in vascular smooth muscle, we blocked the existing Ca^{2+} conductance and influx of Ca^{2+} by pre-treating vascular preparations with a non-selective cation channels blocker $LaCl_3$. $LaCl_3$ non-selectively prevents Ca^{2+} influx from the extracellular space through both voltage dependent L-type calcium channels (VDCC) and non-selective cation channels (NSCC), by blocking Ca^{2+} influx through the cell membrane (Hogestatt and Andersson, 1984; Kasai and Neher, 1992). Since it has been shown that both natural poly-APS and synthetic APS12-2 increase Ca^{2+} activity in human embryonic kidney cell line (HEK 293) cells and rat hippocampal and DRG neurons, due to the formation of large transient pores in cellular membranes (Houssen et al., 2010; McClelland et al., 2003), we anticipated that APS12-2 could permeabilize the cellular membrane of vascular smooth muscle cells, causing Ca^{2+} influx and producing contraction. $LaCl_3$ completely abolished APS12-2–evoked contractions of vascular smooth muscle, suggesting either the formation of conductive pores by APS12-2, which can be blocked by $LaCl_3$, or activation of NSCC and/or VDCC, which may contribute to the contractile effect of APS12-2. In subsequent experiments, in which vascular smooth muscles were pretreated with verapamil (10 μM), the selective antagonist of L-type of VDCC, contractions were also prevented, which thus clearly demonstrated that the high potency of APS12-2 to contract vascular smooth muscle is mediated *via* activation of voltage-dependent L-type Ca^{2+} channels. One possible mechanism might be membrane depolarization, as has been shown for poly-APS and activation of VDCC. However, the direct effects of APS12-2 on L-type VDCC cannot be excluded. It is also worth mentioning that the relaxing response to substance P, which induces relaxation in smooth muscle *via* the NK1 receptor (Cathieni et al., 1999), was neutralized by APS12-2. In order to reveal a possible vasoconstrictive role of eicosanoids in APS12-2–induced smooth muscle contraction, coronary arteries were pre-incubated with a non-selective inhibitor of cyclooxygenases (COX-1,2), enzymes that convert arachidonic acid into prostaglandins. Constitutively expressed COX-1 is a preferential enzyme that mediates endothelium-dependent contractions in large arteries of rats and mice induced by synthesis of endothelium-derived contracting factors (EDCFs) or contractile prostaglandins, which can also be synthesized in smooth muscle cells (for detailed review, see Wong and Vanhoutte, 2010). Pre-incubation of coronary arteries with the non-selective COXs inhibitor indomethacin had no effect on APS12-2–induced smooth muscle contraction, indicating that prostanoids have no important role in vasoconstriction induced by this compound.

In conclusion, the APS12-2–induced contraction of porcine coronary arterial ring preparation is mainly associated with Ca^{2+} influx from the extracellular space through L-type voltage-dependent calcium channels. Cation non-selective conductances in coronary smooth muscle cells that are La^{3+}-sensitive probably do not contribute significantly to the contractile response of APS12-2, since the maximum amplitude of the APS12-2 contractile response can be completely blocked by verapamil, an L-type calcium channel blocker. Another possibility, according to which APS12-2 produces new cation-permeable pores in smooth muscle cells sensitive to $LaCl_3$ and verapamil, seems less likely. Moreover, in addition to the contractile response, APS12-2 can also abolish the functional relaxing response of vascular muscle preparation to substance-P, suggesting that APS12-2 induces an alteration in this response mechanism. The final maximum concentration of APS12-2 (13.60 μM), producing a significant increase in coronary ring tension *in vitro*, is comparable to the maximum calculated concentration of APS12-2 in the blood (plasma) *in vivo* after injection of one LD_{50}, which produced arrhythmias and cardiorespiratory arrest. Vascular muscle contraction leads to vasospasm and related myocardial hypoxia. Additionally, the relaxing response of arterial smooth muscle is compromised during and after its exposure to APS12-2. All these mechanisms play an important role in the cardiotoxicity of APS12-2.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors gratefully acknowledge the Slovenian Research Agency (Grants P4-0053-0406-04). The authors would like to thank Katarina Babnik and Boštjan Drolc for their excellent technical assistance and Martin Cregeen for English proofreading of the manuscript.

References

Bunc, M., Strupi Šuput, J., Vodovnik, A., Šuput, D., 2002. Toxic effects of head-to-tail 3-alkylpiridinium polymers isolated from the marine sponge Raniera sarai in rat. Toxicon 40, 84–849.

Cathieni, M.M., Taban, C.H., Sors, P., Mastrangelo, D., 1999. Binding of gold– protein ligand complexes to neurokinin receptors. Visualization with the electron microscope on pig coronary artery and rat portal vein. J. Vasc. Res. 36, 59–67.

Elmoselhi, A.B., Grover, A.K., 1997. Endothelin contraction in pig coronary artery: receptor types and Ca(2+)-mobilization. Mol. Cell. Biochem. 176, 29–33.

Frieden, M., Sollini, M., Bény, J.L., 1999. Substance P and bradykinin activate different types of KCa currents to hyperpolarize cultured porcine coronary artery endothelial cells. J Physiol. 519, 361–371.

Grandič, M., Sepčić, K., Turk, T., Juntes, P., Frangež, R., 2011. In vivo toxic and lethal cardiovascular effects of a synthetic polymeric 1,3-dodecylpyridinium salt in rodents. Toxicol. Appl. Pharmacol. 255, 86–93.

Grandič, M., Zovko, A., Frangež, R., Turk, T., Sepčić, K., 2012a. Binding and permeabilization of lipid bilayers by natural and synthetic 3-alkylpyridinium polymers. Bioorg. Med. Chem. 20, 1659–1664.

Grandič, M., Araoz, R., Molgo, J., Turk, T., Sepčić, K., Benoit, E., Frangež, R., 2012b. Toxicity of the synthetic polymeric 3-alkylpyridinium salt (APS3) is due to specific block of nicotinic acetylcholine receptors. Toxicology. http://dx.doi.org/10.1016/j.tox.2012.10.013.

Hogestatt, E.D., Andersson, K.E., 1984. Mechanisms behind the biphasic contractile response to potassium depolarization in isolated rat cerebral arteries. J. Pharmacol. Exp. Ther. 228, 187–195.

Houssen, W.E., Lu, Z., Edrada-Ebel, R., Chatzi, C., Tucker, S.J., Sepčić, K., Turk, T., Zovko, A., Shen, S., Mancini, I., Scott, R.H., Jaspars, M., 2010. Chemical synthesis and biological activities of 3-alkyl pyridinium polymeric analogues of marine toxins. J. Chem. Biol. 3, 113–125.

Kasai, H., Neher, E., 1992. Dihydropyridine-sensitive and omega-conotoxin-sensitive calcium channels in a mammalian neuroblastoma-glioma cell line. J. Physiol. 448, 161–188.

Kuriyama, K., Kitamura, T., Itoh, R., 1998. Physiological features of visceral smooth muscle cells, with special reference to receptors and ion channels. Physiol. Rev. 78, 811–920.

Kužner, J., Drevenšek, G., Geršak, B., Budihna, M., 2004. Hypoxic and pharmacological preconditioning preserves vasomotor response of porcine coronary artery. Pol. J. Pharmacol. 56, 789–797.

Lunder, M., Drevenšek, G., Hawlina, S., Sepčić, K., Žiberna, L., 2012. Cardiovascular effects induced by polymeric 3-alkylpyridinium salts from the marine sponge Reniera sarai. Toxicon 2012 (60), 1041–1048.

Mancini, I., Sicurelli, A., Guella, G., Turk, T., Maček, P., Sepčić, K., 2004. Synthesis and bioactivity of linear oligomers related to polymeric alkylpyridinium metabolites from the Mediterranean sponge Reniera sarai. Org. Biomol. Chem. 2, 1368–1375.

McClelland, D., Evans, R.M., Abidin, I., Sharma, S., Choudhry, F.Z., Jaspars, M., Sepčić, K., Scott, R.H., 2003. Irreversible and reversible pore formation by polymeric alkylpyridinium salts (poly-APS) from the sponge Reniera sarai. Brit. J. Pharmacol 139, 1399–1408.

Paleari, L., Trombino, S., Falugi, C., Gallus, L., Carlone, S., Angelini, C., Sepčić, K., Turk, T., Faimali, M., Noonan, D.M., Albini, A., 2006. Marine sponge-derived polymeric alkylpyridinium salts as a novel tumor chemotherapeutic targeting the cholinergic system in lung tumors. Int. J. Oncol. 29, 138–1388.

Rattmann, Y.D., Anselm, E., Kim, J.H., Dal-Ros, S., Auger, C., Miguel, O.G., Santos, A.R., Chataigneau, T., Schini-Kerth, V.B., 2012. Natural product extract of dicksonia sellowiana induces endothelium-dependent relaxations by a redox-sensitive Src- and Akt-dependent activation of eNOS in porcine coronary arteries. J. Vasc. Res. 49, 284–298.

Sepčić, K., Guella, G., Mancini, I., Pietra, F., Dalla Serra, M., Menestrina, G., Tubbs, K., Maček, P., Turk, T., 1997. Characterization of anticholinesterase-active 3-alkylpyridinium polymers from the marine sponge Reniera sarai in aqueous solutions. J. Nat. Prod. 60, 991–996.

Turk, T., Frangež, R., Sepčić, K., 2007. Mechanisms of toxicity of 3-alkylpyridinium polymers from marine sponge Reniera sarai. Mar. Drugs 5, 157–167.

Turk, T., Sepčić, K., Mancini, I., Guella, G., 2008. 3-alkylpyridinium and 3-alkylpyridine compounds from marine sponges, their synthesis, biological activities and potential use. In: Rahman, Atta-ur- (Ed.), Studies in Natural Product Chemistry; Bioactive natural products (Part O), Studies in Natural Products Chemistry, vol. 3. Elsevier, Amsterdam (etc.), pp. 355–397.

Wong, M.S., Vanhoutte, P.M., 2010. COX-mediated endothelium-dependent contractions: from the past to recent discoveries. Acta Pharmacol. Sin. 31, 1095–1102.

Zovko, A., Vaukner Gabric, M., Sepčić, K., Pohleven, F., Jaklič, D., Gunde-Cimerman, N., Lu, Z., Edrada-Ebel, R., Houssen, W.E., Mancini, I., Defant, A., Jaspars, M., Turk, T., 2012. Antifungal and antibacterial activity of 3-alkylpyridinium polymeric analogs of marine toxins. Int. Biodeter. Biodegr. 68, 71–77.