Chemical synthesis and biological activities of 3-alkyl pyridinium polymeric analogues of marine toxins

Wael E. Houssen · Zhibao Lu · RuAngelie Edrada-Ebel · Christina Chatzi · Steven J. Tucker · Kristina Sepčić · Tom Turk · Ana Zovko · Sanbing Shen · Ines Mancini · Roderick H. Scott · Marcel Jaspars

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Abstract Two new large poly-1,3-dodecylpyridinium salts, APS12 and APS12-2 of 12.5- and 14.7-kDa size, respectively, were synthesised and tested for their pore-forming and transfection capabilities in HEK 293 and undifferentiated mouse ES cells using patch-clamp recording, Ca^{2+} imaging and flow cytometry. Polymerisation reactions were enhanced by microwaves, and the product sizes were controlled by altering the irradiation time. This method can also be applied to obtain polymers with variable linking chains as shown by the preparation of poly-(1,3-octylpyridinium) salt of 11.9-kDa size. Molecular weights of the final products were determined using ESIMS analysis, which also indicated the products to be amongst the largest macro-cycles ever recorded, up to a 900-membered ring. Anti-bacterial, haemolytic and anti-acetylcholinesterase activities were also reported for the two dodecyl pyridinium polymers. These

biological activities are characteristic to the structurally related marine toxin, poly-APS.

Keywords Marine toxins · Transfection · Poly-APS · Pore formers · Microwave-assisted polymerisation

Abbreviations

AChE	Acetylcholinesterase
bFGF	Basic fibroblast growth factor
DRG	Dorsal root ganglion
EB	Embryoid body
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EMEM	Eagle's minimum essential medium
ES	Embryonic stem cell
ESIMS	Electrospray ionisation mass spectrometry

Wael E. Houssen and Zhibao Lu equally contributed to this work.

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W. E. Houssen · C. Chatzi · S. J. Tucker · S. Shen · R. H. Scott (✉)
Institute of Medical Sciences, University of Aberdeen,
Aberdeen, AB25 2ZD Scotland, UK
e-mail: rod.scott@abdn.ac.uk

W. E. Houssen · Z. Lu · M. Jaspars (✉)
Marine Biodiscovery Centre, Department of Chemistry,
University of Aberdeen,
Aberdeen, AB24 3UE Scotland, UK
e-mail: m.jaspars@abdn.ac.uk

R. Edrada-Ebel
Strathclyde Institute of Pharmacy and Biomedical Sciences,
University of Strathclyde,
John Arbuthnott Building, 27 Taylor Street, Glasgow G4 0NR,
UK

K. Sepčić · T. Turk · A. Zovko
Department of Biology, Biotechnical Faculty,
University of Ljubljana,
Večna pot 111, 1111 Ljubljana, Slovenia

I. Mancini (✉)
Laboratorio di Chimica Bioorganica, Università di Trento,
via Sommarive 14, 38100 Trento, Italy
e-mail: mancini@science.unitn.it

Present Address:
C. Chatzi
Burnham Institute for Medical Research,
Development and Ageing,
10901, North Torrey Pines Rd, La Jolla, CA 92037, USA

FCS	Foetal calf serum
HEK 293	Human embryonic kidney cell line
LIF	Leukaemia inhibitory factor
MALDI-TOF	Matrix-assisted laser desorption ionisation-time of flight
MEF	Mouse embryonic fibroblast
MIC	Minimal inhibitory concentration
NEAA	Non-essential amino acids
NSCLC	Non-small-cell lung cancer
RA	Retinoic acid

Introduction

The water-soluble marine toxin, poly-APS (**1**), has been isolated from the Mediterranean sponge *Reniera sarai* (now *Haliclona sarai*, family Haliclonidae, order Haplosclerida). Matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) spectrometric analysis of **1** showed an ion cluster centred at m/z 5,520 Da with a low degree of polydispersity and a less intense and broader one at 18,900 Da [28]. Poly-APS (**1**) is related structurally to the epidermal growth factor (EGF)-active alkaloid from the sponge *Callyspongia fibrosa* [8] and to the halitoxins and amphitoxin isolated from several haplosclerid sponges [1, 3, 24, 25]. Halitoxins and amphitoxin include polymeric compounds containing pyridinium rings with wide variations in chemical structure mainly with respect to the degree of polymerisation and to the monomer structure, which shows a linear, branched or unsaturated linking C5–C12 alkyl chains.

Poly-APS (**1**) showed a broad spectrum of biological activities [31]. This includes anti-bacterial [6], anti-fouling [9, 11], potent anti-acetylcholinesterase (anti-AChE) activity with a rather unusual pattern of inhibition [29] and both haemolytic [18] and cytotoxic [27] activities. In addition, it showed selective toxicity toward NSCLC cells whilst having no apparent toxicity towards normal lung fibroblast cells and tissue in vitro and in vivo [22].

Electrophysiological and Ca^{2+} imaging experiments on human embryonic kidney cell line (HEK 293) cells and rat hippocampal and DRG neurons indicated that poly-APS (**1**) at a concentration below its lethal level [4] can form large transient pores in cellular membranes. Small and macromolecules including cDNA and peptides can diffuse into intracellular compartments through the pores formed. Poly-APS (**1**) can, thus, act as a transfecting tool that is mechanistically distinct from lipofection systems, where genetic material is introduced into the cell via liposomes [17, 20, 30]. However, the development of this transfecting agent has been hampered by the lack of knowledge about the structure–activity relationship (SAR) and the difficulties

in getting a sustainable and consistent supply of a single bioactive compound from the natural resource. Structurally well-defined analogues to poly-APS (**1**) are, thus, required to develop such a molecular delivery tool.

In 1993, Davies-Coleman and colleagues managed to synthesise cyclic alkylpyridinium oligomers with different alkyl chains and small numbers (two to five) of monomer units. Their approach involved the synthesis of the (3-pyridyl)-alkyl alcohol and introduction of triflate as a good leaving group at the end of the alkyl chain. This monomer was then refluxed in dichloromethane to give cyclic dimers and oligomers. Otherwise, refluxing a monomer bearing a terminal chlorine and a linking ether bridge in the alkyl chain, in acetonitrile in the presence of KI for a sustained period, resulted in the production of a mixture of cyclic and linear oligomers with a maximum of 15 monomer units [12]. A rather more sophisticated approach is the formation and subsequent reaction of an *N*-(2,4-dinitrobenzene) pyridinium salt (Zincke salt) to yield poly-alkylpyridinium salts with up to eight monomer units [15]. The degree of polymerisation can be increased stepwise by the controlled and iterative process of oligomerisation, providing general and practical access to a wide range of linear poly-cationic compounds. A more successful strategy to obtain linear derivatives with a defined grade of oligo/polymerisation is based on the reaction of an *N*-nucleophile monomer deactivated to alkyl substitution on the chain with an *N*-protected/activated monomer. This strategy was successfully applied to the production of linear tetramers related to poly-APS (**1**) [19]. A method for producing disubstituted alkylpyridinium oligomers based on the use of organic resins as a solid support was also patented [14].

Biological testing of the oligomers has shown that, for many activities, including transient pore-forming capability exhibited by poly-APS (**1**), the degree of polymerisation played a key role [17, 19, 20]. For these reasons and based on the known synthetic strategies previously applied and recently reviewed (Turk et al. [32]), a new method which allows the straightforward formation of large polymers with consistent degree of polymerisation (narrow dispersity) is needed.

In this study, a new method for preparation of high molecular weight poly-alkylpyridinium salts with low degree of poly-dispersity is presented. The method relies on the microwave irradiation of the 3-alkylpyridine monomer with a good leaving group (e.g. bromide) at the free terminal of the alkyl chain. Microwave irradiation of organic reaction mixtures is an effective tool in organic synthesis. It is able to drastically reduce reaction times when compared with conventional heating and to improve product yields, as well as to adopt safe, economical and eco-friendly conditions [16].

Two new large poly-1,3-dodecylpyridinium salts, APS12 (**9a**) and APS12-2 (**9b**) of 12.5- and 14.7-kDa size, respectively, were prepared. The method is generally applicable to prepare poly-alkylpyridinium salts with variable linking chains which can be used to tune the biological properties for specific applications. This was shown by the preparation of a poly-octylpyridinium salt, APS8 (**10**), of 11.9-kDa size. Compound **9b** is one of the largest macro-cycles ever recorded in the literature with a ring size of 900.

APS12 (**9a**) and APS12-2 (**9b**) were screened for a variety of biological activities including anti-bacterial, haemolytic and anti-AChE activities. Additionally, undifferentiated mouse embryonic stem (ES) cells were used to compare the ability of the two compounds to make transient pores in cell membranes. Undifferentiated mouse ES cells were selected as a model for this study because, in these cells, no voltage-activated membrane ionic current can be detected before induction of differentiation [33], making them an ideal model to test pore formation which allows ionic current passage through membranes. Moreover, these cells can be used to assess the effects of these molecules on the complex process of differentiation into immature GABAergic neurons [5].

Having proven their pore-forming capability, compounds **9a** and **9b** were then tested at concentrations below their IC_{50} s for their ability to transfect cDNA encoding enhanced green fluorescent protein (EGFP) into HEK 293 and undifferentiated mouse ES cells using flow cytometry.

Results

Synthesis

Poly-(alkylpyridinium) salts APS12 (**9a**), APS12-2 (**9b**) and APS8 (**10**) were synthesised using microwave-assisted polymerisation and following the scheme in Fig. 1. The monomer units were prepared according to the method reported by Davies-Coleman et al. in 1993 with minor modifications. Briefly, the bromoalcohol (**2**) was protected by a silyl group and coupled with 3-picoline. Deprotection

using tetrabutylammonium fluoride gave the pyridyl alcohol (**5**) which could be converted into the monomer unit (**7**) by refluxing with hydrobromic acid followed by neutralisation. Alternatively, the dibromoheptane (**6**) was coupled with 3-picoline to give the monomer (**8**). Initially, solutions of monomer in acetonitrile were refluxed to generate small oligomers [12], which was followed by concentration of the materials and subjecting them to microwave irradiation. The size of the polymers was controlled by altering the irradiation time (Fig. 1). Mass spectrometric analysis of the dodecyl pyridinium polymers showed that, after 30 min, the polymerisation reached 51 monomer units. Lengthening the irradiation to 48 h on a separate batch of the material increased polymerisation to 60 monomer units. This behaviour is consistent with the known mechanism of self-condensation in polymerisation in which increasing the reaction time to a great extent makes little impact on the degree of polymerisation [7].

Structural assignments of synthetic polymers

Analysis of nuclear magnetic resonance (NMR) spectra of the synthetic polymers was not helpful to establish the degree of polymerisation or to define if they are linear or macro-cyclic. On the other hand, MS was diagnostic for the determination of molecular weights and degree of polymerisation. For poly-APS (**1**), molecular weight determination was carried out using MALDI-TOF mass spectrometry which gave reliable and consistent results with those published before [28]. However, for the synthetic polymers (**9a**, **9b** and **10**), this method gave variable results, possibly due to the intricacy of matrix sample preparation and MALDI measurements of poly-electrolyte polymers. The nature and the size of halogen counterion influence electrostatic interactions, so that bromide ions could show a different behaviour if compared with chloride ions present in the natural polymers, probably responsible for multi-charged species not detected by MALDI ionisation system. For this reason, the synthetic materials were analysed by electrospray ionisation mass spectrometry (ESIMS) with an LTQ/Orbitrap FT mass analyser. The data indicate the narrow dispersity and high degree of polymerisation of the products obtained, e.g. APS12 (**9a**) and APS12-2 (**9b**) were shown to be 12.5 and 14.7 kDa, respectively. This is consistent with structures of 51 and 60 monomer units, respectively (Fig. 2a, b). Similarly, APS8 (**10**; Fig. 2c) was proven to be of 11.9 kDa which is consistent with 63 monomer units. Deconvolution with charge state analysis for each compound was consistent with its accurate mass analysis and gave the same degree of polymerisation. This also showed that, under the conditions used, the polymer was fully ionised. Analysis of the molecular formulae showed that there was no terminal halogen, and the charge

Fig. 1 Synthesis of poly-(1,3-alkylpyridinium) bromide salts. *Reagents, conditions and yields:* (i) *tert*-butyldimethylsilyl chloride, triethylamine, 4-dimethyl aminopyridine, DCM, stirring overnight, 95%; (ii) 3-picoline, diisopropylamine, THF, *n*BuLi, -78 to 0 °C, stirring, 80% of **4** and 35% of **8**; (iii) tetrabutylammonium fluoride, THF, 90%; (iv) HBr, toluene, reflux overnight followed by neutralisation, 60%; (v) reflux in

acetonitrile in the presence of KI followed by microwave irradiation either at 130 °C/8 bar/40 W/30 min for compound **9a** or by additional treatment at 130 °C/8 bar/40 W/48 h for compound **9b**; reflux in methanol followed by microwave irradiation at 130 °C/8 bar/30 W/60 h for compound **10**. Numbering for compounds **9a**, **9b** and **10** is for convenience in the description of NMR data

state equalled the number of monomers, indicating that the compounds had cyclised. The largest of these, APS12-2 (**9b**), with 60 monomer units thus forms a 900-membered macro-cycle, which is one of the largest ever reported.

Anti-bacterial, haemolytic and anti-AChE activities

Anti-bacterial, haemolytic and anti-AChE activities have been previously reported for poly-APS (**1**) [6, 18, 29] and together represent a characteristic biological profile of the natural toxin. Similarly, APS12 (**9a**) and APS12-2 (**9b**) showed these activities with different potencies.

Anti-bacterial activities of APS12 (**9a**) and APS12-2 (**9b**) against *Escherichia coli* EXB-V1 strain (Gram $-$) and *Staphylococcus aureus* EXB-V54 (Gram $+$) were determined. The minimal inhibitory concentration (MIC) values of APS12 (**9a**) on *E. coli* EXB-V1 and *S. aureus* EXB-V54 were 5 and 0.3 mg ml^{-1} , respectively, whilst the corresponding values for APS12-2 (**9b**) were 0.5 and 0.1 mg ml^{-1} , respectively. Additionally, poly-APS (**1**) showed less anti-bacterial activity compared to the synthetic compounds with MIC values against *E. coli* and *S. aureus* of >10 and 0.89 mg ml^{-1} , respectively [19].

The haemolytic activity of APS12 (**9a**) and APS12-2 (**9b**) was analysed to obtain the time course of haemolysis for each compound. Both polymers showed similar extents of haemolysis. The time course of the reaction was sigmoidal (not shown), which indicated the colloid-osmotic mechanism of cell lysis. This mechanism is usually

observed when the formation of transient pores with defined radius occurs. In Fig. 3a, the reciprocal t_{50} values of haemolysis, induced by active compounds, are plotted against their concentrations. The haemolysis rates ($1/t_{50}$) produced by 1 μg ml^{-1} of APS12 (**9a**) and APS12-2 (**9b**) were 0.1 and 0.12 s^{-1} , respectively.

The synthetic analogues, APS12 (**9a**) and APS12-2 (**9b**), also showed potent reversible non-competitive inhibition of AChE activity (Fig. 3b) with inhibitory constant (K_i) values of 0.2 and 0.5 ng ml^{-1} , respectively.

Preliminary experiments using compound APS8 (**10**) showed that it had anti-bacterial, haemolytic and anti-cholinesterase activities that were similar to activities obtained with poly-APS (**1**). APS8 (**10**) gave MIC values against *E. coli* EXB-V1 and *S. aureus* EXB-V54 of 0.3 and 0.05 mg ml^{-1} respectively, gave a haemolysis rate of 0.02 s^{-1} and a K_i for inhibition of AChE activity of 15 ng ml^{-1} . No further biological evaluation has been carried out with APS8 (**10**).

Electrophysiology and Ca^{2+} imaging

Initially, basic electrophysiological experiments were conducted to determine whether poly-APS (**1**; 5 μg ml^{-1}) applied for ~ 20 s caused a reversible collapse of the resting membrane potential and input resistance of undifferentiated mouse ES cells, similar to effects previously described in other cell types [17, 20, 30]. The mean resting membrane potential was -43 ± 3 mV under control conditions, and at

Fig. 2 Mass spectra for the synthetic polymers APS12 (**9a**), APS12-2 (**9b**) and APS8 (**10**). **a, b** Left panels show centroid mode spectra for polymers APS12 (**9a**) and APS12-2 (**9b**), respectively. Right panels show the deconvoluted MS spectra exhibiting 51 monomer units with a molecular weight of 12,557.303 (12.5 kDa) and 60 monomer units at 14,773.298 (14.7 kDa) for the respective polymers both exhibiting a monomer unit of $C_{17}H_{28}N$ as confirmed from their HRFTMS data. Both polymers are cyclic compounds where the number of nitrogen atoms is equivalent to the

number of positive charges with no halogens. **c** Left panel show centroid mode spectrum for APS8 (**10**) at $z=63$ m/z 190.2. As confirmed by its HRFTMS data, it gave the molecular formula $C_{819}H_{1250}N_{63}$ which implied the presence of the same number of $C_{13}H_{20}N$ monomer units as the number of charges, suggesting that the polymer is cyclic. HRFTMS established a molecular weight of 11,980.0186 Da for 63 monomer units. Right panels show the deconvoluted MS spectra exhibiting 63 charged monomer units for m/z 190.2 with an MW of 11.9 kDa

Fig. 3 Haemolytic (a) and acetylcholinesterase-inhibitory activity (b) of the synthetic poly-APS analogues, APS12 (9a) and APS12-2 (9b). $1/t_{50}$ = the rate of haemolysis as described by the reciprocal value of the time necessary for the lysis of 50% erythrocytes. K_i = inhibitory constant obtained by using 0.125-mM (triangles), 0.25-mM (circles) and 0.5-mM (squares) concentrations of the substrate acetylthiocholine

the peak of the response to poly-APS (**1**; $5 \mu\text{g ml}^{-1}$) this value was significantly reduced to $-13 \pm 3 \text{ mV}$ ($n=6$; $P < 0.005$). Cells were held at -70 mV by constant current injection prior to applying a current step command to evoke an electronic potential to standardise the measurement of input resistance. The reduction in input resistance was from a mean control value of 909 ± 210 to $216 \pm 127 \text{ M}\Omega$ in the presence of poly-APS (**1**; $n=8$ and 5 ; $P < 0.03$). Partial recovery (50% or more) of both the membrane potential and input resistance was observed 10–20 min after application of poly-APS (**1**). Similarly, under voltage clamp ($V_h = -70 \text{ mV}$), leak currents were evoked by 100-ms voltage step commands to clamp potentials between -140 and $+80 \text{ mV}$. All cells studied had linear current–voltage relationships, confirming that voltage-activated channels were not expressed in these undifferentiated cells. Poly-APS (**1**), APS12 (**9a**) and APS12-2 (**9b**) applied at a concentration of $5 \mu\text{g ml}^{-1}$ evoked inward currents. Figure 4a shows the values of the mean currents required to hold cells at -70 mV under control conditions and the significantly larger mean current observed in the presence of poly-APS (**1**) and the synthetic compounds (**9a** and **9b**). All responses were at least partially reversible, and Fig. 4b

shows an example current record of a response to poly-APS (**1**). Figure 4c shows example records of current responses to voltage step commands of $+130 \text{ mV}$ applied under control conditions during the peak response and after 10 min recovery. Poly-APS (**1**) and its two synthetic analogues, **9a** and **9b**, did not cause the current–voltage relationships to deviate from linearity and did not shift the reversal potential.

Ca^{2+} imaging experiments showed that mean dose-dependent response relationships to poly-APS (**1**), APS12 (**9a**) and APS12-2 (**9b**; 0.05 , 0.5 and $5 \mu\text{g ml}^{-1}$) could be obtained (Fig. 4d). The proportion of responding cells also increased as the concentration of compound was increased. However, example cells that failed to respond to all concentrations of poly-APS (**1**) were found. Overall, cells were more responsive to the synthetic compounds compared with poly-APS (**1**) applied at the lowest concentration ($0.05 \mu\text{g ml}^{-1}$) tested, with larger responses in greater proportions of cells being observed with the synthetic compounds (Fig. 4d). There was, however, a considerable amount of variability in the responses to all three compounds, and the records from individual cells in Fig. 4e show this.

Fig. 4 Acute applications of poly-APS (**1**), APS12 (**9a**) or APS12-2 (**9b**) evoked inward currents and intracellular Ca^{2+} transients $[\text{Ca}^{2+}]_i$ in undifferentiated mouse ES cells. **a** Bar chart showing resting holding current at -70 mV (open bars) and holding currents after application of $5 \mu\text{g/ml}$ of poly-APS (**1**; $n=6$; $*P<0.02$), APS12 (**9a**; $n=10$; $*P<0.01$) or APS12-2 (**9b**; $n=10$; $**P<0.002$), filled bars. **b** Current record showing a reversible inward current activated by poly-APS (**1**; $5 \mu\text{g/ml}$) from a holding potential of -70 mV. **c** Example records of current responses to 100-ms voltage step commands of $+130$ mV ($V_C +60$ mV), under control conditions, during the peak response to the application of $5 \mu\text{g/ml}$ of poly-APS (**1**), APS12 (**9a**) or APS12-2 (**9b**) and after 10–20-min recovery. The resting holding voltage was -70 mV, and the dotted lines denote 0 pA. **d** Bar chart showing dose–response Ca^{2+} imaging data for the whole population of ES cells studied (responders and non-responders included) for poly-APS (**1**; $n=67$; filled bars), APS12 (**9a**; $n=47$; hatched bars) and APS12-2 (**9b**; $n=51$; open bars); for each compound, statistical analysis was carried out to compare 0.05, 0.5 and $5 \mu\text{g/ml}$, $*P<0.0001$. The values in square brackets give the percentage of cells responding to each concentration. **e** Example traces showing the variations in the changes in fluorescence ratio induced by poly-APS (**1**), APS12 (**9a**) and APS12-2 (**9b**) in single cells

A feature of the differentiation of the ES cells used in this study was that the cells developed properties of immature neurones. Specifically, the cells synthesised GABA, and although they did not fire action potentials, they expressed voltage-activated potassium channels [5]. To determine whether exposure to poly-APS (**1**) had long-term effects, cells in EBs were exposed to retinoic acid ($1 \mu\text{M}$) and then incubated with $5 \mu\text{g ml}^{-1}$ poly-APS (**1**) for 5 to 20 min before being placed in neurobasal medium. These

cells differentiated, and after 3 weeks had the same electrophysiological properties as the differentiated cells that were not exposed to poly-APS (**1**). The mean currents required to hold differentiated cells at -70 mV were -156 ± 75 and -63 ± 64 pA ($n=6$; NS) for controls and poly-APS-treated cells, respectively. Additionally, potassium currents were expressed in both control differentiated cells and poly-APS-treated differentiated cells. From a holding potential of -70 mV, the mean peak potassium current activated at

+30 mV under control conditions was $1,360 \pm 720$ pA ($n=3$). This value was not significantly different from $1,010 \pm 240$ pA ($n=3$) for the mean peak potassium current activated at +30 mV in differentiated cells treated with poly-APS (**1**).

Cytotoxicity and transfection

Experiments were conducted to compare the cytotoxic activities of poly-APS (**1**), APS12 (**9a**) and APS12-2 (**9b**) to HEK 293 and undifferentiated mouse ES cells and to determine the highest concentrations of the compounds that can be used in the transfection experiments. The mean IC_{50} values in HEK 293 cells for poly-APS (**1**), APS12 (**9a**) and APS12-2 (**9b**) were 3.2, 9.5 and $8 \mu\text{g ml}^{-1}$ ($n=3$), respectively. The corresponding values in undifferentiated ES cells were 7.5, 22.5 and $26 \mu\text{g ml}^{-1}$ ($n=3$), respectively.

Cytotoxicity experiments indicated that HEK 293 cells were more sensitive to poly-APS (**1**) than to its two synthetic analogues (**9a** and **9b**). This allowed the use of both APS12 (**9a**) and APS12-2 (**9b**) at a higher concentration ($5 \mu\text{g ml}^{-1}$) than that previously used for poly-APS (**1**; $0.5 \mu\text{g ml}^{-1}$) [30] in the transfection experiments. Transfection efficiency was determined by flow cytometry as a percentage of the number of cells expressing EGFP to the total number of cell population. Data analysis indicated that APS12 (**9a**) and APS12-2 (**9b**) showed transfection efficiencies in HEK 293 cells of $8.67\% \pm 0.49$ ($n=5$, $P < 0.001$) and $27.81\% \pm 2.68$ ($n=5$, $P < 0.001$), respectively, whilst lipofectamine showed an efficiency of $87.84\% \pm 1.38$ ($n=5$, $P < 0.001$; Fig. 5). The percentage of cells showing background fluorescence in control population was only $0.07\% \pm 0.04$ ($n=5$). Undifferentiated ES cells proved more difficult to be transfected with cDNA. APS12 (**9a**) and APS12-2 (**9b**) showed mean transfection efficiencies of $0.48\% \pm 0.2$ ($n=5$, $P=0.05$) and $0.42\% \pm 0.15$ ($n=5$, $P < 0.05$), respectively, whilst lipofectamine showed an efficiency of $10.7\% \pm 1.45$ ($n=5$, $P < 0.01$; Fig. 5). The background or control transfection efficiency observed was $0.08\% \pm 0.02$.

Discussion

In this study, a successful method for the production of high molecular weight monodisperse poly-alkylpyridinium salts analogous to the bioactive marine toxin, poly-APS (**1**), is presented. Different biological activities were described for poly-APS (**1**) [26] of which the transient pore-forming capability below its lethal concentration [30] is considered the most promising for developing a new molecular delivery tool to the wide area of biomedical research. In spite of the success of using poly-APS (**1**) as a delivery tool

for large molecules including proteins and cDNA [30], its development was hampered by a lack of knowledge of how structural modifications affected the biological activity and the difficulties in getting a sustainable supply of a single bioactive compound from the natural resource.

Previous studies [8, 12, 15] managed to synthesise only oligo-alkylpyridinium salts, but biological screening of some of them indicated that the pore-forming capability was abolished [19]. This highlighted the importance of high degree of polymerisation for this kind of biological activity.

The method of synthesis reported in this study relies on the use of microwave irradiation to enhance the polymerisation. The size of the product obtained is dependent on the irradiation time and was shown to give products with a remarkably low dispersity. Polymers with different linkers can also be synthesised. Two poly-(1,3-dodecylpyridinium) salts, APS12 (**9a**) and APS12-2 (**9b**), and one poly-(1,3-octylpyridinium) salt, APS8 (**10**), were synthesised. MALDI-TOF mass analysis was ineffective for size determination. ESIMS analysis indicated the masses to be of 12.5, 14.7 and 11.9 kDa, respectively, showing that the polymers had high polymerisation states. The cyclic nature of these polymers has been further confirmed by mass spectrometric analysis.

The two dodecyl pyridinium polymers were shown to have haemolytic, anti-AChE and weak anti-bacterial activities. These activities are well-documented for the natural poly-APS (**1**) [6, 18, 29]. The anti-bacterial activities observed in this study are low for 3-alkylpyridinium cyclic oligomers and thus they are unlikely to be of value as anti-bacterial agents, since these compounds are more toxic to eukaryotic than prokaryotic cells. Moreover, both compounds have been tested for their ability to form large transient pores in HEK 293 and undifferentiated mouse ES cells. The pore-forming capability of poly-APS (**1**) on ES cells has also been assessed and compared with those of the two synthetic polymers. Moreover, poly-APS (**1**) has been shown to not interfere with the complex process of differentiation of mouse ES cells into neurons. The two synthetic polymers were found to be more potent than poly-APS (**1**) as pore formers. Both compounds have also been tested for their abilities to transfect cDNA encoding EGFP into HEK 293 and mouse ES cells. Both toxins were found to be active with overall higher efficiency in HEK 293 cells. The variation in transfection efficiency seen with different cells reflect the different membrane composition as well as the need to understand the molecular mechanism by which transfection is taking place. The results also indicated that the larger polymer **9b** was much more active as a transfecting agent. Interestingly, in contrast to the membrane activity on erythrocytes, both polymers were less cytotoxic than poly-APS (**1**) to both HEK 293 and

Fig. 5 Transient transfection of undifferentiated mouse ES and HEK 293 with pMAX-GFP using lipofectamine, APS12 (**9a**) and APS12-2 (**9b**). **a** Confocal images of undifferentiated mouse ES cells (*upper panels*) and HEK 293 (*lower panels*) treated, from the left panels to the right ones, with no transfection vehicle, lipofectamine (2 mg/ml), APS12 (**9a**; 5 μ g/ml) and APS12-2 (**9b**; 5 μ g/ml), respectively, in the presence of pMAX-GFP. Images were captured 48 h post-transfection, and each is representative of five experimental repeats. **b** Representative flow cytometry dot plots show populations (gated) of ES (*upper panels*) and HEK 293 (*lower panels*) that can express EGFP after 48-h transfection using, from *left panels* to *right ones*, no transfection vehicle, lipofectamine (2 mg/ml), APS12 (**9a**; 5 μ g/ml) and APS12-2 (**9b**; 5 μ g/ml) respectively. **c** Bar chart shows percent transfection of mouse ES and HEK 293 cells as analysed by flow cytometry. Data shown are the mean $\pm$ SEM

mouse ES cells, and this allowed the use of higher concentrations of both polymers in the transfection experiments. It should be noted that the transfection efficiency achieved even in HEK 293 cells with pore-forming polymers is much lower than that obtained with lipofectamine and related reagents.

In summary, the use of microwave-assisted polymerisation and the synthetic sequence described in Fig. 1 allowed the production of large monodisperse poly-alkylpyridinium salts. This method can be used to synthesise compounds with different degrees of polymerisation and different alkyl chain linkers with the aim of studying the SAR of these

bioactive compounds. The two new chemical entities APS12 (**9a**) and APS12-2 (**9b**) represent more potent and less toxic transfecting agents than poly-APS (**1**). Their mechanism of action is through making large transient pores in cell membranes through which small and large molecules can diffuse into the intercellular compartment. This mechanism is quite different from the mechanism of the widely used lipofectamine and thus may be promising to test with model systems where lipofectamine proved inefficient. Moreover, the two compounds can be obtained on a large scale with high degree of purity using the method reported here.

Materials and methods

General methods

All starting materials were purchased from Aldrich. NMR spectra were recorded in CDCl_3 or CD_3OD (as specified) on a Bruker AC 250 MHz NMR spectrometer with Tecmag acquisition system or on Varian Unity-INOVA 400 MHz system. Chemical shifts were reported as δ values relative to an internal standard, tetramethylsilane (^1H , $\delta=0.00$ ppm). Coupling constants are given in hertz. Electrospray mass spectra of the synthesised compounds were measured on an LTQ/Orbitrap mass spectrometer equipped with a high-resolution FT mass analyser set up at 100,000 and externally calibrated at 3 ppm. The samples were dissolved in $\text{MeOH}/\text{H}_2\text{O}$ (1:1 v/v) and were injected at a volume of 20 μl , run with direct infusion of acetonitrile/ H_2O (90:10 v/v with 0.1% formic acid) at a flow rate of 200 $\mu\text{l min}^{-1}$. Positive-mode ionisation was accomplished at a capillary temperature of 200 $^\circ\text{C}$, a capillary voltage of 35.5 V and a source voltage of 4.0 kV. MS data were processed utilising the centroid algorithm mode of the LTQ at a merge width of 5.0. High-resolution MS data were processed using Xcalibur 2.1, and the deconvolution software used was Promass. MALDI-TOF analysis experiments were carried out on a Shimadzu or an Axima CFR MALDI-TOF mass spectrometer (m/z range 1–20 kDa; positive-ion reflectron and linear mode using sinapinic acid as matrix). The microwave-assisted polymerisations were performed on Biotage Initiator Microwave Synthesiser (Uppsala, Sweden).

Synthesis

Compounds 2–8

See [Supplemental Experimental Procedures](#).

Compounds 9a and 9b

A solution of **7** (0.5 g, 1.53 mmol) in acetonitrile (6 ml) was heated under reflux for 2 days to generate short-chain oligomers. At this stage, a small amount of potassium iodide (0.1 g) was added to speed up the reaction, and the solution was heated under reflux for 2 more days. Another amount of potassium iodide (0.5 g, excess) was added, and the reflux was continued for another 3 days. After this, more acetonitrile (5 ml) was added, and the solution was filtered. The acetonitrile solution was concentrated to give a yellowish waxy solid. Microwave irradiation (130 $^\circ\text{C}$, 8 bar, 40 W) of one batch of the waxy solid (0.2 g) in methanol (5 ml) over 30 min followed by purification on Sephadex LH-20 gave the viscous polymer **9a**. A separate batch of the waxy solid (0.3 g) was subjected to microwave

irradiation (130 $^\circ\text{C}$, 8 bar, 40 W) in methanol (5 ml) for a 2-day period followed by purification on Sephadex LH-20 to give the viscous polymer **9b** (0.24 g).

NMR resonances for polymers **9a** and **9b** were very broad due to their high molecular weights, and their spectra appeared virtually identical. The data given here are for compound **9a**. ^1H -NMR (250 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$) δ 1.0–2.04 (20H, m, H_2 –8– H_2 -17), 2.93 (2H, m, H_2 -7), 4.68 (2H, m, H_2 -18), 8.03 (1H, m, H-5), 8.48 (1H, m, H-4), 8.88 (1H, m, H-6), 9.07 (1H, brs, H-2).

Compound 10

A solution of **8** (0.42 g, 1.55 mmol) in methanol (8.0 ml) was subjected to microwave irradiation (130 $^\circ\text{C}$, 8 bar, 30 W) for 60 h. The resulting mixture was concentrated and extracted with a mixture of petroleum ether (40–60 $^\circ\text{C}$) and DCM (1:1 v/v) to remove un-reacted monomer and some oligomers. Final purification was carried out by size exclusion on Sephadex LH-20 to give **10** as brown oil (0.30 g).

^1H -NMR (250 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$) δ 1.0–1.70 (12H, m, H_2 –8– H_2 -13), 2.78 (2H, m, H_2 -7), 4.65 (2H, m, H_2 -14), 7.88 (1H, m, H-5), 8.20 (1H, m, H-4), 8.97 (1H, m, H-6), 9.18 (1H, brs, H-2).

Anti-bacterial, haemolytic and anti-AChE activities

Bacteria (*E. coli*, strain EXB-V1, and *S. aureus*, strain EXB-V54) were obtained from the local collection at the Department of Biology, University of Ljubljana. Anti-bacterial activity was evaluated using the standard agar diffusion test, as described in [19], in order to determine the MIC of each compound against each microorganism.

Haemolytic activity was measured by a turbidimetric method [2]. The compounds were progressively diluted in deionized water, and 20 μl of the resulting solutions or pure water (control) was added to 180 μl of bovine erythrocyte suspension with an apparent absorbance of 0.5 at 650 nm. The decrease of apparent absorbance was recorded for 20 min at 650 nm using a Kinetic Microplate Reader (Dynex Technologies, USA) to determine the time necessary for 50% haemolysis, t_{50} . Each experiment was repeated at least three times. The rate of haemolysis was expressed as $1/t_{50}$ (s^{-1}).

The activity of AChE was measured by Ellman's method [10], using acetylthiocholine iodide (0.125, 0.25 or 0.5 mM) as a substrate in 100 mM potassium phosphate buffer pH 7.4 at 25 $^\circ\text{C}$ and electric eel AChE as a source of enzyme (Sigma, 6.25 U/ml). Hydrolysis of acetylthiocholine iodide was followed on a Kinetic Microplate Reader (Dynex Technologies, USA) at 412 nm. AChE inhibition was monitored for 5 min for each compound, which has

been progressively diluted in deionized water and added to final 200 μ l of the reaction mixture. All readings were corrected for their appropriate blanks, and every measurement was repeated at least three times. The inhibitory constants (K_i) and the types of enzyme inhibition for each tested compound were determined from Dixon plots.

Cell culture

HEK 293 cells were maintained in culture as previously reported [21]. Briefly, cells were cultured in EMEM (Sigma M2279), supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin and 1% non-essential amino acids (NEAA).

The mouse ES cell line, Abdn2, was derived from C57Bl/6JCrI mouse and used in this study [5]. Cells were maintained on mitotically inactivated mouse embryonic fibroblast (MEF), in the knockout serum replacement–knockout Dulbecco's modified Eagle's Medium (KDMEM) medium comprising of KDMEM (Invitrogen) supplemented with 20% Knockout Serum Replacement (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma), purified recombinant mouse LIF (equivalent to 1,000 U/ml), 0.1 mM NEAA (Sigma), 4 mM GlutaMAXTM-I (Invitrogen), 50 U/ml penicillin and 50 μ g/ml streptomycin (Invitrogen). MEFs were usually removed before carrying out experiments. The media were renewed every 2 days. The cultures were passaged at ~70% of confluence by trypsinisation.

RA-based neuronal differentiation protocol of ES cells through EB formation

MEFs were firstly removed prior to EB formation by sub-culturing on gelatinised (0.1%) plates. Sub-confluent cells were transferred on non-adherent 10-cm Petri dishes at a concentration of $1\sim2\times10^5$ cells per millilitre in EB growth medium comprising of DMEM supplemented with 10% FCS, 0.1 mM NEAA, 4 mM GlutaMAXTM-I, 50 U/ml penicillin and 50 μ g/ml streptomycin. The dishes were shaken at a 37 °C incubator at 50 rpm to allow cell aggregation. After 3 days of suspension cultures, uniformly sized EBs suitable for RA induction were formed. EBs were induced for differentiation by shaking on non-adherent 10-cm Petri dishes in fresh EB growth medium with *all-trans* RA at a final concentration of 10^{-6} M. After 3 days, EBs were harvested and plated on culture dishes pre-coated with poly-L-ornithine and fibronectin. The cultures were left overnight in EB formation medium (without RA) to enhance EB attachment.

Cells were washed three times with NaCl-recording medium before poly-APS (**1**) was applied at a final concentration of 5 μ g/ml for 5, 10 or 20 min. Cells were then washed with neuronal induction medium comprising of

neurobasal medium supplemented with B27, bFGF (10 ng/ml) and EGF (10 ng/ml) and containing 10% FCS for 30 s. Serum was added to inactivate the poly-APS (**1**). The cells were then cultured in serum-free neuronal induction medium for 3 weeks before carrying out the electrophysiological experiments. The medium was renewed every 3 days.

Electrophysiology

The electrophysiological actions of poly-APS (**1**) and its two synthetic analogues, APS12 (**9a**) and APS12-2 (**9b**), on undifferentiated mouse ES cells were characterised at room temperature (18–20 °C) using the whole-cell patch-clamp techniques [13]. Resting membrane potentials, input resistances and current–voltage relationships under voltage clamp were measured. Patch pipettes with resistances of 3–9 M Ω were made from Pyrex borosilicate glass capillary (Plowden and Thompson Ltd., Dial Glass Works) using a two-stage vertical microelectrode puller (David Kopf Instruments, Tujunga, CA, USA, Model 730). An Axoclamp 2A switching amplifier (Axon Instruments) operated at 18 kHz was used. Patch pipettes were filled with KCl-based solution containing 140 mM KCl, 0.1 mM CaCl₂, 5 mM EGTA, 2 mM MgCl₂, 2 mM ATP and 10 mM HEPES. The pH and osmolarity of the patch pipette solution were corrected to 7.2 and 310–320 mOsm l⁻¹ with Tris and sucrose, respectively. The extracellular bathing solution used contained 130 mM NaCl, 2 mM CaCl₂, 3 mM KCl, 0.6 mM MgCl₂, 1 mM NaHCO₃, 10 mM HEPES and 5 mM glucose. The pH and osmolarity of this extracellular bathing solution were corrected to 7.4 and 320 mOsm l⁻¹ with NaOH and sucrose, respectively. Data were captured and stored on digital audiotape using a biologic digital tape recorder (DTR 1200). Analysis of data was performed off-line using Cambridge Electronic Design voltage clamp analysis software (version 6.0). All voltage-activated K⁺ currents recorded from differentiated stem cells had scaled linear leakage and capacitance currents subtracted to obtain values for the net current. Data are given as mean $\pm$ standard error of the mean (SEM), and statistical significance was determined using a paired or independent Student's *t* test as appropriate.

Intracellular Ca²⁺ measurement

Intracellular Ca²⁺ transients [Ca²⁺]_i evoked in undifferentiated mouse ES cells by poly-APS (**1**) and its synthetic analogues APS12 (**9a**) and APS12-2 (**9b**) were measured as previously reported for studies on halitoxin [25] and poly-APS [20]. Cells were incubated in the dark for 1 h in NaCl-based extracellular solution containing 0.01 mM fura-2AM (Sigma, 1 mM stock in dimethylformamide). This period allowed for cytoplasmic de-esterification of the Ca²⁺-sensitive fluorescent dye. The cells were then washed for

10–20 min with NaCl-based extracellular solution to remove excess fura-2AM. The cells were constantly perfused (1–2 ml min⁻¹) with NaCl-based extracellular solution and viewed using an inverted Olympus BX50W1 microscope with a KAI-1001 S/N 5B7890-4201 Olympus camera attached. The fluorescence ratiometric images from data obtained at excitation wavelengths of 340 and 380 nm were viewed and analysed using OraCal pro, Merlin morphometry temporal mode (Life Sciences resources, version 1.20). One-minute applications of 0.05, 0.5 and 5 µg/ml of poly-APS (**1**), APS12 (**9a**) and APS12-2 (**9b**) in NaCl-based extracellular solution were conducted. [Ca²⁺]_i levels were allowed to return to baseline prior to an additional application of drug-containing solution. Regions of interest (ROI; one per cell) within a given field were pre-selected by means of a transmission image overlay. For data analysis, ratiometric values obtained from Openlab (V. 4.02, Improvion, Coventry, UK) were plotted against time, and the peak rise in fluorescence for each ROI was determined, shown as arbitrary fluorescence units in sample traces. All values were converted into (% $\Delta F/F$) with F defined as an average of ten baseline values before drug application. The mean % $\Delta F/F$ was calculated for each dose and statistically compared with that for other drugs. The percentage of cells which responded towards a given drug application was determined in the following way. The mean of the ten baseline fluorescence values was subtracted from the maximum baseline fluorescence to give a value for variance in fluorescence prior to drug application. A drug response was considered to have occurred if the change in fluorescence was greater than four times the value of the baseline variance plus the mean baseline fluorescence. Each experiment was repeated at least two times using different culture batches. Intracellular Ca²⁺ imaging data are given as means $\pm$ SEM, and statistical significances were determined using a paired or independent Student's t test or ANOVA as appropriate. One-way ANOVA followed by Newman Keuls post-test was used for multiple comparisons. Significance was set at $P < 0.05$ = significant, $P < 0.01$ = highly significant and $P < 0.001$ = very highly significant.

Crystal violet cytotoxicity assay

HEK 293 and Abdn2 cells were seeded in 96-well plates at 8,000 cells per well and incubated for 24 h at 37 °C in 5% CO₂. The media were replaced with serum-free media with or without one of the test materials, and cells were incubated for a further 48 h. Each test material was added to eight final concentrations from 0.5 µg ml⁻¹ to 1 mg ml⁻¹ in triplicate. After incubation, adherent cells were fixed in paraformaldehyde and stained in crystal violet dye as previously described [23]. Subsequent elution of the dye and spectrophotometric analysis quantified the number of adherent cells.

Transfection experiments

HEK 293 and Abdn2 cells were seeded at 1×10^5 cell per well in six-well plates in 2 ml media for 24 h to reach a plate confluency of 50–60% on the day of transfection. Control transfections were carried out using lipofectamine (Invitrogen Life Technologies) transfection protocol as previously reported [21]. Briefly, cells were incubated with 1 µg pMAX-GFP plasmid vector (3,486 bp; Lonza) and lipofectamine in the absence of serum for 3 h prior to reintroduction to serum-containing medium. The toxin transfection protocol developed by Tucker and colleagues in 2003 was used in this study. The protocol involved 5-min serum-free cell incubation with the toxin preparation, followed by addition of 2.5 µg pMAX-GFP. After a further 3-h incubation, medium was replaced by standard serum-containing medium. The cells were then cultured for a further 48 h. Levels of EGFP in the transfected cells were detected and corrected for background fluorescence of the non-transfected cells using flow cytometry. The transfection efficiency was calculated based on the percentage of the cells that expressed EGFP (positive cells) in the total number of cells.

Flow cytometry

A FACSCalibur or BD LSRII (BD Biosciences) was used for data acquisition, and DiVA or CellQuest Pro (BD Biosciences) software was used for data analysis. Gates and instrument settings were set according to forward and side scatter characteristics, and populations were gated to exclude dead or clumped cells. A total of 10,000 events were collected. Data were collected from five different cultures and expressed as means and SEMs and compared using unpaired Student t test.

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