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

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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.

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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

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

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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 6). 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, antimicrobial, 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
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. Recent studies of poly-APS induced transfection showed that this process was, surprisingly, more effective at lower temperatures (7–12 °C). 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 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. 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 C12H18N+.12 APS8 and APS12-2 were proven to be of 11.9 and 14.7 kDa, respectively, which is consistent with 63 and 60 monomer units, respectively. 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-<i>i</i>-serine (POPS) and 1-hexadecanoyl-2-(9Z-octadecanoyl)-sn-glycero-3-ethyolphosphocholine (POPC<sub>2</sub>) 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](image-url). Chemical structures of 3-alkylpyridinium compounds used in this study.
Percent of hemolysis was expressed as 100 – % of inhibition of hemolysis (= \(A_{t12}/A_{max} - A_{min}\)), where \(A_{t12}\) 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 \(\mu\)L of SV at various lipid concentrations (0–2.5 mg/mL) in erythrocyte buffer were pipetted onto a multiwell-plate. 50 \(\mu\)L 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 \(\mu\)L 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 a 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 \(\mu\)L 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. 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 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. 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.

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 \(\mu\)g/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 \(\mu\)g/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 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
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. 26 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. 12–24 Our combined results suggest that 3-APS are excluded from lipid rafts when interacting with cell membranes.
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. 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.

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. 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
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), 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 and natural lipid membranes, these compounds appear to prefer and increase their permeabilization rates (Fig. 9).

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