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Squalamine is not a proton ionophore

Barry S. Selinsky [a,*], Rob Smith [a], Andrea Frangiosi [a], Beth Vonbaur [a], Larry Pedersen [b]

[a] *Department of Chemistry, Villanova University, Villanova, PA 19085, USA*
[b] *Chemistry Department, College Misericordia, Dallas, PA, USA*

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Abstract

Squalamine, an aminosterol antibiotic isolated from the dogfish shark, creates relatively large defects in phospholipid bilayers, allowing the unrestricted translocation of small molecules across these compromised membranes (B.S. Selinsky, Z. Zhou, K.G. Fotjik, S.R. Jones, N.R. Dollahon, A.E. Shinnar, Biochim. Biophys. Acta 1370 (1998) 218–234). However, an aminosterol structurally similar to squalamine was found to act as a proton ionophore in anionic phospholipid vesicles. In contrast with squalamine, gross membrane disruption was not observed with this synthetic analog (G. Deng, T. Dewa, S.L. Regen, J. Am. Chem. Soc. 118 (1996) 8975–8976). In this report, the ionophoric activity of squalamine was tested in anionic and zwitterionic phospholipid vesicles. No ionophoric activity was observed for squalamine in vesicles comprised of phosphatidylglycerol (PG), phosphatidylcholine (PC), or a mixture of the two lipids. Experiments using radiolabeled squalamine indicated that all of the squalamine added to PG vesicles remained with the vesicles, while approximately one-half of the squalamine added to PC vesicles was incorporated. We have synthesized the aminosterol analog of squalamine possessing ionophoric activity, and its ionophoric activity in PG vesicles was confirmed. The synthetic compound possessed no measurable lytic activity when added to preformed phospholipid vesicles. As both compounds possess significant antimicrobial activity, these results suggest that either multiple mechanisms for the antimicrobial activity of aminosterols exist, depending upon the aminosterol structure, or possibly an unrelated common mechanism for antimicrobial activity remains to be discovered. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Squalamine; Aminosterol; Antimicrobial agent; Proton ionophore

1. Introduction

Squalamine is a novel aminosterol sulfate isolated from the liver of the dogfish shark [1]. The structure of squalamine (shown in Fig. 1) has been determined by NMR spectroscopy, mass spectroscopy, and by synthesis of the authentic compound [2–4]. Squalamine exhibits antimicrobial activity against a wide variety of microorganisms [1]. Squalamine also demonstrates promise as an antiangiogenic agent, preventing the formation of new blood vessels in developing malignant tumors [5].

A large number of aminosterols similar to squalamine have been synthesized and tested for antimicrobial activity [6–9]. While in our measurements squalamine remains the most effective antimicrobial agent, many similar compounds also exhibit significant activity. The minimum structural requirements

* Corresponding author. Fax: (610) 519-7167;
E-mail: selinsky@chem.vill.edu

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for antimicrobial activity appear to be a steroid ring (or other similar large hydrophobic backbone) to which is attached an amine at one site and some type of charged or polar group (carboxylic acid, carboxylic acid ester, or sulfate group) at a second site. Antimicrobial activity is modified by the nature of the acidic group, the length, charge, and structure of the amine or polyamine group, and the addition of hydroxyl substituents to the steroid ring. However, the existence of antimicrobial activity seems to require only the presence of the requisite components, and not their precise location.

When added externally to preformed phospholipid vesicles, squalamine effects the leakage of fluorescent dyes entrapped within the vesicles [10]. Defects of approx. 2 nm in diameter are formed when squalamine is added to vesicles. Significant morphological changes in phospholipid vesicles are induced by the addition of squalamine. Based upon these and other experiments, a model to explain the antimicrobial activity of squalamine was proposed. In this model, squalamine interacts with acidic phospholipids in microbial membranes, forming squalamine/phospholipid aggregates. These aggregates disrupt the membrane and form transient defects, allowing the translocation of materials across the membrane. If the defect persists, the cell loses significant resources and dies.

Regen's group has synthesized an aminosterol with potent antimicrobial activity structurally different from the types synthesized in our laboratory [8]. The structure of the aminosterol synthesized by Regen's group (hereafter referred to as compound **1**) is given in Fig. 1. Compound **1**, when incorporated into large unilamellar phospholipid vesicles during their preparation, acts as a proton ionophore in anionic phospholipid membranes [11]. No ionophoric activity is observed when the compound is incorporated into zwitterionic phosphatidylcholine membranes. Based upon these observations, Regen proposed that squalamine acts as a proton ionophore, dissipating the proton gradient across bacterial membranes and disabling ATP synthesis in bacterial cells, resulting in cell death.

In this paper, the ionophoric model for the antimicrobial activity of squalamine was tested. No ionophoric activity was found for squalamine under a variety of different assay conditions. We have synthesized compound **1**, and have confirmed the ionophoric activity of this material. In contrast with squalamine, compound **1** is not lytic when tested against anionic or zwitterionic phospholipid vesicles. These results indicate that small differences in structure may lead to vastly different ways in which aminosterols interact with membranes. Multiple mechanisms for antimicrobial activity may exist, or a mechanism not yet explored might be possible.

2. Methods

2.1. Chemicals

Egg yolk phosphatidylcholine (PC), phosphatidylglycerol derived from egg yolk PC (PG), and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL). 1-Hydroxypyrene-3,6,8-trisulfonic acid (HPTS) and *p*-xylene-bis-pyridinium bromide (DPX) were purchased from Molecular Probes (Eugene, OR). Calcein was purchased from Fluka (Ronkonkoma, NY) and purified by Sephadex LH-20 column chromatography [12].

Squalamine was isolated from the liver of the shark *Squalus acanthias* as previously described [1], and purified at the trifluoroacetate salt. The squalamine analog **1** (Fig. 1) was synthesized essentially as described [8]. The identity of the squalamine analog was confirmed by NMR spectroscopy, mass spectroscopy, and CHN elemental analysis, which agreed with published values [8] within experimental error. Tritiated squalamine was the kind gift of Dr. William Kinney of Magainin Pharmaceuticals.

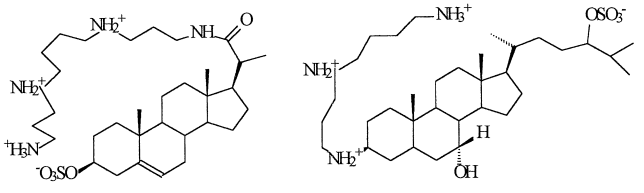

Fig. 1. The structures of the aminosterols used in this study. Squalamine is the naturally occurring compound isolated from the shark, while compound **1** is a synthetic analog.

2.2. Measurements of ionophoric activity

Large unilamellar vesicles (LUVs) were prepared by extrusion [13] in the presence of HPTS. Five milligrams of phospholipid (PC, PG, POPG, or a 7/3 mixture of PC/PG) were suspended in 2/1 chloroform/methanol. When desired, a small aliquot of either squalamine or compound **1**, dissolved in methanol, was added to the lipid solution. The organic solvent was removed under a stream of nitrogen gas, and then under vacuum for at least 2 h. The dry lipid was resuspended into 0.5 ml of buffer (25 mM HEPES, 50 mM NaCl, 0.1 mM HPTS, pH 7.0), subjected to ten freeze-thaw cycles, and then extruded 29 times through a 100 nm polycarbonate filter. External HPTS was removed by passage over a (1×15 cm) Sephadex G-75 column, eluting with the same buffer without HPTS. Harvested vesicles were stored on ice and used the same day of preparation.

To test for ionophoric activity, an aliquot of vesicles (20–200 μl) containing encapsulated HPTS at pH 7.0 was added to a cuvette containing 3.0 ml of buffer (25 mM HEPES, 50 mM NaCl, pH 8.0) maintained at 25°C. The fluorescence of HPTS (λ_{ex} = 469 nm; λ_{em} = 508 nm) was monitored as a function of time on a Quantamaster fluorescence spectrometer (Photon Technologies, Princeton, NJ). The fluorescence intensity of HPTS is a linear function of pH in the range between pH 7.0 and 8.0 (Fig. 2). The increase in fluorescence intensity after addition of vesicles to pH 8.0 buffer is interpreted to result from hydrogen ion leakage across the vesicular membrane.

The fluorescence intensity at 508 nm was converted into the hydrogen ion concentration. A plot of $\ln([H^+]/[H^+]_{init})$ versus time is linear for at least 30 s after the pH jump, suggesting that proton translocation is a pseudo-first order process. The plot deviates from linearity at later times after the pH jump due to proton translocation back into the vesicles through the ionophore. The increase in fluorescence during the first 30 s after the pH jump was used to calculate a leakage rate, assuming a first order process, and first order rate constants calculated.

To control for vesicle lysis resulting from the pH jump, in some experiments 5 mM DPX is included in the pH 8.0 buffer. When DPX is included, vesicle lysis would expose HPTS to the quenching agent, resulting in a decrease in the observed fluorescence. The ability of DPX to quench HPTS fluorescence was confirmed by lysing vesicles with 0.1% Triton X-100. Hydrogen ion leakage without lysis will always generate an increase in fluorescence [14].

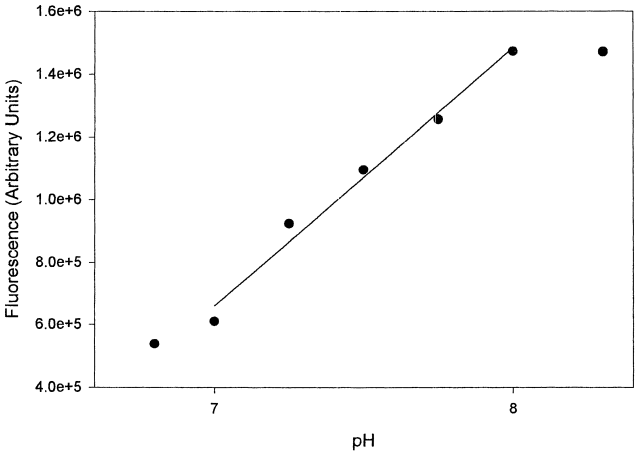

Fig. 2. The relationship between the fluorescence intensity of HPTS (λ_{ex} = 469 nm; λ_{em} = 508 nm) and pH. A linear relationship between fluorescence intensity and pH (r^2 = 0.98) is observed between pH 7.0 and 8.0.

2.3. Binding of squalamine to phospholipid vesicles

Radiolabeled squalamine was used to determine the amount of squalamine that remained associated with LUVs after extrusion. Vesicles were prepared from either PC or PG in the presence of ^{3}H-squalamine. After extrusion, the vesicles were separated from non-vesicular components by a (1×15 cm) Sephadex G-75 column. One milliliter fractions were collected and radioactivity measured by scintillation counting.

2.4. Contents leakage

The leakage of calcein from LUVs caused by externally added aminosterol was measured as previously described [10]. Squalamine or compound **1** did not affect the entrapment of calcein when added before vesicle extrusion. Vesicles containing entrapped calcein were added to buffer (20 mM PIPES, 150 mM NaCl, 1 mM EDTA, pH 7.0) at 30°C. A small aliquot of either squalamine (from an aqueous 1 mg/ml stock) or compound **1** (from a 1 mg/ml solution in methanol) was added, and the calcein leak-

age recorded as an increase in fluorescence ($\lambda_{ex} = 490$ nm; $\lambda_{em} = 520$ nm).

3. Results and discussion

3.1. Ionophoric activity of squalamine

The ionophoric activity of squalamine was tested in extruded LUVs prepared from egg yolk PC, PG derived from egg yolk PC, POPG, and a PC/PG (7/3) mixture. Squalamine was incorporated into LUVs by cosolubilization in organic solvent (2/1 chloroform/methanol), followed by solvent evaporation and hydration into aqueous buffer at pH 7.0. The resuspension buffer contained 0.1 mM HPTS, a pH-sensitive fluorescent dye whose quantum yield increases with increasing pH in the 6.5–8.5 pH range. The use of HPTS as a pH indicator in liposomes was carefully characterized by Daleke et al. [14]. We used an excitation maximum of 469 nm, which is slightly higher than the excitation maximum of 450 nm reported by Daleke et al. [14], and an emission maximum of 508 nm. We find that the fluorescence of HPTS increased in a linear fashion as a function of pH between 7.0 and 8.0 (Fig. 2), in agreement with Daleke et al. In the calculation of proton leakage rates, a linear relationship between pH and fluorescence was assumed.

Vesicles were prepared by extrusion and external HPTS separated from entrapped dye by size exclusion chromatography. To test for ionophoric activity, the vesicles containing entrapped HPTS at pH 7.0 were added to pH 8.0 buffer in a fluorescence cuvette, which was isoosmotic to the buffer within the vesicles. The change in fluorescence was followed as a function of time, and *pseudo*-first order rate constants for proton translocation were calculated.

The first order rate constants describing the proton ionophoric activity of squalamine are presented in Fig. 3. There is no significant difference in proton translocation across any of the membranes tested when squalamine is included in the LUVs at concentrations of up to 2 mole%. We conclude that squalamine does not act as a proton ionophore under these conditions.

One possible explanation for the absence of ionophoric activity would be the incomplete incorporation of squalamine into phospholipid vesicles during extrusion. We have tested the ability of radiolabeled squalamine to associate with LUVs, and have found that $100 \pm 10\%$ of squalamine added to PG in organic solvent remains associated with the vesicles after extrusion. Only $50 \pm 10\%$ of radiolabeled squalamine added to PC in organic solvent remains associated with PC vesicles after extrusion, while vesicles of mixed lipid composition were not tested. The lack of observed ionophoric activity of squalamine in PG vesicles was not due to loss of squalamine from the vesicles during preparation.

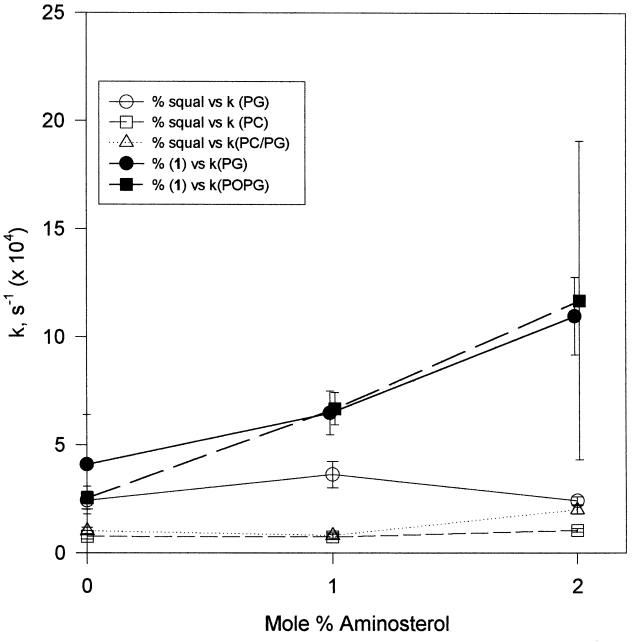

Fig. 3. The first order rate constants describing proton translocation across phospholipid membranes as a function of added aminosterol. Vesicle preparation and aminosterol incorporation are described in Section 2. The symbol identities are described in the inset. Open symbols describe experiments carried out with squalamine, while closed symbols describe experiments carried out with the analog compound **1**. PC/PG refers to vesicles comprised of 70% PC/30% PG (mol/mol). Error bars represent 1 S.D. ($n = 3$–4). When error bars are not shown, the measured error is less than the size of the symbol.

Regen and co-workers have synthesized an analog of squalamine (compound **1**) which exhibited proton ionophoric activity [11]. We have synthesized compound **1**, prepared LUVs containing the compound, and tested these for proton translocation. In agreement with previous studies, we find that compound **1** acts as a proton ionophore in LUVs prepared either from egg PG or from POPG (Fig. 3). Identical pro-

ton translocation rates are obtained in the presence of the fluorescence quencher DPX in the pH 8.0 buffer solution. The inaccessibility of HPTS to added DPX indicates that the change in fluorescence intensity after the pH jump is due to proton transport out of the vesicles, and not the result of HPTS leakage from compromised vesicles. Significant quenching of HPTS fluorescence by DPX is observed after lysis of the vesicles with Triton X-100. We also confirm the lack of ionophoric activity of compound **1** when incorporated into PC vesicles (data not shown). By confirming the ionophoric activity of the squalamine analog, we also demonstrate our ability to observe proton translocation using the HPTS fluorescence method. Therefore, our inability to observe additional proton translocation in the presence of squalamine is not due to experimental difficulties with the ionophore assay.

In our experiments, we have not been able to prepare PG LUVs with a passive proton leakage rate described by a rate constant of less than 2.0×10^{-4} s^{-1}. Regen's group reports a passive proton permeability in PG vesicles with a first order rate constant of less than 1.0×10^{-4} s^{-1} [11]. We have prepared vesicles from both PG derived from egg PC and POPG, with approximately the same proton permeability in the absence of incorporated aminosterol. Our observations are similar to that reported by Komatsu and Chong [15], who have also found that vesicles prepared from egg PG are more permeable to protons than vesicles prepared from egg PC. We see no increase in proton translocation with up to 2 mole% squalamine, while the addition of compound **1** increases the rate constant describing proton translocation to greater than 10×10^{-4} s^{-1}. Therefore, we are confident that squalamine does not act as an ionophore in PG vesicles.

An alternative explanation for the results shown in Fig. 3 is the formation of two populations of vesicles during lipid extrusion. One population would sequester most if not all of the HPTS, while the second population would not contain entrapped HPTS. This possibility is unlikely for the following two reasons. First, vesicles prepared under similar conditions have homogeneous morphology, as characterized by scanning electron microscopy [10]. If two populations of vesicles were formed, the vesicles would expect to differ in size or morphology. Second, both this study and work previously published by Regen [11] demonstrate that the proton translocation rate increases with increasing ionophore concentration, a relationship not necessarily expected if two populations of vesicles lead to the fluorescence changes.

When a mixture of egg PC and egg PG (7/3) is used, vesicles with low passive proton permeability can be prepared. Squalamine does not exhibit proton ionophoric activity in those vesicles. In vesicles prepared from egg PC, the rate constant describing passive proton leakage is approx. 0.7×10^{-4} s^{-1}, similar to that reported by Regen et al. for the same experimental system [11].

When added to preformed phospholipid vesicles, squalamine is a potent lytic agent, effecting the release of entrapped fluorescent dye from LUVs at low squalamine/phospholipid ratios [10]. The ability of compound **1** to promote the leakage of calcein from PG LUVs was tested. The squalamine analog **1**, when added at molar ratios of up to 4/1 relative to vesicle phospholipid, is totally ineffective as a lytic agent (data not shown). Squalamine will effect the quantitative leakage of calcein from anionic lipid vesicles at molar ratios of less than 1/1 [10].

To summarize our comparison of the two antimicrobial agents, squalamine lyses LUVs but is not ionophoric while compound **1** acts as a proton ionophore but possesses no lytic activity. In our previous report, we concluded that the lytic activity of squalamine was absolutely required for antimicrobial activity. Since compound **1**, which possesses no lytic activity, is an effective antimicrobial agent, there may be no relationship between vesicle lysis and antimicrobial activity. Our laboratory has synthesized a wide variety of squalamine analogs [6,7], and we have begun to test these compounds for lytic activity. In our initial tests, we find that several of our analogs exhibit no lytic activity against LUVs prepared from anionic phospholipid. Therefore, the lytic activity of squalamine may not be related to its mechanism for antimicrobial activity. However, we do observe that squalamine will effectively create defects in bacterial plasma membranes, at squalamine/phospholipid ratios similar to those required to generate defects in PG LUVs (D.W. Crawford III, B.S. Selinsky, manuscript in preparation). This suggests that bacterial plasma membrane lysis may contribute to

bacterial cell death, and might at least partially explain the antimicrobial activity of squalamine.

On the other hand, the ability of some aminosterols to act as proton ionophores must be unrelated to their antimicrobial activity. Compound **1** will only act as a proton ionophore when incorporated into vesicles by co-extrusion with phospholipid. The same analog added to preformed LUVs neither lyses the vesicles nor increases the rate of proton translocation. Since the antimicrobial activity of the aminosterols is measured by exposing intact bacterial cells to the antimicrobial agent, the ability of aminosterol antibiotics must not be related to ionophoric activity. The antimicrobial activity of squalamine must be caused by some other mechanism.

Curiously, when squalamine is incorporated into PG vesicles during their formation, the resulting vesicles retain the ability to encapsulate fluorescent dyes. When added to preformed vesicles at the same squalamine/phospholipid ratios, squalamine completely lyses PG vesicles. Squalamine may incorporate differently into phospholipid vesicles after cosolubilization than when added to preformed vesicles in aqueous systems. Alternatively, squalamine forms transient pores when added to preformed phospholipid vesicles, allowing for the leakage of encapsulated fluorescent dyes. The rate of dye leakage is initially large, but decreases to zero within several minutes. At low squalamine/phospholipid ratios, some of the encapsulated dye is retained, and the vesicles retain the remaining dye for at least several hours. The reclosure of vesicles compromised with squalamine suggests that after the initial membrane insult, squalamine finds an environment in the membranes which does not adversely affect vesicle integrity. During cosolubilization, squalamine may more easily find the same position in the membrane, allowing for its presence without membrane destabilization.

To summarize, squalamine has been shown to cause the leakage of the fluorescent dye calcein from lipid vesicles comprised of either anionic or zwitterionic phospholipid. Squalamine does not act as a proton ionophore when incorporated into phospholipid LUVs either when added externally to preformed vesicles or when cosolubilized with lipid during vesicle preparation. While squalamine does not exhibit proton ionophoric activity, the ability of a different aminosterol to effect proton translocation in membranes has been confirmed. However, the ability of some aminosterols to act as proton ionophores is likely unrelated to their antimicrobial activity.

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