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Squalamine and aminosterol mimics inhibit the peptidoglycan glycosyltransferase activity of PBP1b

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Abstract

Peptidoglycan (PG) is an essential polymer of the bacterial cell wall and a major antibacterial target. Its synthesis requires glycosyltransferase (GTase) and transpeptidase enzymes that, respectively, catalyze glycan chain elongation and their cross-linking to form the protective sacculus of the bacterial cell. The GTase domain of bifunctional PBPs of class A, such as *E. coli* PBP1b, belong to the GTase 51 family. They play essential role in PG synthesis and their specific inhibition by moenomycin lead to bacterial cell death. In this work, we report that the aminosterol squalamine and mimic compounds presented an unexpected additional mode of action consisting in the inhibition of the GTase activity of the model enzyme PBP1b. In addition, selected compounds were able to specifically displace the lipid II from the active site in a fluorescence anisotropy assay suggesting that they act as competitive inhibitors.

Introduction

Peptidoglycan (PG) is an essential polymer of the bacterial cell wall and a major antibacterial target; it surrounds the cytoplasmic membrane, determines the cell shape and protects the cell from lysis. The PG structure consists of glycan strands made of alternating β -1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues cross-linked by peptides [1]. It is assembled using the lipid II precursor (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc) by the glycosyltransferases (GTases) activities of the class A penicillin-binding proteins (aPBPs) and SEDS proteins and cross-linked by the transpeptidases (TPases) activities of aPBPs and class B PBPs (bPBPs) [2–4]. The inhibition of each one of those activities lead to cell lysis and the death of the bacteria [5].

The glycosyltransferases (GTase) domain of bifunction PBPs of class A, such as *E. coli* PBP1b, belong to the GTase 51 family. The enzymatic cavity of these proteins can be divided into two substrate binding sites: a donor site for the lipid-bound growing glycan chain, and an acceptor site for lipid II [6]. Moenomycin A is the only known natural product that specifically binds to the GTase 51 donor site and competitively inhibits PG synthesis [7]. It is a potent antibiotic that is not used in human therapy but was used in animal feedstock for decades without reported resistance [8].

Squalamine is a cationic aminosterol antibiotic, isolated from the dogfish shark (*Squalus acanthias*) [9], exhibiting a broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria and potentiate the activity of several antibiotic classes at subinhibitory concentrations, against both susceptible and resistant bacteria [10]. Its structure is composed of a steroid linked to a polyamino spermidine moiety, which taken independently exhibit

considerably low antibiotic activity and are both required for the observed antimicrobial activity of squalamine [9].

Since more than one decade, numerous aminosterol derivatives of squalamine have been synthesized and characterized [12–16]. Their mode of action was proposed to act by disrupting the outer membrane integrity of Gram-negative bacteria and by depolarizing the cytoplasmic membrane of Gram-positive bacteria [17,18]. Some of them display good membrane selectivity with high activity on bacterial membrane and low hemolytic activity, which make them promising candidate as new antibiotics or as adjuvants to sensitize resistant bacteria to the well-known antibiotics [17,18].

Herein, we will demonstrate that squalamine and other aminosterol mimics present an unexpected additional mode of action, consisting of the inhibition of a bacterial peptidoglycan polymerase. By using *in vitro* activity assays based on the processing of lipid II substrate into peptidoglycan polymers by *E. coli* PBP1b, we found that squalamine and six other aminosterol analogs inhibit the GTase activity of this enzyme. In addition, the selected compounds were able to displace the lipid II from the active site in a fluorescence anisotropy assay indicating that they act as competitive inhibitors.

Results

Squalamine inhibits the GTase activity of PBP1b

The crystal structure of the glycosyltransferase domain of the class A PBP1a from *Aquifex aeolicus* presents a steroidal CHAPS detergent molecule bound to a hydrophobic patch that lead to the active site [19]. Its side chain was suggested to mimic and partly overlap with diphospholipid chain of the lipid II substrate at the acceptor site. We noticed that the aminosterol antibiotic squalamine and CHAPS have some structural feature in common, consisting essentially of a sterane core (**Fig. 2**). We were curious to know if squalamine could affect the peptidoglycan polymerase activity of *E. coli* PBP1b. Surprisingly, by using radioactive lipid II as substrate in an endpoint TLC assay we found that squalamine was able to almost completely inhibit the glycosyltransferase activity of PBP1b (1.1 ± 0.6 % residual activity (RA) at 0.8 mM) (**Fig. 1A**). Variable concentrations of squalamine were then tested using both an endpoint TLC assay and a continuous fluorescence assay that monitor the consumption of dansyl-lipid II substrate over time. The inhibition result shows concentrations-dependent profile and the efficacy of squalamine was rather modest in the high μ molar range (IC_{50} value of 291 ± 26 μ M) (**Fig. 1B, Table 1**). When the zwitterionic CHAPS detergent was tested using the same assay at high concentrations and below the critical micelle concentration (CMC 6-10 mM) value, no inhibition of PBP1b GTase activity was observed up to 5 mM (**Fig. 1C**). In addition, steroid progesterone **11** or octanediamine **12** tested alone do not inhibit GTase activity of PBP1b (**Fig. 2, Table 1**).

Inhibition of the GTase activity of PBP1b by aminosterol mimics of squalamine

Based on these encouraging results we have analyzed twelve additional aminosterol compounds with one or more substitutions at C-3, C-6, C-7 and C-17 positions of the sterane rings. To determine the relative activity of the compounds, they were tested at 400 and 800 μM and ranked according to their effect on the residual activity (RA) of the enzyme. Six compounds were found to inhibit the glycosyltransferase activity of PBP1b. Thus, the residual GTase activity of PBP1b ranged from 0.3 to 8.5 % and from 1.2 to 39 % by using compound concentrations of 400 and 800 μM , respectively (**Fig. 2, Table 1**). The active compounds were investigated further to determine the IC_{50} values (**Table 1**) and the best derivatives **6** and **2** were found about three time more efficient than squalamine with IC_{50} values of $111 \pm 6 \mu\text{M}$ and $114 \pm 6 \mu\text{M}$, respectively. Interestingly, compound **9**, a close analog of compound **2** which is a stereoisomer at position C-7, was almost inactive (RA 98 % at 800 μM). A second observation that can be made is that the active compounds (except for squalamine) possess a nonpolar moiety in C-17. In contrast, the presence of polar groups at this position tends to abolish the activity of the compounds (e.g. amino group in **10, 13, 14, 15**). Altogether, these results indicate that the observed inhibitions of the GTase activity of PBP1b by squalamine and analogs are specific and exclude the possibility of a promiscuous effect of these series of compounds.

Mode of action of aminosterol compounds

The inhibition of the GTase activity can be achieved either by compounds that bind to the active site of the enzyme (e.g. moenomycin A) or through binding to the lipid II substrate (e.g. nisin, ramoplanin). To distinguish between these two mechanisms, we further characterized the mode of action of squalamine, the best inhibitory compound (**6**) and an inactive compound (**15**) by studying their interaction with PBP1b and lipid II, using a recently developed fluorescence anisotropy assay based on a fluorescent lipid II as a probe [20]. The assay was previously validated for direct interaction of the probe with PBP1b and with the antibiotics nisin and

ramoplanin (FA increase upon binding), as well as the ability of unlabeled-lipid II, nisin or ramoplanin to displace the probe from the enzyme active site (FA decrease in the presence of competitive compound) [20]. While no significant interaction was observed between lipid II and squalamine or compounds **6** and **15**, the active compounds were able to compete with lipid II for binding to PBP1b, whereas the inactive compound **15** had no effect. These results clearly indicate that the active aminosterol compounds directly interact with the glycosyltransferase active site of PBP1b (**Fig. 3**). All together the data reveal that squalamine and active analogs have multiple antibacterial mode of action, in addition to their potent membranes damaging activities, they also have modest cell wall synthesis inhibition activity.

Discussion

The prevalence of antibacterial resistance is increasing and new solutions should be developed urgently to deal with the resistant pathogens. The GTase activity of bifunctional PBPs is essential for bacterial viability and constitute a proven antibacterial target [21,22]. However, only one natural product antibiotic, moenomycin, that specifically bind to the active site of these enzymes has been discovered more than 50 years ago [7,23], and therefore finding a new chemical scaffold able to bind and inhibits this class of enzymes is of great importance. In this regard, the discovery of the natural product squalamine and synthetic analogues that inhibit the GTase activity of the model class A PBP1b enzyme open new perspective for the development of new class of GTase inhibitors. The identification of aminosterol derivatives with improved activity against the GTase, while maintaining some specific bacterial membranes damaging activity, could result in potent antibacterial agents, particularly against resistant Gram-negative bacteria.

Interestingly, FA experiments indicate that these compounds are competitive inhibitors able to displace the lipid II substrate from the GTase active site, but in contrast to moenomycin which

bind only to the donor site and shows an increase in FA upon cooperative binding with lipid II substrate to PBP1b [20,24], the titration of the PBP1b-probe complex by aminosterol compounds induce a decrease in FA signal, similar to that observed in the presence of unlabeled lipid II, indicating that they prevent lipid II probe binding to both donor and acceptor sites.

The observation that the presence of amino group at the C17 substitution abolish the activity of the compounds (**10**, **13**, **14**, **15**), may indicate that this part of the molecules interact with the positively charged residues of the active site, potential candidates would be the positive pocket in the donor site formed by residues (K274, R282, R286, R287) [6,25]. Positioning of a CHAPS molecule into the active site of PBP1b by homology to PBP1a-CHAPS structure [19] suggest that this may be the case (**Fig 4**).

Moenomycin has an excellent antibacterial potency and no reported resistance despite extensive use in animal feeds, but it is not used in human therapy because of its undesirable physical properties that result in poor bioavailability and long serum half-life, probably due to its lipidic C25 moenocinol moiety [8,26,27]. This lipid tail is necessary for moenomycin activity and its replacement by an alternative one could improve the properties of the resulting molecule. The C25 lipid tail of moenomycin was not resolved in any crystal structure of GTase-moenomycin complexes, indicating that it is not bound in an ordered conformation [6,28]. In contrast, CHAPS was well defined in the structure of the GTase domain of PBP1a from *A. aeolicus* [19] suggesting that binding of the cyclic steroid-substituted compounds would provide a defined structure and facilitate structure-based optimization of compounds.

Most of GTase inhibitors (moenomycin and synthetics compounds) are inactive against Gram-negative bacteria [22]. On the other hand, aminosterols have the capacity to cause lesions in the outer membrane and either kill or sensitize Gram-negative resistant strains to common antibiotics. Therefore, it is tempting to speculate that aminosterol compounds may be useful substitute to the moenocinol moiety of the moenomycin or analogs (or to the lipid tail of lipid

II analogs [29]) to generate useful new hybrid compounds, consisting of the carbohydrate and phosphoglycerate moieties or the minimal moenomycin pharmacophore (disaccharide (EF)-phosphoglycerate [22]) and an aminosterol tail (**Fig. 4**). Such compounds would have two functions: *i*) to replace the lipid tail of moenomycin derivatives while maintaining their binding to the GTase active site; and *ii*) to allow these compounds to cross the outer membrane (via the polyamine cationic moiety) and gain effectiveness against Gram-negative pathogens.

Material and Methods

Reagents and protein. Labelled lipid II [N-acetylglucosaminyl-N-acetylmuramoyl (L-Ala- γ -DGlu-(L)-Lys-(L)-D-Ala-D-Ala)-pyrophosphate-undecaprenol] variants were prepared as previously described: Dansyl-lipid II [30], NBD-lipid II [31], [^{14}C]-Lipid II ($0.06 \mu\text{Ci nmol}^{-1}$) [32].

PBP1 γ (M46-N844) was purified as previously described [33].

Compounds synthesis. The procedures for the synthesis of compounds are described in the supplementary material section.

All the solvents were purified according to reported procedures, and the reagents used were commercially available. Methanol, ethyl acetate and dichloromethane were purchased from SDS and used without further purification. Column chromatography was performed on SDS silica gel (70-230 mesh). ^1H NMR and ^{13}C NMR spectra were recorded in MeOD on a Bruker AC 300 spectrometer working at 300 MHz and 75 MHz, respectively (the usual abbreviations are used: s: singlet, d: doublet, t: triplet, q: quadruplet, m: multiplet). Tetramethylsilane was used as the internal standard. All chemical shifts are given in ppm. Mass spectroscopy analysis have been performed by the Spectropole (Analytical Laboratory) of Aix-Marseille Université (Marseille). The purity of the compounds was checked by analytical HPLC (C18 column, eluent CH_3CN -water-TFA (90:10:0.025, v/v/v), 0.5-1 mL/Min) with PDA detector spanning from 210 nm to 310 nm. All compounds possessed purity above 95%, as determined by analytical LCMS Agilent.

Octanediamine **11** and progesterone **12** were purchased from Sigma-aldrich (Saint Quentin Falavier, France). Squalamine **1** was prepared according to reported procedures [34,35]. Derivatives **2**, **3** and **9** were prepared according [36]. Derivative **4** was prepared according [37]. Derivatives **10**, **13** and **14** were prepared according [14]. Claramine A1 **8** was prepared according [38].

General procedure for the titanium-mediated reductive amination reaction:

6 β -(spermine)-cholestan-3 β -ol 6: A mixture of 6-ketocholestanol (157 mg, 0.39 mmol), titanium(IV) isopropoxide (573 μ L, 2.02 mmol) and spermine (202 mg, 1 mmol) in absolute methanol (5 mL) was stirred under argon at room temperature for 12 h. Sodium borohydride (38 mg, 1 mmol) was then added at -78 $^{\circ}$ C and the resulting mixture was stirred for an additional 2 hours. The reaction was then quenched by adding water (1 mL) and stirring was maintained at room temperature for 20 minutes. The resulting inorganic precipitate was filtered off over a pad of Celite and washed with methanol and ethylacetate. The combined organic extracts were dried over Na₂SO₄, filtered and concentrated in vacuo to afford the expected crude amino derivative which was purified by flash chromatography affording the expected amino derivative. Purification by column chromatography (silica gel; CH₂Cl₂/ MeOH/ NH₄OH(32%), 7:3:1) afforded a pale yellow solid in 45% yield; this compound can be converted subsequently into its hydrochloride salt as white solid ¹H NMR (300 MHz, CD₃OD) : δ = 0.67-0.76 (m, 4H), 0.90-1.17 (m, 24H), 1.20-1.46 (m, 7H), 1.47-1.92 (m, 20H), 2.03-2.07 (m, 1H), 2.50-2.54 (m, 1H), 2.63-2.73 (m, 13H), 3.56-3.63 (m, 1H) ¹³C NMR (75 MHz, CD₃OD) : δ = 71.50, 58.96, 56.27, 56.05, 54.78, 49.98, 49.21, 47.99, 47.84, 47.34, 42.62, 40.47, 39.94, 39.49, 39.06, 36.44, 36.14, 35.86, 35.77, 35.63, 33.58, 31.61, 30.45, 28.18, 27.97, 24.37, 23.78, 22.78, 22.52, 21.03, 18.63, 16.30.12.13. C₃₇H₇₂N₄O ; MS (ESI) m/z = 589.5 [M+H]⁺

6 β -(1,4-diaminobutane)-cholestan-3 β -ol 5: Purification by column chromatography (silica gel; CH₂Cl₂/ MeOH/ NH₄OH(32%), 7:3:1) afforded a pale yellow solid in 73% yield; ¹H NMR (300 MHz, MeOD): δ = 0.66-3.57 (m, 58H) ¹³C NMR (75 MHz, MeOD): δ = 71.65, 59.88, 58.54, 56.29, 56.04, 54.75, 48.18, 47.29, 42.71, 42.64, 39.94, 39.50, 39.04, 36.16, 35.78, 35.65, 31.56, 31.03, 30.40, 29.67, 27.99, 25.96, 24.35, 23.81, 22.79, 22.54, 21.05, 18.65, 16.33, 14.09, 12.15. C₃₁H₅₈N₂O ; MS (ESI) m/z = 475.4 [M+H]⁺

N1,N1'-((3S,7R,10R,13R,17R)-10,13-dimethyl-17-((R)-5-methylhexan-2-yl)-tetradecahydro-1H-cyclopenta[a]phenanthrene-3,7-diyl)bis(pentane-1,5-diamine) 7

Synthesis according a similar protocol than for derivative **5** but using 3,7-diketocholestene and 1,5-diaminopentane as starting materials. Purification by column chromatography (silica gel; CH₂Cl₂/ MeOH/ NH₄OH(32%), 7:3:1) afforded a pale yellow solid in 48% yield; – ¹H NMR (300 MHz, MeOD): δ = 0.68-0.70 (m, 3H), 0.83-0.87 (m, 7H), 0.91-0.93 (m, 3H), 1.01-1.03 (m, 3H), 1.06-1.08 (m, 2H), 1.09-1.18 (m, 4 H), 1.19- 1.25 (m, 4H), 1.27-1.35 (m, 8H), 1.36-1.55 (m, 8H), 1.57-2.03 (m, 13H), 2.31-2.37 (m, 1H), 2.59-2.87 (m, 7H), 2.94-3.01 (m, 2H), 5.36-5.38 (m, 1H). – ¹³C NMR (75 MHz, MeOD): δ = 140.88, 125.41, 55.81, 55.47, 55.07, 52.16, 47.41, 47.13, 47.02, 42.85, 40.71, 40.61, 40.45, 39.50, 39.40, 38.40, 37.52, 37.25, 36.22, 35.70, 32.97, 32.87, 30.36, 30.26, 29.05, 28.87, 28.19, 24.78, 24.24, 24.14, 24.06, 22.66, 21.32, 18.76, 18.65, 12.35. C₃₆H₆₈N₄ ; MS (ESI) m/z = 557.54 [M+H]⁺

(13S,17S)-17-((4-aminobutyl)amino)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-3-ol 15

Synthesis according a similar protocol than for derivative **5** but using oestrone and 1,4-butanediamine as starting materials. Purification by column chromatography (silica gel; CH₂Cl₂/ MeOH/ NH₄OH(32%), 7:3:1) afforded a pale yellow solid in 37% yield; – ¹H NMR (300 MHz, MeOD): δ = 0.77-1.58 (m, 17H), 2.02-2.50 (m, 3H), 2.66-2.74 (m, 5H), 3.13-3.30 (m, 3H), 3.43-3.63 (m, 2H), 6.50-7.08 (m, 3H), 8.06 (s, 1H). – ¹³C NMR (75 MHz, MeOD): δ = 156.55, 139.17, 132.80, 127.63, 116.65, 114.32, 70.45, 53.94, 45.69, 44.57, 42.29, 40.75, 40.60, 36.24, 31.15, 30.62, 29.13, 28.10, 24.87, 23.05, 20.46, 14.74, 12.77, 12.69. C₂₂H₃₄N₂O, M = 342.53 g.mol⁻¹, Calc. C 77.15, H 10.1, N 8.18, Exp.C 77.12, H 11.3, N 8.21.

Glycosyltransferase assays

Continuous fluorescence assay. The GTase activity of PBP1b was monitored by a continuous fluorescent assay using dansyl lipid II as substrate. It was performed in a medium binding black 96-well microplate at 30°C (Greiner Bio One) as described [39,40]. The samples (50 μl) contained 10 μM dansyl-lipid II, 50 mM HEPES-NaOH pH 7.5, 200 mM NaCl, 10 mM CaCl₂, 0.085% of decyl-PEG, 20 % of dimethylsulfoxide (DMSO), 1 unit of *N*-acetylmuramidase of *Streptomyces globisporus* (Sigma) and variable concentrations of compounds. The reactions

were initiated by the addition of 100 nM PBP1b and monitored by following the fluorescence decrease over 15-20 min at 30°C using an Infinite 200 PRO Microplate reader (Tecan, Männedorf, Switzerland) with excitation wavelength at 340 nm and emission at 520 nm.

Glycosyltransferase activity assay using TLC. The end-point assay, based on radiolabeled lipid II ($[^{14}\text{C}]$ lipid II), was performed in 30 μl in the same conditions as the fluorescence assay using 4 μM $[^{14}\text{C}]$ lipid II (0.06 μCi / nmol) instead of the fluorescent substrate and by omitting the muramidase. The reaction was incubated 15 min at 30°C, stopped by heating at 80°C for 10min and the products were separated by thin-layer chromatography (TLC) on silica gel plates in 2-propanol–ammonium hydroxide (25%)– H_2O (6:3:1; V/V/V). The TLC plates were exposed to a storage phosphor screen (GE Healthcare) for 16 h, and images were revealed using a Typhoon Trio imager and Image Quant TL software (GE Healthcare).

The GTase inhibition assay was carried out by measuring the residual activity (RA) of *E. coli* PBP1b in the presence of 400 and 800 μM compounds. All the assays were repeated at least three times. The IC_{50} values were determined for the active compounds using variable concentrations of inhibitor (0–1000 μM) by fitting the data using GraphPad Prism 6.0 software.

Fluorescent anisotropy (FA) assay

Fixed concentrations of NBD-lipid II (0.33 μM) and PBP1b (~1 μM) in the 25 mM Tris–HCl pH 7.5, NaCl 0.1 M, CHAPS 0.14 % w/v, were mixed with serial dilutions of the compounds in 384-well plates. All the experiments were performed in triplicates and blank samples without proteins were also prepared for background fluorescence estimation. The mixtures (30 μL) were allowed to reach equilibrium for 20 min at room temperature with gentle shaking and the FA signals were recorded at 21°C using an Infinite® F Plex (Tecan, Männedorf, Switzerland) microplate reader equipped with polarization filters with excitation and emission wavelengths at 485 nm and 535 nm respectively. For direct binding evaluation of the compounds with the

probe, the experiments were performed in the same condition in the absence of the protein. FA values in millianisotropy units (mA) were plotted as a function of compound concentrations in μM as previously described [20].

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Figure legend

Figure 1.

Inhibition of the GTase activity of PBP1b by squalamine. A) TLC analysis of peptidoglycan polymerase activity of PBP1b using radioactive lipid II as substrate, in the presence of squalamine and aminosterol compounds. Lanes: 1, lipid II incubated without enzyme; 2-3, lipid II incubated with 100 nM PBP1b with (2) or without (3) addition of methanol (because some compounds tested were solubilized in methanol); and 4-5, the reaction was incubated in the presence of 1 mM squalamine, or compounds 13, 14, 10, respectively. Inhibition of PBP1b GTase activity using increasing concentration of squalamine (200-1000 μ M) (B) or CHAPS (1-5 mM) (C), monitored by dansyl-lipid II based continuous fluorescence assay.

Figure 2.

Structures of the aminosterol compounds used in this study.

Figure 3.

Fluorescence anisotropy assay using the NBD-lipid II as a probe. FA (in mA units) is plotted as a function of compound concentrations. The error bars represent the FA values as mean \pm s.d. of triplicate experiments. (A) Competition of squalamine and compound **6** for PBP1b/NBD-lipid II binding induce a decrease of the FA of the probe while the inactive compound **15** does not affect the FA. (B) Measurements of direct binding between the probe and the compounds show no significant variation of FA, indicating absence of interactions.

Figure 4.

Ligand bound to the GTase domain. Crystal structure of PBP1b GTase domain in complex with moenomycin A (yellow) with a bound CHAPS molecule (green) in the active site positioned by homology to PBP1a GTase domain of *Aquifex aeolicus*-CHAPS structure (PDB code 2OQO).

Table 1. GTase inhibition activities of squalamine and aminosterol analogues. RA = the residual GTase activities values of PBP1b in % of inhibition compared to non-treated control are shown for two compounds concentrations (400 and 800 μ M). Both RA and IC₅₀ data represent mean values of three independent experiments \pm s.d.nd, not determined.

Compounds	RA % (0.8 mM)	RA % (0.4 mM)	IC₅₀ (μM)
Squalamine (1)	1.1 \pm 0.6	6 \pm 2	291 \pm 26
2	0.3 \pm 0.1	1.2 \pm 0.3	114 \pm 6
3	8.5 \pm 3.1	16 \pm 2	220 \pm 10
4	4 \pm 1	39 \pm 2	303 \pm 15
5	3 \pm 1	16 \pm 1.4	221 \pm 26
6	0.8 \pm 0.4	3.9 \pm 0.1	111 \pm 6
7	1.5 \pm 0.8	12.5 \pm 4.4	229 \pm 17
8	97 \pm 0.2	nd	nd
9	98 \pm 1	nd	nd
10	99 \pm 0.5	nd	nd
11	96 \pm 2	nd	nd
12	98 \pm 1	nd	nd
13	100	nd	nd
14	100	nd	nd

15	105 ± 3	nd	nd
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Figure 1

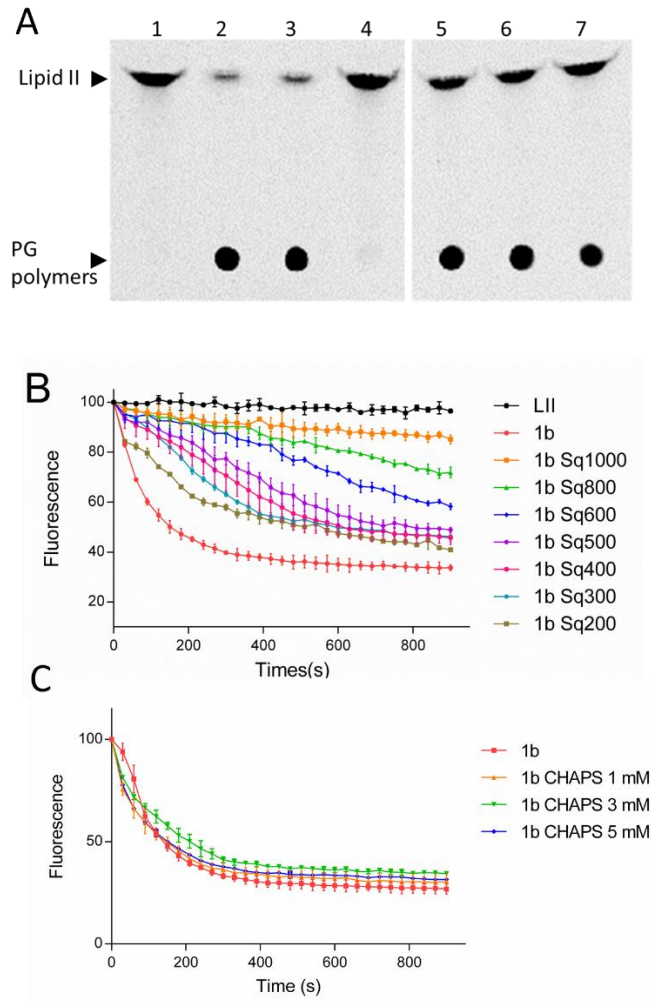


Figure 2

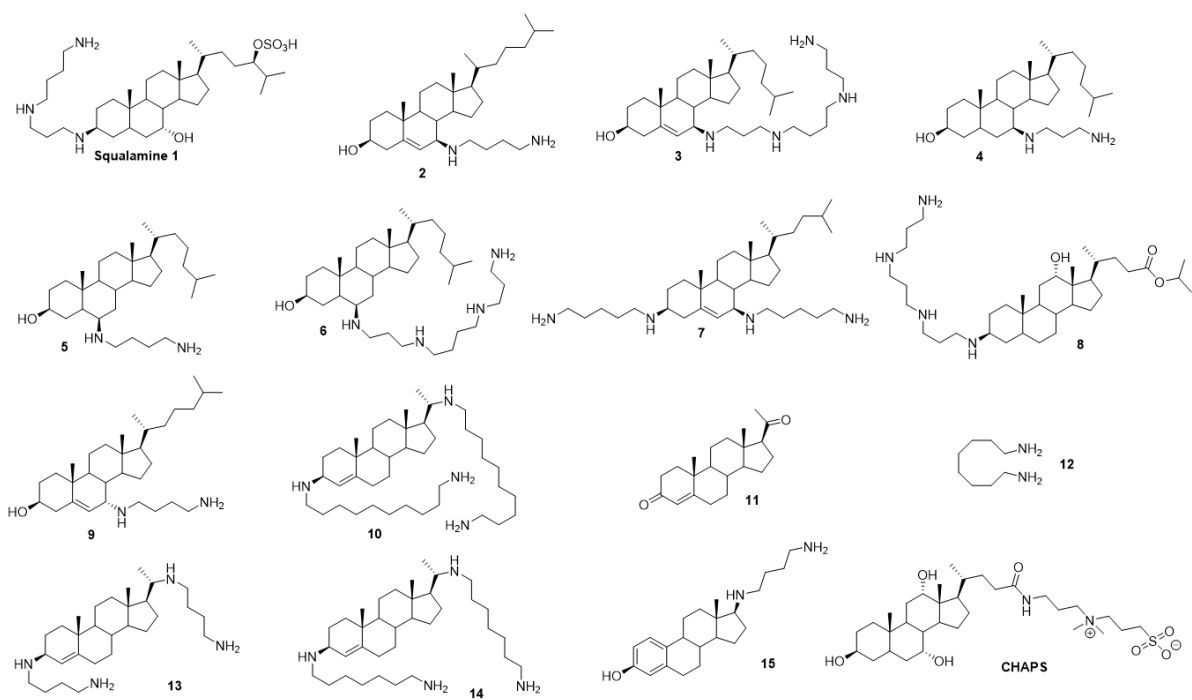


Figure 3

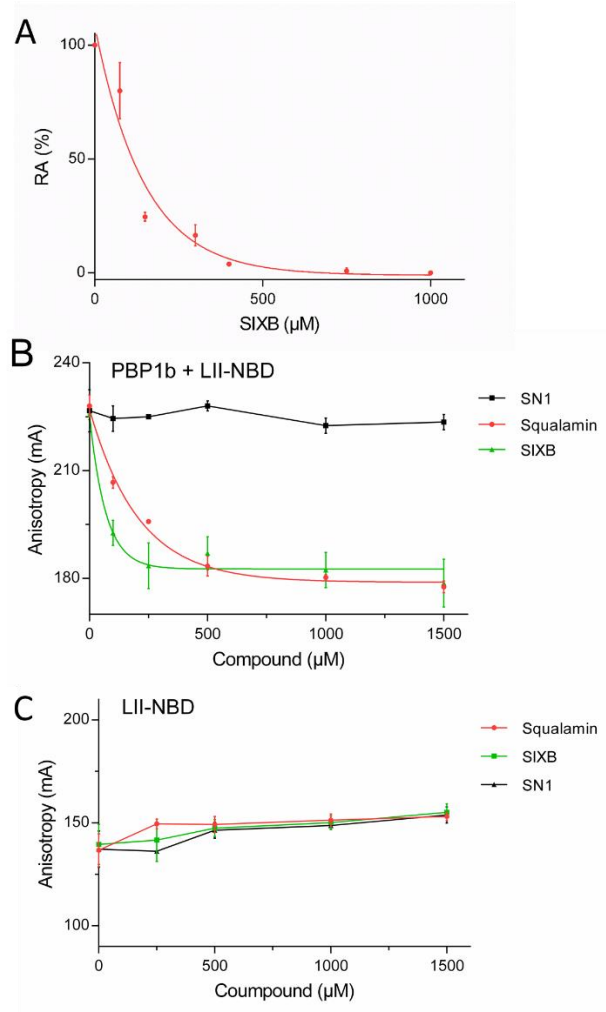


Figure 4

