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ARTICLE



Membrane permeabilization and perturbation induced by alkyl- biguanidium salts

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ABSTRACT

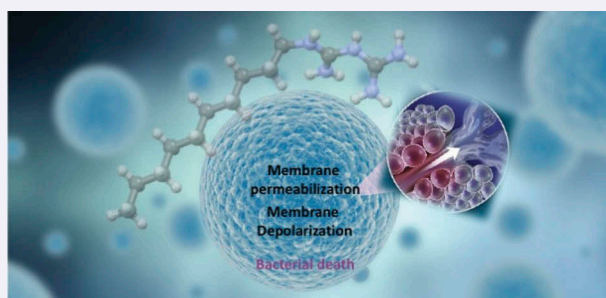
Alkylbiguanidium salts show membrane perturbing properties toward simple phospholipid bilayer (liposomes) and complex phospholipid membranes (bacterial membranes). Compared to metformin and phenformin, alkylbiguanidium salts induce a more significant permeabilization of the phospholipid membranes, leading to highly selective H^+/OH^- ion transport and subsequent depolarization of the membrane. Although alkylbiguanidium salts with shorter **C**₆ and **C**₈ chains are too hydrophilic to be inserted into the membrane and perturb it, those with **C**₁₀, **C**₁₂, **C**₁₄, and **C**₁₆ alkyl chains show high membrane permeabilization and depolarization activity. In addition, the **C**₁₀, **C**₁₂, and **C**₁₄ compounds possess interesting antimicrobial activity, and, at concentrations close to their minimal inhibitory concentrations, they show low hemolytic activity and toxicity.

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Alkylbiguanidium salts;
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Introduction

Biguanides in general, particularly *N,N*-dimethylbiguanide, commonly known as metformin (Figure 1), have been used worldwide for decades for the treatment of type-II diabetes and have recently drawn attention for their antitumorigenic effects. Pollak *et al* (1), reported evidence of the antineoplastic activity of metformin, confirming a reduction of 37% in the incidence of certain types of cancer in diabetic patients treated with metformin (2). Unfortunately, phase II clinical trials on patients with advanced pancreatic cancer treated with metformin did not show any improvement in their survival rate. The poor *in vivo* results were attributed to the high concentrations of metformin required (*in vitro* activity at millimolar concentrations), in addition to a low internalization rate and the need for accumulation (3). Metformin, which has pK_a s of 2.8

and 11.5, is monoprotonated at physiological pH, very hydrophilic, and unable to permeate biological membranes through passive diffusion (4). Recently, Tomic *et al* (5), reported that alkyl and alkene-substituted biguanides and their salts have good antiproliferative activity for lung, breast, and pancreatic cancer cells. Unfortunately, no evidence of improved cell penetration and internal accumulation of these biguanide derivatives has been reported to date.

Alkylbiguanidium salts are expected to influence cellular membrane permeability because their structures are very similar to those of quaternary ammonium compounds (QACs), surfactants with membrane perturbing properties that are widely used as antibacterial agents (6). The positively charged ammonium group of QACs interacts with the negatively charged phosphate groups of phospholipids, facilitating the insertion of the

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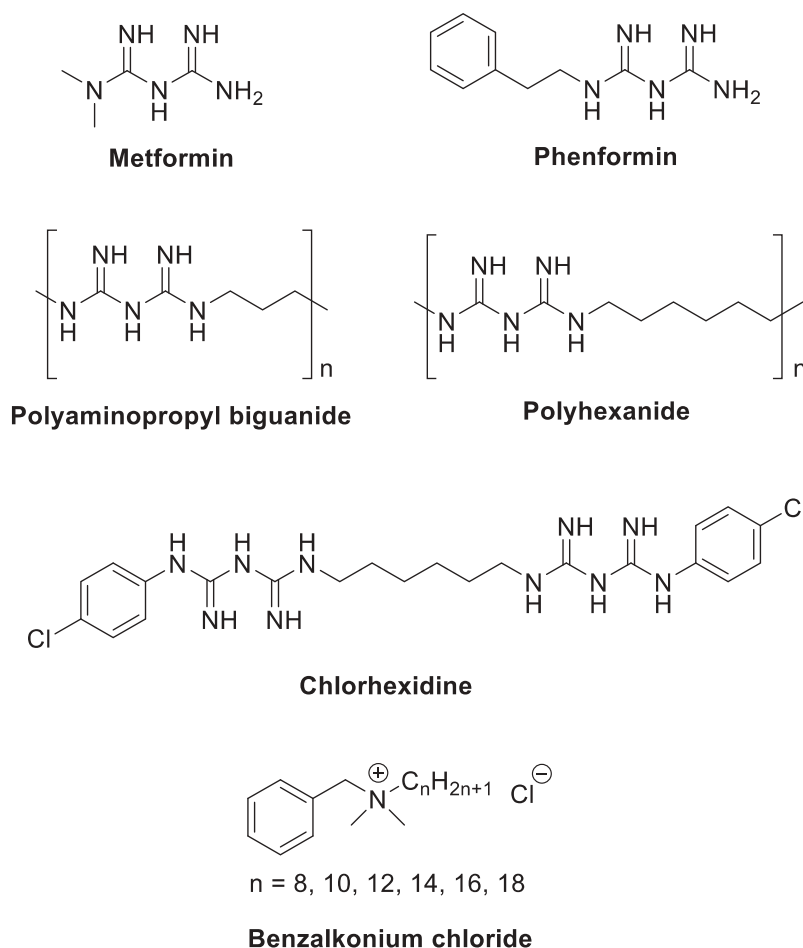


Figure 1. Structures of commercially available biguanides and benzalkonium chloride.

hydrophobic tail of the QAC into the bacterial membrane and the formation of transmembrane pores (7, 8). The resulting membrane lysis and bacterial death explain the use of these compounds since the 1930s as antibacterial agents in hospitals and industry. Possessing a similar structure, alkylbiguanidium salts can be expected to show important membrane disrupting properties and improved antibacterial activity compared to metformin. Additionally, when biguanide derivatives such as chlorhexidine (8), polyhexanide (8, 9), or polyaminopropyl biguanide (8), (Figure 1) are used as antibacterial agents, they superficially interact with bacterial membranes and displace membrane-bound Ca^{2+} and Mg^{2+} , resulting in increased rigidity and loss of membrane structural integrity. As commercially available QACs such as benzalkonium chloride (BAC, Figure 1) have been linked to an increased expression of the membrane-affiliated efflux pump QacA in resistant bacteria (10), new compounds are required to compensate for the development of bacterial resistance.

Thus, we wanted to address whether alkylbiguanidium salts act as membrane disruptors or membrane

perturbators (through permeabilization, depolarization, or ion transport) and identify whether this effect can be correlated to their antibacterial activity. To address these questions, we synthesized simple alkylbiguanidium salts (Figure 2) possessing different alkyl chains and studied their interactions with model membranes, Gram-positive and Gram-negative bacteria, and yeast membranes.

Materials and methods

All chemicals were purchased from Aldrich Chemicals in their highest purity and used without further purification. Deuterated dimethylsulfoxide ($\text{DMSO}-d_6$) was purchased from CDN Isotopes. NMR spectra were recorded on a Bruker Avance 400. Coupling constants are given in hertz (Hz) and chemical shifts are given in parts per million (ppm, δ) measured relative to the residual solvent (the multiplicity of the signals are given as s: singlet, d: doublet, t: triplet, and m: multiplet). High-resolution mass spectra (HRMS) were recorded on a TSQ Quantum Ultra (Thermo Scientific) triple quadrupole

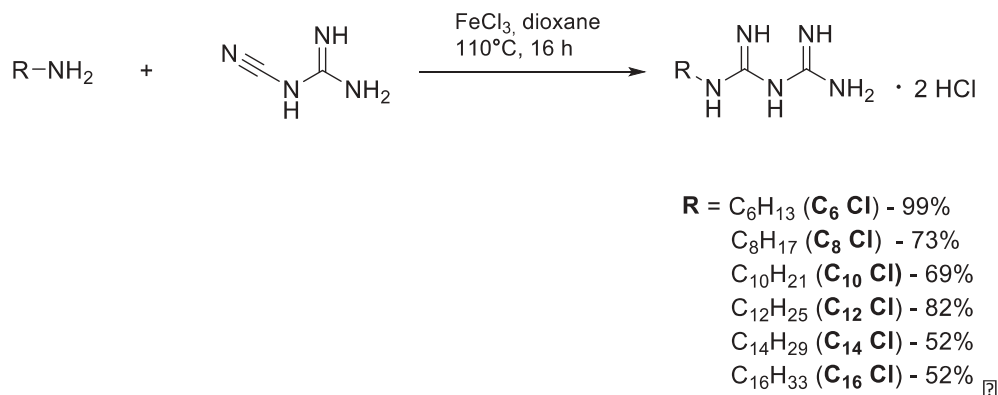


Figure 2. Synthesis of alkylbiguanidinium salts **C₆–C₁₆Cl**.

with accurate mass option instrument. (Université de Montréal Mass Spectrometry Facility). L- α -Phosphatidylcholine was purchased from Avanti Polar Lipids. Transport and depolarization studies were performed on a Varian Cary Eclipse fluorescence spectrophotometer. The antimicrobial (minimum inhibitory concentration (MIC)) assays were performed on a Fluostar Optima plate reader. UV-vis permeabilization experiments were performed on a Tecan Infinite M200 microplate reader. Bacterial cell lysis was carried out by sonication using a Branson sonicator (5 to 15 rounds of 30-s sonication until the optical density at 600 nm ($\text{OD}_{600\text{nm}}$) was equal to 0). The *Escherichia coli* (MG1655, DH5 α and SK037) and *Bacillus thuringiensis* (HD73) strains were provided by Prof. J. Pelletier, Chemistry Department, Université de Montreal. Dynamic light scattering (DLS) experiments were performed on a Mobius Mobility Laser Photometer.

General procedure for the synthesis of alkylbiguanidinium salts

The alkylamine (1.19 mmol), dicyandiamide (1.19 mmol), and FeCl₃ (1.19 mmol) were dissolved in dioxane (1.19 mL, 1 M) and heated to 100°C overnight. After the reaction had cooled down, HCl 12 M (0.3 mL) was added to the mixture and a white precipitate formed. The solid was filtered and washed with ethyl acetate to afford the alkylbiguanidinium compounds (**C₆**, **C₈**, **C₁₀**, **C₁₂**, **C₁₄**, and **C₁₆**) as chloride salts (52% to 99% yields).

1-Hexylbiguanidinium chloride (**C₆Cl**) Yield: 99%

¹H NMR (400 MHz, DMSO-*d*₆, 25°C, TMS): δ = 0.86 (t, ³J(H, H) = 9.0 Hz, 3H; CH₃), 1.26 (s, 6H; (CH₂)₃), 1.49 (s, 2H; CH₂), 3.18 (d, ³J(H, H) = 5.7 Hz, 2H; CH₂), 7.35 (s, 1H; NH), 8.29 (s, 3H; NH), 8.87 (s, 1H; NH), 9.28 (s, 1H; NH).

¹³C NMR (75 MHz, CD₃OD, 25°C, TMS): δ = 13.0 (CH₃), 22.2 (CH₂), 26.1 (CH₂), 27.4 (CH₂), 31.1 (CH₂), 42.9 (CH₂), 152.4 (CNH), 155.2 (CNH).

MS (120.0V, ES+): *m/z* (%) = 186.17 (100) ([C₈H₁₉N₅]+ H)⁺, 187.17 (**11**) (M + H)⁺.

Elemental analysis calcd (%) for [C₁₄H₃₁N₅] 2 HCl: N = 20.46, C = 49.12, H = 9.72; found: N = 21.14, C = 47.28, H = 10.35.

1-Octylbiguanidinium chloride (**C₈Cl**) Yield: 73%

¹H NMR (400 MHz, DMSO-*d*₆, 25°C, TMS): δ = 0.84 (t, ³J(H, H) = 8.0 Hz, 3H; CH₃), 1.24 (s, 10H; (CH₂)₅), 1.50 (s, 2H; CH₂), 3.19 (s, 2H; CH₂), 7.50 (s, 1H; NH), 8.42 (s, 3H; NH), 8.97 (s, 1H; NH), 9.38 (s, 1H; NH).

¹³C NMR (75 MHz, CD₃OD, 25°C, TMS): δ = 13.0 (CH₃), 22.2 (CH₂), 26.4 (CH₂), 27.4 (CH₂), 28.9 ((CH₂)₂), 31.5 (CH₂), 42.9 (CH₂), 152.4 (CNH), 155.2 (CNH).

MS (120.0V, ES+): *m/z* (%) = 214.20 (100) ([C₁₀H₂₃N₅]+ H)⁺, 215.20 (**20**) (M + H)⁺.

Elemental analysis calcd (%) for [C₁₀H₂₃N₅] 2 HCl: N = 23.02, C = 39.48, H = 8.94; found: N = 23.72, C = 39.03, H = 9.03.

1-Decylbiguanidinium chloride (**C₁₀Cl**) Yield: 69%

¹H NMR (400 MHz, DMSO-*d*₆, 25°C, TMS): δ = 0.85 (t, ³J(H, H) = 9.4 Hz, 3H; CH₃), 1.19 (s, 14H; (CH₂)₇), 1.49 (s, 2H; CH₂), 3.15 (d, ³J(H, H) = 5.9 Hz, 2H; CH₂), 7.27 (s, 1H; NH), 8.18 (s, 3H; NH), 8.77 (s, 1H; NH), 9.25 (s, 1H; NH).

¹³C NMR (75 MHz, CD₃OD, 25°C, TMS): δ = 13.0 (CH₃), 22.3 (CH₂), 26.4 (CH₂), 27.5 (CH₂), 28.9 (CH₂), 29.0 (CH₂), 29.2 (CH₂), 29.3 (CH₂), 31.6 (CH₂), 42.9 (CH₂), 152.4 (CNH), 155.2 (CNH).

MS (120.0V, ES+): *m/z* (%) = 242.20 (100) ([C₁₂H₂₇N₅]+ H)⁺, 243.20 (**17**) (M + H)⁺.

Elemental analysis calcd (%) for [C₁₂H₂₇N₅] 2 HCl H₂O: N = 21.07, C = 43.37, H = 9.40; found: N = 22.27, C = 43.83, H = 9.92.

1-Dodecylbiguanidinium chloride (**C₁₂Cl**) Yield: 82%

^1H NMR (400 MHz, DMSO- d_6 , 25°C, TMS d_6): δ = 0.85 (t, $^3J(\text{H}, \text{H})$ = 9.4 Hz, 3H; CH_3), 1.25 (s, 18H; $(\text{CH}_2)_9$), 1.49 (s, 2H; CH_2), 3.15 (d, $^3J(\text{H}, \text{H})$ = 9.4 Hz, 2H; CH_2), 7.34 (s, 1H; NH), 8.17 (s, 3H; NH), 8.77 (s, 1H; NH), 9.27 (s, 1H; NH).

^{13}C NMR (75 MHz, CD_3OD , 25°C, TMS): δ = 15.2 (CH_3), 23.4 (CH_2), 27.4 (CH_2), 28.6 (CH_2), 29.9 (CH_2), 30.0 (CH_2), 30.2 (CH_2), 30.3 ($(\text{CH}_2)_2$), 30.4 (CH_2), 32.5 (CH_2), 43.7 (CH_2), 153.2 (CNH), 156.3 (CNH).

MS (120.0V, ES+): m/z (%) = 270.20 (100) ($[\text{C}_{14}\text{H}_{31}\text{N}_5]^+ \text{H}^+$), 271.26 (25) ($(\text{M} + \text{H})^+$).

Elemental analysis calcd (%) for $[\text{C}_{14}\text{H}_{31}\text{N}_5] \cdot 2 \text{HCl}$: N = 20.46, C = 49.12, H = 9.72; found: N = 21.14, C = 47.28, H = 10.35.

1-Tetradecylbiguanidium (C_{14}Cl) Yield: 52%

^1H NMR (400 MHz, DMSO- d_6 , 25°C, TMS d_6): δ = 0.84 (t, $^3J(\text{H}, \text{H})$ = 9.1 Hz, 3H; CH_3), 1.23 (s, 22H; $(\text{CH}_2)_{11}$), 1.49 (s, 2H; CH_2), 3.15 (d, $^3J(\text{H}, \text{H})$ = 6.2 Hz, 2H; CH_2), 7.17 (s, 1H; NH), 8.13 (s, 3H; NH), 8.73 (s, 1H; NH), 9.15 (s, 1H; NH).

^{13}C NMR (75 MHz, CD_3OD , 25°C, TMS): δ = 13.0 (CH_3), 22.3 (CH_2), 26.5 (CH_2), 27.5 (CH_2), 28.9 (CH_2), 29.0 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 ($(\text{CH}_2)_4$), 31.7 (CH_2), 42.9 (CH_2), 152.5 (CNH), 155.2 (CNH).

MS (120.0V, ES+): m/z (%) = 298.30 (100) ($[\text{C}_{16}\text{H}_{35}\text{N}_5]^+ \text{H}^+$), 299.30 (20) ($(\text{M} + \text{H})^+$).

Elemental analysis calcd (%) for $[\text{C}_{16}\text{H}_{35}\text{N}_5] \cdot 2 \text{HCl}$: N = 16.48, C = 45.23, H = 9.49; found: N = 17.16, C = 45.82, H = 9.81.

1-Hexadecylbiguanidium (C_{16}Cl) Yield: 52%

^1H NMR (400 MHz, DMSO- d_6 , 25°C, TMS d_6): δ = 0.85 (t, $^3J(\text{H}, \text{H})$ = 8.9 Hz, 3H; CH_3), 1.23 (s, 26H; $(\text{CH}_2)_{13}$), 1.49 (s, 2H; CH_2), 3.15 (s, 2H; CH_2), 7.25 (s, 1H; NH), 8.22 (s, 3H; NH), 8.80 (s, 1H; NH), 9.21 (s, 1H; NH).

^{13}C NMR (75 MHz, CD_3OD , 25°C, TMS): δ = 13.0 (CH_3), 22.3 (CH_2), 26.4 (CH_2), 27.5 (CH_2), 28.7 (CH_2), 28.9 (CH_2), 29.0 (CH_2), 29.2 (CH_2), 29.4 ($(\text{CH}_2)_6$), 31.7 (CH_2), 42.9 (CH_2), 152.4 (CNH), 155.2 (CNH).

MS (120.0V, ES+): m/z (%) = 326.32 (100) ($[\text{C}_{18}\text{H}_{39}\text{N}_5]^+ \text{H}^+$), 327.33 (20) ($(\text{M} + \text{H})^+$).

Elemental analysis calcd (%) for $[\text{C}_{18}\text{H}_{39}\text{N}_5] \cdot 2 \text{HCl}$: N = 15.46, C = 47.73, H = 9.79; Found: N = 15.87, C = 46.73, H = 9.93.

Dynamic light scattering

A 2 mL volume of a 25 mg/mL solution of egg-yolk phosphatidyl choline (EYPC) in chloroform was slowly reduced *in vacuo* to form a thin film on the side of the flask. Then, 1 mL of phosphate buffered saline (PBS buffer, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 , adjusted to pH = 7.4) was added, and the resulting suspension was subjected to 10

freeze/thaw cycles (1 cycle = 1 min at -20°C followed by 1 min at 37°C). The mixture was extruded onto a 100-nm polycarbonate membrane 21 times and diluted to obtain a final concentration of 10 mM. To a plastic cuvette, 2 mL of ultrapure water, 20 μL of the liposome solution, and the alkylbiguanidium salt in DMSO were added to yield a concentration of 1 $\mu\text{g}/\text{mL}$ or 100 $\mu\text{g}/\text{mL}$.

Lucigenin assay

A 2 mL volume of a 25 mg/mL solution of EYPC in chloroform was slowly reduced *in vacuo* to form a thin film on the side of the flask. Then, 1 mL of a lucigenin solution (2 mM lucigenin, 10 mM Na_2HPO_4 , 10 mM NaH_2PO_4 , and 100 mM NaCl) was added, and the resulting suspension was subjected to 10 freeze/thaw cycles (1 cycle = 1 min at -20°C and 1 min at 37°C). The mixture was extruded onto a 100-nm polycarbonate membrane 21 times and passed through a Sephadex G-25 column to remove the extravesicular lucigenin. The eluent used for the column was a phosphate buffer with sodium chloride (10 mM Na_2HPO_4 , 10 mM NaH_2PO_4 , and 100 mM NaCl), and the resulting liposome solution was diluted to obtain a final concentration of 10 mM. To a quartz cuvette, 2.5 mL phosphate buffer with sodium nitrate (10 mM Na_2HPO_4 , 10 mM NaH_2PO_4 , and 100 mM NaNO_3) and 40 μL of liposome solution were added with light stirring ($\lambda_{\text{ex.}}$ = 372 nm, $\lambda_{\text{em.}}$ = 503 nm). At t = 50 s, a solution of the alkylbiguanidium salt in methanol was added to the cuvette to obtain a 50 mM final solution (50 mol% relative to the concentration of EYPC). At t = 350 s, Triton-X 10% v/v was added to lyse the liposomes. The fluorescence was monitored for 400 s.

HPTS assay

Here, 2 mL of a 25 mg/mL solution of EYPC in chloroform was slowly reduced *in vacuo* to form a thin film on the side of the flask. Then, 1 mL of a solution of the trisodium salt of 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) (1 mM HPTS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) salt, and 100 mM NaCl, adjusted to pH = 7.4) was added, and the resulting suspension was subjected to 10 freeze/thaw cycles (1 cycle = 1 min at -20°C and 1 min at 37°C). The mixture was extruded onto a 100-nm polycarbonate membrane 21 times and passed through a Sephadex G-25 column to remove the extravesicular HPTS. HEPES buffer (10 mM HEPES and 100 mM NaCl, adjusted to pH = 7.4) was used as the eluent for the column and the resulting liposome solution was diluted to obtain a

final concentration of 10 mM. To a quartz cuvette, 1.9 mL of HEPES buffer and 25 μL of liposome solution were added with light stirring ($\lambda_{\text{ex.}} = 405/450 \text{ nm}$, $\lambda_{\text{em.}} = 510 \text{ nm}$). At $t = 50 \text{ s}$, a solution of alkylbiguanidium salt in methanol was added to obtain a 5 mM final concentration. At $t = 300 \text{ s}$, NaOH was added to obtain a 5 mM final concentration, and, at $t = 350 \text{ s}$, Triton-X 10% v/v was added to lyse the liposomes. The fluorescence was monitored for 600 s. As a control, we monitored the variation of HPTS fluorescence after the addition of a 100 $\mu\text{g/mL}$ solution of alkylbiguanidium salts. No variation was observed.

Safranin O assay

Here, 2 mL of a 25 mg/mL solution of EYPC in chloroform was slowly reduced *in vacuo* to form a thin film on the side of the flask. Then, 1 mL of HEPES buffer (10 mM HEPES salt, 100 mM KCl, adjusted to pH = 7.4) was added, and the resulting suspension was subjected to 10 freeze/thaw cycles (1 cycle = 1 min at -20°C and 1 min at 37°C). The mixture was extruded on a 100-nm polycarbonate membrane 21 times, and the resulting liposome solution was diluted to obtain a 10 mM final concentration. To a quartz cuvette, 1.9 mL of HEPES buffer with sodium chloride (10 mM HEPES salt and 100 mM NaCl, adjusted to pH = 7.4) and 100 μL of liposome solution were added with light stirring. Safranin O dye was added to a 60 nM final concentration ($\lambda_{\text{ex.}} = 522 \text{ nm}$, $\lambda_{\text{em.}} = 581 \text{ nm}$). At $t = 50 \text{ s}$, a solution of alkylbiguanidium salt in DMSO was added to obtain a 100 $\mu\text{g/mL}$ final solution in the cuvette, and the fluorescence was monitored for 300 s. As a control, we monitored the variation in safranin O fluorescence in the solution by adding 100 $\mu\text{g/mL}$ alkylbiguanidium salts. No change was observed.

Bacterial membrane depolarization

A bacterial preculture was prepared by the inoculation of 1 mL of lysogeny broth (LB) with *E. coli* (DH5 α strain) or *B. thuringiensis* (HD73 strain). The preculture was incubated overnight at 37°C with agitation and was then added to 75 mL of LB and incubated for 2 h at 37°C with agitation. The culture broth was centrifuged at 1000g for 10 min, rinsed twice with a glucose buffer (20 mM glucose and 5 mM HEPES), and resuspended in glucose buffer to $\text{OD}_{600\text{nm}} = 0.15$. The solution was incubated with 1 μM 3,3'-diethylthiadicarbocyanine iodide dye (DISC₂5) at 37°C for 1 h. The experiment was carried out with 2.5 mL of bacterial solution in a quartz cuvette with light stirring ($\lambda_{\text{exc}} = 600 \text{ nm}$, $\lambda_{\text{em}} = 660 \text{ nm}$). The alkylbiguanidium salt in DMSO was added after 50 s, yielding a final

concentration of 100 $\mu\text{g/mL}$. As a control, we monitored the variation of DISC₂5 fluorescence in solution after the addition of a 100 $\mu\text{g/mL}$ alkylbiguanidium salt solution.

Bacterial growth and MIC determination

A bacterial preculture was prepared by the inoculation of 1 mL lysogeny broth (LB) with *E. coli* (DH5 α or SK037) or *B. thuringiensis* (HD073). The preculture was incubated overnight at 37°C with agitation and then added to 75 mL of LB and incubated for 2 h at 37°C with agitation. The bacterial culture was then diluted in LB to $\text{OD}_{600\text{nm}} = 0.15$. To each well of a 96-well plate, 5 μL of alkylbiguanidium salt solution in DMSO with 195 μL of bacterial culture were added. Plates were incubated at 37°C with light agitation for 24 h and then analyzed by UV-vis spectroscopy. The MIC was considered the minimal concentration of alkylbiguanidium salt at which no bacterial growth was observed after 24 h.

Yeast growth and MIC determination

A yeast preculture was prepared by the inoculation of 1 mL YPDA (yeast, peptone, dextrose and adenine) broth with *Candida albicans* (SC5314) or *C. krusei* (UM680). The preculture was incubated overnight at 30°C with agitation and then diluted in YPDA to $\text{OD}_{600\text{nm}} = 0.015$. To each well of a 96-well plate, 5 μL of alkylbiguanidium salt solution in DMSO with 195 μL of yeast culture were added. The plates were incubated at 30°C with light agitation for 24 h and then analyzed by UV-vis spectroscopy. The MIC was considered the minimal concentration of alkylbiguanidium salt at which no yeast growth was observed after 24 h.

Hemolytic activity

Red blood cells in Alsever's solution were centrifuged for 10 min at 300g, washed 3 times with PBS buffer, and resuspended in PBS at 2% v/v. To each well of a 96-well plate, 195 μL of red blood cell solution and 5 μL of alkylbiguanidium salt in DMSO were added, and the plate was incubated with light agitation for 1 h at 37°C . The plate was then centrifuged for 10 min at 300g, and 50 μL of the supernatant solution of each well was transferred to another plate. Absorbance was measured at $\lambda = 405 \text{ nm}$. Each measurement was performed in triplicate in three different experiments.

Results and discussion

Synthesis

The alkylbiguanidium salts were synthesized following the procedure developed by Suyama *et al.* starting with the corresponding alkylamine and dicyandiamide (Figure 2) (11a). All alkylbiguanidium salts were obtained as doubly protonated chloride salts (11b).

Integrity of the phospholipid bilayer

EYPC large unilamellar liposomes (LUVs) were first used as simple phospholipid models to assess the membrane perturbation properties of alkylbiguanidium salts (12). DLS measurements were performed to determine the variation in the size of the LUVs when in contact with the alkylbiguanidium chlorides. The EYPC LUVs initially had a radius of 60 nm and a high internal ion concentration (mainly Na^+ , K^+ , and Cl^-) when bathing in a salt-free ultrapure water environment (13). The results summarized in Table 1 show that there was a slight swelling of the liposomes when the alkylbiguanidium chlorides were added at concentrations of 10 or 100 $\mu\text{g/mL}$, indicating the maintenance of the integrity of the phospholipid membrane. A 10% increase in liposome size was observed for **C₁₆Cl** at 100 $\mu\text{g/mL}$; this is similar to the results obtained with benzalkonium chloride (**BAC**), which is known for its membrane perturbing properties (13). The swelling observed for **C₁₆Cl** could be a consequence of membrane insertion and accumulation because of its structural similarity to EYPC. The slight swelling observed for the other alkylbiguanidium chlorides indicates the modest movement of water molecules and ions from the salt-free extravesicular solution to the highly concentrated intravesicular solution. Although the liposomes were completely disassembled into small aggregates by the lysing agent, Triton-X, they were still intact even after exposure to a concentration

of 100 $\mu\text{g/mL}$ of alkylbiguanidium chlorides, indicating the perturbation and permeabilization of the phospholipid membrane (14), as previously shown for other membrane-depolarizing compounds (15, 16). The behavior of the alkylbiguanidium salts as selective permeabilizing agents is unexpected behavior considering that most QACs, which are structurally similar to alkylbiguanidium salts, act as lysing agents (17). This could provide an interesting solution to the toxicity of QACs toward mammalian cells (18).

Ion transport

When the permeabilization of liposomal membranes occurs, it is important to know which small molecules or ions can cross the perturbed phospholipid bilayers. To investigate this, we used EYPC LUVs containing HPTS, a pH-sensitive probe having two different excitation wavelengths corresponding to its protonated and neutral forms but having only one emission wavelength. By measuring the intensity of fluorescence for each excitation wavelength and comparing the ratios, it is possible to calculate the ratio of the protonated and neutral form of the probe and determine the internal pH of the LUV (19). At the beginning of this experiment the pHs of the extra- and intravesicular solutions were neutral. After 300 s, a pH pulse was induced by the addition of NaOH to the external solution. This created a driving force for the movement of ions across the membranes. The addition of alkylbiguanidium chlorides to the HPTS-containing liposomes resulted in the basification of the liposome internal solution, either induced by the efflux of protons or the influx of hydroxide anions (Figure 3(a)). Although no significant basification of the liposomes was observed for metformin and phenformin, all the alkylbiguanidium chlorides tested, particularly **C₁₂Cl**, **C₁₄Cl**, and **C₁₆Cl**, induced pronounced H^+/OH^- transport across the phospholipid membrane.

Ion transport through phospholipid membranes can be achieved through electrogenic processes, where the movement of ions results in charge transfer, or electro-neutral processes, through either a symport or antiport process. Because the electroneutral process involves the transport of another ion across the membrane, we first investigated the transport of Na^+ by alkylbiguanidium salts. The same EYPC-LUVs with the HPTS probe were used, but the salt in the external solution was changed from NaCl to LiCl and KCl at the same internal and external pH (Figure 3(b) for **C₁₂Cl**). No variation in the internal pH was observed in the presence of these different cations, which have different sizes and hydration properties, showing that the external cation is not

Table 1. Radius of EYPC liposomes after exposure to alkylbiguanidium chlorides.^a

Radius (nm)	At 10 $\mu\text{g/mL}$	At 100 $\mu\text{g/mL}$
DMSO	60.6 $\pm$ 0.8	60.6 $\pm$ 0.8
Metformin	62.8 $\pm$ 0.7	61.5 $\pm$ 0.4
Phenformin	64.5 $\pm$ 0.5	63.6 $\pm$ 1.0
C ₆ Cl	63.7 $\pm$ 1.8	61.4 $\pm$ 0.7
C ₈ Cl	61.5 $\pm$ 0.2	62.1 $\pm$ 0.4
C ₁₀ Cl	61.6 $\pm$ 0.3	62.4 $\pm$ 0.3
C ₁₂ Cl	63.6 $\pm$ 0.4	62.7 $\pm$ 0.2
C ₁₄ Cl	62.2 $\pm$ 0.9	62.3 $\pm$ 0.3
C ₁₆ Cl	62.3 $\pm$ 0.4	70.8 $\pm$ 1.9
BAC	61.9 $\pm$ 1	67.9 $\pm$ 0.1
Triton-X	4.2 $\pm$ 0.1	4.2 $\pm$ 0.1

^aIntravesicular solution of PBS 1X (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 , adjusted to pH = 7.4) and extravesicular solution of ultrapure water.

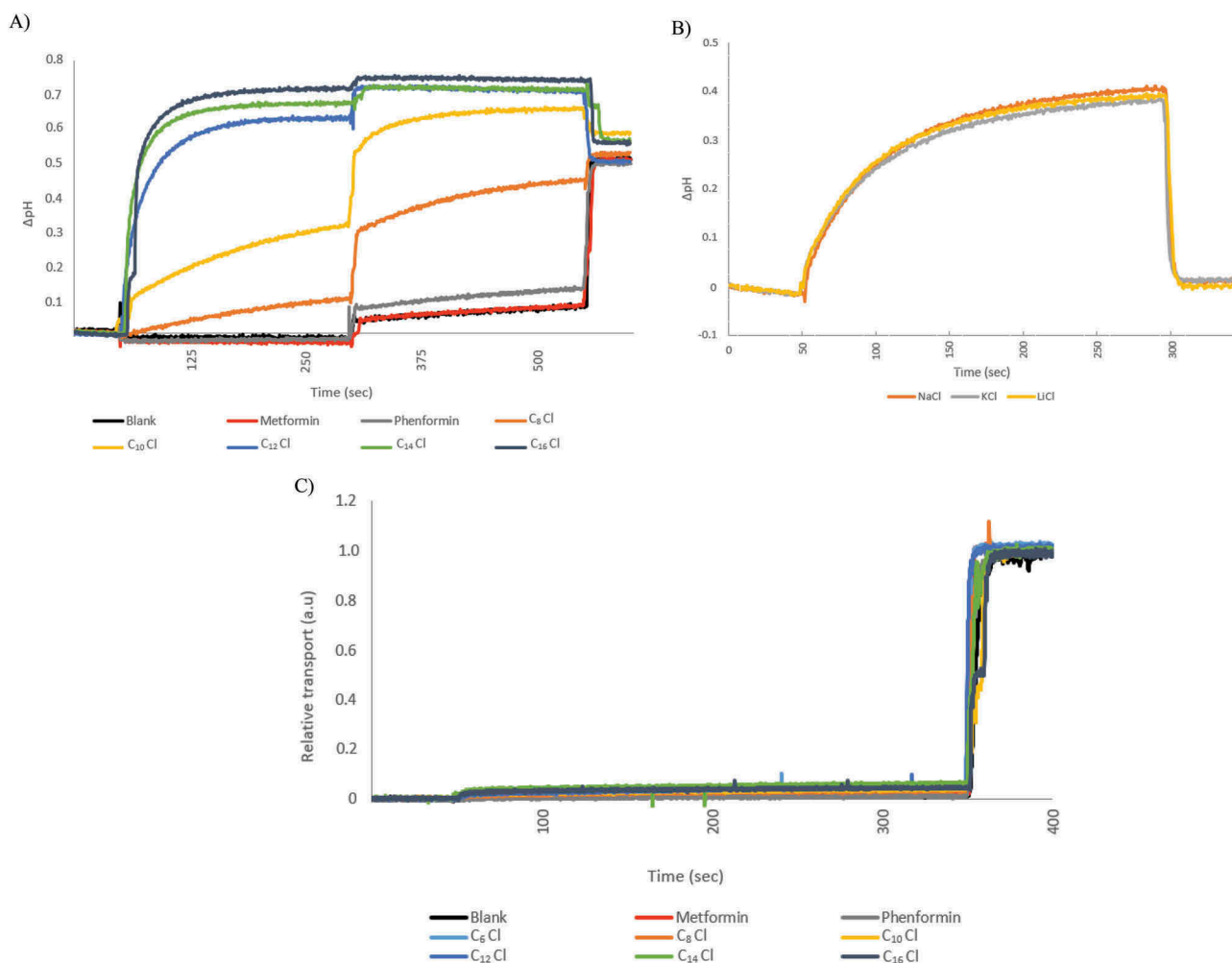


Figure 3. (Colour online) (a) Variation in the internal pH of HPTS-containing EYPC liposomes. Intravesicular solution: 1 mM HPTS, 10 mM HEPES, and 100 mM NaCl, adjusted to pH = 7.4, and extravesicular solution: 10 mM HEPES and 100 mM NaCl, adjusted to pH = 7.4. Alkylbiguanidium chlorides were injected after 50 s at 5 mM (50 mol% relative to the 10 mM EYPC concentration), a NaOH pulse was induced at 300 s, and the liposomes were lysed with Triton-X at 550 s. (b) Variation in the internal pH of the HPTS-containing EYPC liposomes with different external salt solutions. Intravesicular solution: 1 mM HPTS, 10 mM HEPES, and 100 mM NaCl, adjusted to pH = 7.4, and extravesicular solution: 10 mM HEPES, 100 mM of the corresponding salt (LiCl, NaCl, or KCl), adjusted to pH = 7.4. Alkylbiguanidium chloride **C12Cl** was injected after 50 s at 5 mM (50 mol% relative to the 10 mM EYPC concentration), and the liposomes were lysed with Triton-X after 300 s. (c) Chloride efflux in lucigenin-containing EYPC liposomes. Intravesicular solution: 2 mM lucigenin, 10 mM Na_2HPO_4 , 10 mM NaH_2PO_4 , and 100 mM NaCl, and extravesicular solution: 10 mM Na_2HPO_4 , 10 mM NaH_2PO_4 , and 100 mM NaNO_3 . Alkylbiguanidium chlorides were injected after 50 s at 50 mM (50 mol% relative to the 100 mM EYPC concentration), and the liposomes were lysed with Triton-X at 350 s. Each curve is the average of three independent measurements.

involved in the overall transport process. These results show that alkylbiguanidium salts do not induce an electroneutral H^+/Na^+ antiport or OH^-/Na^+ symport processes.

In a similar study for Cl^- transport, we used EYPC LUVs containing a different fluorophore, lucigenin (10-methyl-9-(10-methylacridin-10-ium-9-yl)acridin-10-ium dinitrate), a probe whose fluorescence is quenched by the presence of chloride. The liposomes are designed to contain a high initial internal concentration of NaCl while suspended in a chloride-free external buffer

such that, at the beginning of the experiment, no fluorescence is observed. The addition of a membrane perturbing agent allowing chloride efflux results in a decrease in the internal chloride concentration and an increase in lucigenin fluorescence, which can be measured and quantified (20). The chloride transport results (Figure 3(c)) reveal no significant increase in fluorescence, regardless of the length of the alkyl chain, indicating that chloride is not transported across the liposomal membrane and that electroneutral H^+/Cl^- symport or OH^-/Cl^- antiport are not possible modes

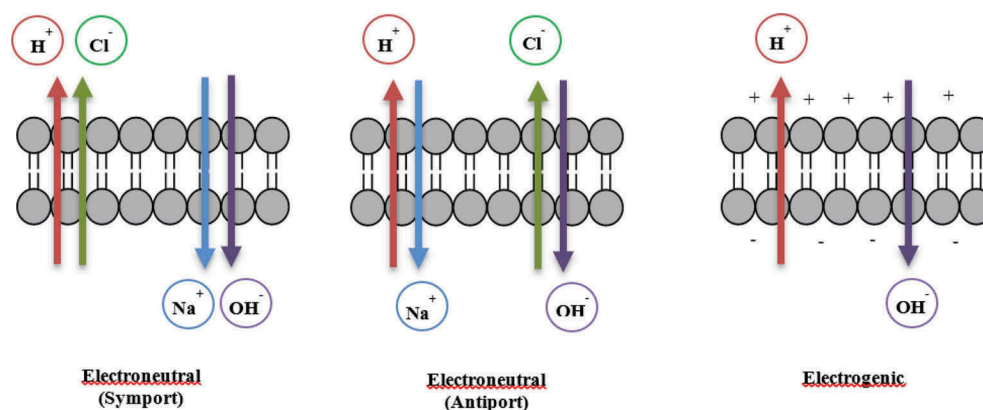


Figure 4. (Colour online) Schematic representation of H^+/OH^- transport induced by alkylbiguanidium salts.

of action. The same lack of chloride transport was observed for metformin and phenformin.

Altogether, the results of the transport experiments indicate that neither chloride or sodium ions were transported through the phospholipid membrane by the alkylbiguanidium salts, eliminating the electroneutral H^+/OH^- transport hypothesis (Figure 4). If this highly selective electrogenic H^+/OH^- transport process (21) is maintained in biological membranes, the disruption of the proton gradient across biological membranes and the resultant membrane potential may lead to cellular regulation problems, such as membrane depolarization and the uncoupling of oxidative phosphorylation.

Depolarization of an artificial phospholipid membrane

The generation of an ionic potential across biological membranes is an important physiological process, crucial for cell homeostasis and survival (22, 23). In any living cell, the disruption of the membrane potential leads to the complete shutdown of the most essential cellular functions and, eventually, cell death (24).

To study whether the alkylbiguanidium chlorides cause the depolarization of phospholipid membranes, we used EYPC LUVs bathing in a solution of safranin O, a probe used as a membrane potential indicator. An increase in its fluorescence intensity indicates hyperpolarization, whereas a decrease in its fluorescence intensity is generally correlated with membrane depolarization. The EYPC liposomes contained intravesicular K^+ and extravesicular Na^+ ions, allowing the generation of a small membrane potential at the beginning of the experiment (which caused a slight fluorescence of the sample). As expected, the addition of valinomycin induced the hyperpolarization of the membrane as a result of K^+ efflux. The addition of alkylbiguanidium chlorides in the external LUV solution led to a rapid decrease in the fluorescence intensity, indicating a

perturbation of the membrane, equilibration of the ion distribution on both sides of the membrane, and depolarization of the phospholipid membrane (Figure 5(a)). Metformin and phenformin had an insignificant effect on the membrane potential, but the C_{12}Cl , C_{14}Cl , and C_{16}Cl alkylbiguanidium salts were able to induce up to 20% membrane depolarization (Figure 5(b)). In the C_{12}Cl -valinomycin experiment, we first observed depolarization induced by the alkylbiguanidium salt, followed by a hyperpolarization induced by valinomycin, lower than that induced when used alone. This indicates that the efflux of K^+ induced by valinomycin competes with the fast re-equilibration of the ion distribution on both sides of the membrane. Because the alkylbiguanidium salts with the best depolarization properties were those exhibiting the most intense ion transport in the HPTS experiment, we believe that the observed membrane depolarization is the result of the transport of H^+/OH^- ions, as a result of the membrane permeabilization.

Permeabilization of bacterial membranes

Because the alkylbiguanidium salts induced the permeabilization of simple model membranes, we were interested to see if the same effect was observed in complex bacterial membranes. For these studies we used the bacterial membrane permeabilization assay initially developed by Lehrer *et al* (25), using the hydrolysis of extracellular *ortho*-nitrophenyl- β -galactoside (ONPG) by the cytoplasmic β -galactosidase in Gram-negative *E. coli* (MG1655). In the presence of membrane permeabilizing agents, ONPG can penetrate the bacterial envelope and is hydrolyzed in the cytoplasm. The enzymatic hydrolysis of ONPG releases *ortho*-nitrophenol, which can be monitored by UV-vis measurements (Figure 6), and a mathematical treatment developed by our group allowed us to determine the rate of permeabilization of the *E. coli* membrane induced by the

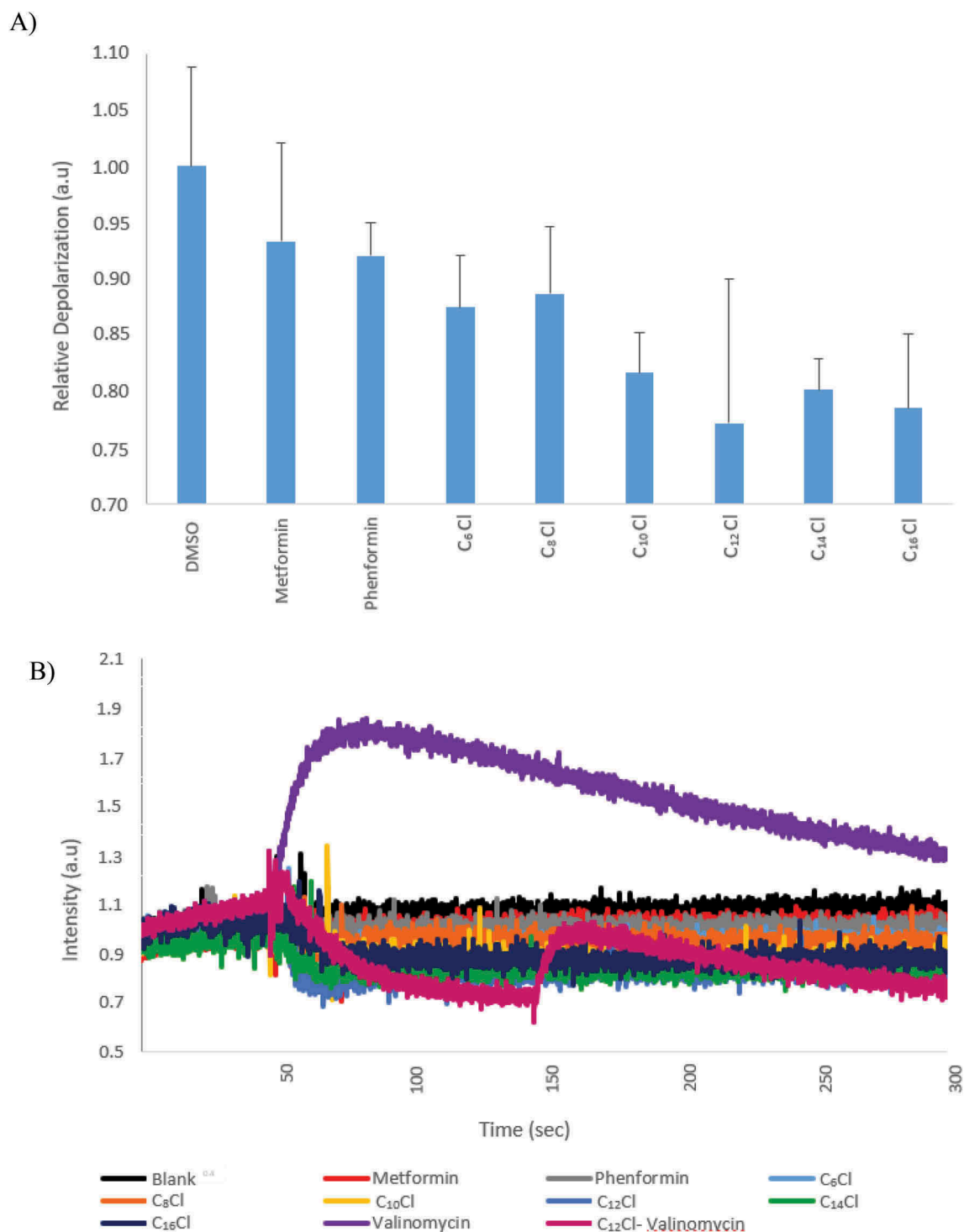


Figure 5. (Colour online) (a) Relative depolarization of the EYPC bilayer. The fluorescence was measured at 300 s, and each value is the average of three independent experiments. (b) Fluorescence of safranin O in the EYPC liposomes. The alkylbiguanidium salt was injected at 50 s, and, in the **C₁₂Cl**-valinomycin experiment, the valinomycin was added at 150 s. Each value is the average of three independent experiments.

alkylbiguanidium salts (26). As summarized in Table 2, **C₆Cl** and **C₈Cl** did not permeabilize the membrane of Gram-negative bacteria, but **C₁₂Cl**, **C₁₄Cl**, and **C₁₆Cl** caused significant membrane permeabilization.

Another interesting general trend observed in the ONPG assay is the fact that the alkylbiguanidium chlorides caused only 50% ONPG hydrolysis. In the case of **BAC**, we previously showed that the complete

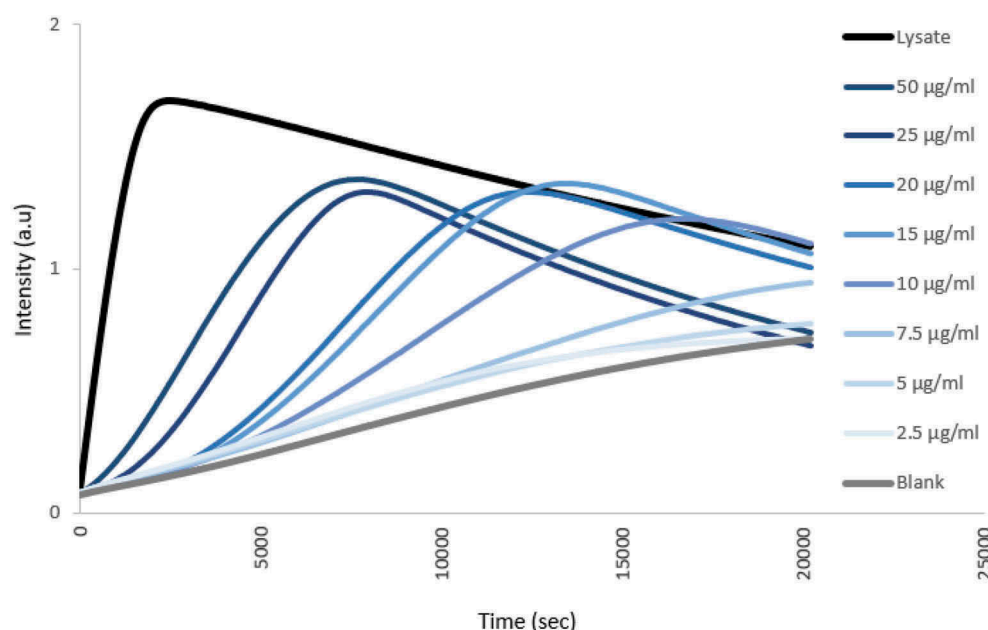


Figure 6. (Colour online) Hydrolysis of ONPG at various concentrations of the **C₁₀Cl** permeabilizing agent in *E. coli* (MG1655). Alkylbiguanidium salts in DMSO were injected at $t = 0$ s, and each curve is the average of three independent measurements.

Table 2. Permeabilization of the *E. coli* (MG1655) membrane by alkylbiguanidium chlorides.

	EC ₅₀ (µg/mL) ^a
Metformin	-
Phenformin	-
C₆Cl	-
C₈Cl	-
C₁₀Cl	15.2 ± 0.9
C₁₂Cl	5.1 ± 0.1
C₁₄Cl	6.7 ± 0.4
C₁₆Cl	7.6 ± 0.9

^aEC₅₀: Alkylbiguanidium salt concentration required to induce 50% ONPG hydrolysis.

permeabilization and lysis of the *E. coli* envelope was a biphasic process (25), corresponding first to the perturbation of the outer membrane and then to that of the inner membrane, inducing complete bacterial lysis and β -galactosidase release. The 50% ONPG hydrolysis indicates that the alkylbiguanidium salts only permeabilize the bacterial envelope and allow ONPG to penetrate the cytosol without β -galactosidase leakage (26).

Depolarization of bacterial membranes

Because membrane permeabilization was observed in the model EYPC membranes, we were interested to see the effect of alkylbiguanidium salts on bacterial membranes composed of a large range of amphiphilic lipids, including phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin. The depolarization of Gram-positive (*B. thuringiensis*) and Gram-negative (*E. coli*) bacterial membranes was studied using bacteria in combination with the

DISC₂₅ dye (20). This probe acts similarly to safranin O, its fluorescence being quenched when inserted into a polarized phospholipid membrane. The change in polarization of the bacterial membrane in the presence of membrane-active compounds can be monitored by fluorescence. The addition of the **C₁₀–C₁₆Cl** alkylbiguanidium salts to a bacterial suspension resulted in the depolarization of the membranes of both Gram-positive and Gram-negative bacteria (Figure 7). The **C₁₂Cl**, **C₁₄Cl**, and **C₁₆Cl** alkylbiguanidium salts induced the strongest depolarization of the membrane because of the important transport of H^+/OH^- ions across the membrane. The depolarization was more pronounced in the case of Gram-positive bacteria, presumably because they possess only one phospholipid bilayer, and a larger electrical potential variation can arise from the direct interaction of the alkylbiguanidium salts with the cytoplasmic membrane. The depolarization was lower in the case of Gram-negative bacteria, which possess an additional phospholipid bilayer.

Because a significant level of membrane potential perturbation was observed for some of the alkylbiguanidium chlorides and knowing that the disruption of the membrane potential may result in cell death, we next investigated the ability of different microorganisms to survive in the presence of different concentrations of the alkylbiguanidium salts.

Antimicrobial activity

The MIC, the lowest concentration at which no microbial growth was observed, of the **C₆–C₁₆Cl**

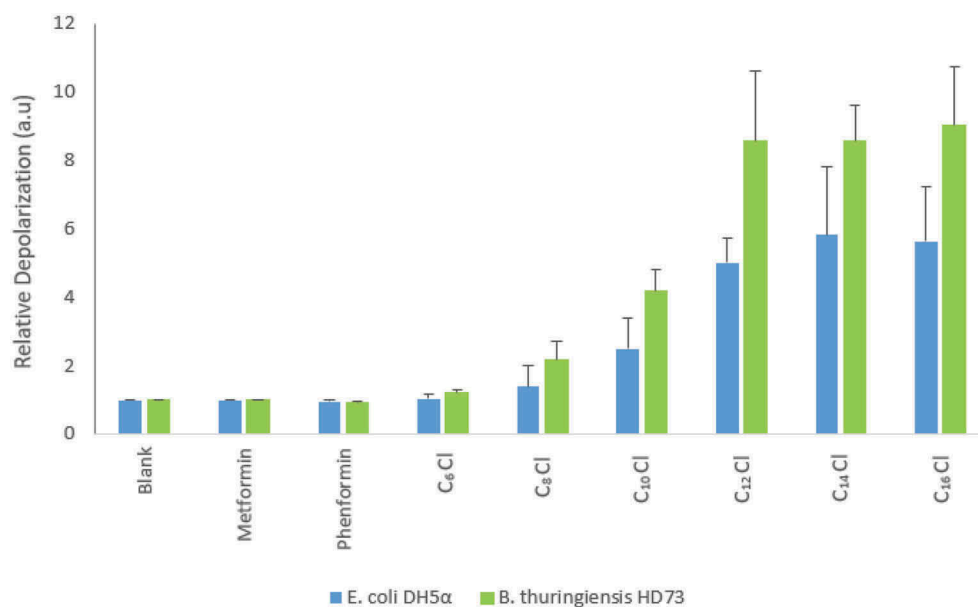


Figure 7. (Colour online) Depolarization of the *E. coli* (DH5α) and *B. thuringiensis* (HD73) membranes. Alkybiguanidium salts were injected at 50 s at 100 μg/mL. The relative fluorescence was taken at 1500 s, and each value is the average of three independent experiments.

Table 3. Minimal inhibitory concentrations (μg/mL).

	<i>E. coli</i> (DH5α)	<i>E. coli</i> (SK037)	<i>B. thuringien-</i> <i>sis</i> (HD73)	<i>C. albicans</i> (SC5314)	<i>C. krusei</i> (UM680)
Metformin	> 50	> 50	> 50	> 50	> 50
Phenformin	> 50	> 50	> 50	> 50	> 50
C ₆ Cl	> 50	> 50	> 50	> 50	> 50
C ₈ Cl	> 50	> 50	> 50	> 50	> 50
C ₁₀ Cl	25	13	> 50	30	30
C ₁₂ Cl	12	6	6	8	8
C ₁₄ Cl	13	13	5	3	3
C ₁₆ Cl	> 50	> 50	13	8	> 50
BAC	36	3	8	n.d.	n.d.

alkybiguanidium salts were measured for Gram-positive *B. thuringiensis* (HD073), Gram-negative *E. coli* (DH5α and SK037), and the yeasts *C. albicans* (SC5314) and *C. krusei* (UM680). The results summarized in Table 3 show that the C₆Cl and C₈Cl alkybiguanidium salts have no antimicrobial activity. The antimicrobial activity of C₁₀Cl, C₁₂Cl, and C₁₄Cl on all the studied strains suggests that the main mechanism of action of these compounds is the permeabilization of the phospholipid bilayer because the most depolarizing compounds are those with better antibiotic activity.

The lower MICs obtained for Gram-positive bacteria indicate the easier penetration of their single phospholipid bilayer and, thus, increased depolarization and antibacterial activity. This has also been observed in the literature for QACs (17). It is well known that Gram-negative bacterial membranes contain many transmembrane efflux pumps that reject molecules that have crossed the outer membrane; this acts as a protection mechanism (27). The

efficiency of alkybiguanidium chlorides in stopping the proliferation of wild-type *E. coli* DH5α and the mutant *E. coli* SK037 strain, in which the *ToiC* efflux pump protein has been deactivated (28), was further investigated. As shown in Table 2, the MIC values are up to four-fold lower for the SK037 strain, indicating that the alkybiguanidium salts are able to penetrate and cross the outer membrane but are effluxed when the *ToiC* pump is present.

Surprisingly, the C₁₆Cl alkybiguanidium salt, which induced a pronounced bacterial membrane depolarization, showed only modest antimicrobial activity. Possible interactions between metformin and the bacterial inner membrane respiratory chain complex NADH-Q oxidoreductase (NDH-I) have been proposed in the past (23). Given the resemblance between bacterial NDH-I and mitochondrial complex I, which metformin is known to inhibit (24), we believe that the alkybiguanidium salts may possess the ability to inhibit the NDH-I complex. The alkybiguanidium salt must first penetrate the outer membrane, becoming partitioned into the periplasmic space and, thus, reaching the inner membrane. Alkybiguanidium C₁₆Cl is probably too hydrophobic and accumulates in the outer membrane of Gram-negative bacteria and does not reach the inner membrane. This possible mechanism of action of alkybiguanidium salts is under investigation by our group.

Hemolytic activity

If the permeabilization of the cellular membrane occurs alone without complete lysis, as suggested by

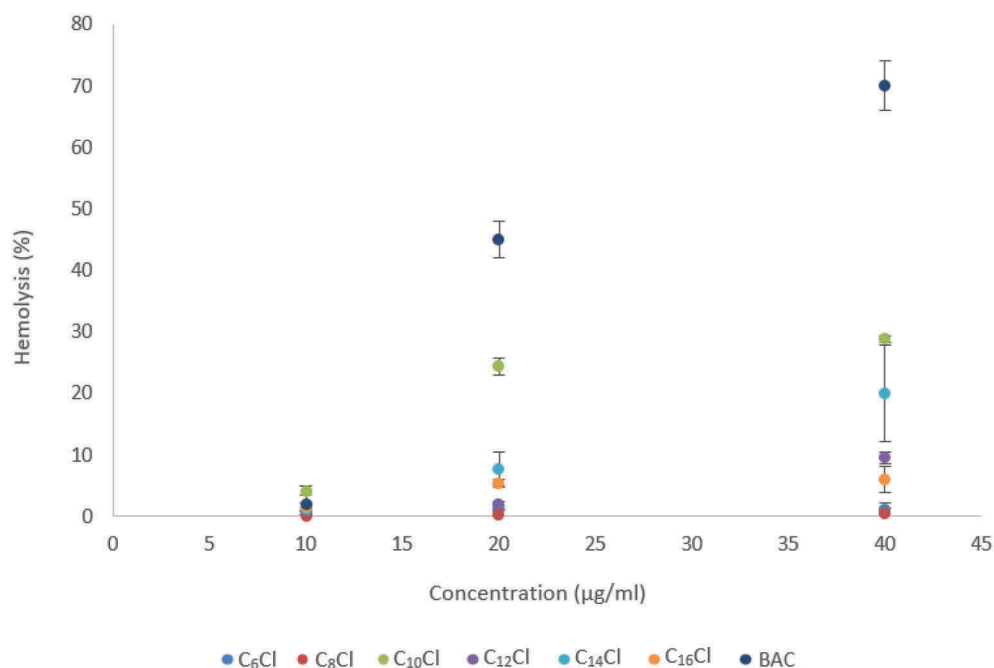


Figure 8. (Colour online) Hemolysis of RBCs at various concentrations of alkylbiguanidium salts after 1 h incubation at 37°C. The values reported are relative to the lysis of RBC induced by Triton-X. Each value is the average of three independent measurements.

the DLS and bacterial permeabilization results, the alkylbiguanidium salts should not induce the hemolysis of red blood cells (RBC) like QACs. RBCs comprise a plasma membrane enveloping hemoglobin. (29) If the integrity of this membrane is altered, as is the case when lysis occurs, hemoglobin is released and is easily detected. When exposed to alkylbiguanidium salts at a concentration of 10 µg/mL, at which they showed relevant antibacterial and antifungal activities, only low levels of hemolysis were observed (under 10%) (Figure 8). The low toxicity of the alkylbiguanidium salts toward RBCs at concentrations close to their MICs and twice their MICs is encouraging for their application as antibacterial and antifungal agents, in particular, when compared to the hemolytic activity of **BAC**, which is much higher at these concentrations.

Conclusions

We have shown that alkylbiguanidium salts have membrane perturbing properties for both simple phospholipid bilayer (liposomes) and complex phospholipid membranes (bacteria and yeast). Compared to metformin and phenformin, the alkylbiguanidium salts induce more significant permeabilization of the phospholipid membranes, leading to highly selective H^+/OH^- transport and subsequent depolarization of the membrane. Although the alkylbiguanidium salts with shorter chains (**C₆** and **C₈**) are too hydrophilic to be inserted into the

membrane and perturb it, those with **C₁₀**, **C₁₂**, **C₁₄**, and **C₁₆** alkyl chains show high membrane permeabilization and depolarization activity. Compounds of the **C₁₀**, **C₁₂**, and **C₁₄** salts possess interesting antimicrobial activity and, at concentrations close to their MICs, have low hemolytic activity and toxicity. The mechanism of action and interaction of the alkylbiguanidium salts with other eukaryotic membranes are currently under study by our group and should shortly bring new insights into their activity toward healthy, as well as cancerous, cells.

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Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. All authors declare no conflict of interest.

Disclosure statement

No potential conflict of interest was reported by the authors.

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