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Original article

Antibacterial properties and mode of action of new triaryl butene citrate compounds

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ABSTRACT

The aim of this study was to evaluate the antibacterial activity of newly synthesized triaryl butene analogues of tamoxifen. Several compounds were synthesized and converted to citrate salts to ensure greater solubility. Four compounds showed significant antibacterial activity at micromolar concentrations against Gram-positive and Gram-negative foodborne pathogens including *Listeria monocytogenes*, *Listeria ivanovii*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Escherichia coli*. Two compounds at 50 μM, caused only 7.8 and 11% hemolysis. One of these as well as the remaining two caused high K^+ and Na^+ efflux from bacterial cells. Ultrastructural alterations were also visible using transmission electron microscopy, which revealed severe damage of the inner or outer membrane of *E. coli*. *L. ivanovii* showed swelling, corrugations and similar damage indicating a loss of cell-wall integrity. Organometallic compounds may offer interesting opportunities for the design of novel classes of antimicrobial compounds.

1. Introduction

The emergence and rapid spread of multidrug-resistant bacterial strains is a serious public health issue. Formerly reliable antibiotics are losing their effectiveness. Development of novel antimicrobial therapies and strategies to overcome the growing problem of resistance has become necessary. In recent years, many libraries of known or newly developed molecules have been screened for their biological activities. This strategy has been adopted for screening antibiotics for inhibiting cancer cells and vice versa many antitumor molecules have been assessed for antimicrobial activity [1]. For instance, the estrogen receptor antagonist tamoxifen, used to treat breast cancer, has been found to have also antibacterial, antifungal and anti-parasitical activities [1–3]. Besides their antitumor potential, derivatives of tamoxifen with metallic moieties have shown excellent antimicrobial activity against pathogenic bacteria [4–6].

Tamoxifen is considered to be a membrane-active drug, since it interacts with lipids in membranes, thereby causing ultrastructural alterations [7]. This molecule and its derivatives act on biomembranes, causing changes in the framework of the erythrocyte membrane and its cytoskeleton, which may result in cell lysis [8,9]. The affinity for membranes is highly influenced by even small modifications in chemical structure. For example, hydroxytamoxifen has been shown to be three times less hemolytic than tamoxifen [8]. Patients treated with tamoxifen often develop hemolytic anemia [10–12].

We have previously synthesized a series of ferrocenic analogues of tamoxifen with anti-proliferative effects [13]. Among these, 1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-ferrocenyl-but-1-ene showed excellent inhibitory effects (at <12.5 μg/mL) against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* [6]. In the present work, we report on the synthesis of new triaryl butene analogues of tamoxifen and their conversion to citrate salts in order to improve their water solubility. The compounds thus synthesized were screened for antibacterial activity against

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foodborne pathogens. Their hemolytic action and mode of antimicrobial action were also investigated.

2. Results

2.1. Synthesis of citrates

The citrates were synthesized using a reaction between citric acid dissolved in dry tetrahydrofuran and the diamine compounds dissolved in dry diethyl ether. The precipitate that immediately formed was recovered by filtration then dried under vacuum.

2.2. Antimicrobial activity

Compounds 1 to 4 (see Experimental methods) were screened for antibacterial activity against Gram-positive and Gram-negative pathogens. As shown in Table 1, all four compounds were more active against tested Gram-positive pathogens than Gram-negative. Compound **1** exhibited the greatest antilisterial activity, with MIC values of 8 and 12.5 μM against *Listeria monocytogenes* and *Listeria ivanovii* respectively. Similarly, the MIC of compound **1** was as low as 8 μM against *Enterococcus faecalis* and *S. aureus*. To a lesser extent, compound **3** was active against all tested Gram-positive strains, with MIC ranging from 8 to 20 μM. In comparison, compounds **2** and **4** inhibited Gram-positive bacteria in the ranges of 25–80 and 25–62 μM respectively. Compounds **1** and **2** were the most active against *Escherichia coli,* with MIC in the range of 50–100 μM. Tested *E. coli* strains were also sensitive to compounds **3** and **4,** with MIC in the ranges of 100 and 100–125 μM respectively. All synthesized compounds were less active against *Salmonella enterica*, with compounds **1** and **3** being the most active (MIC = 625 μM). Compounds **2** and **4** were even less active, with MIC in the millimolar range (1.25 and 2.5 mM respectively). Only compound **4** was active against *P. aeruginosa*, with a MIC of 2.5–5 mM.

Table 1
Antimicrobial and hemolytic activities and therapeutic index of the compounds used in this study.

Strains	Minimum inhibitory concentration MIC (μM)			
	Compound **1**	Compound **2**	Compound **3**	Compound **4**
Listeria ivanovii HPB28	12.5	25	12.5	50–25
Listeria monocytogenes LSD 532	8	31	16	31
Listeria monocytogenes LMA 1045	8	62	8–16	62
Enterococcus faecalis ATCC 27275	8	80	20	40
Staphylococcus aureus ATCC 6538	8	31	8	31
Escherichia coli MC4100	100–50	50	100	100
Escherichia coli ATCC 11229	50	100–50	125	100
Pseudomonas aeruginosa ATCC 15442	>5000	>5000	>5000	5000–2500
Salmonella enterica ATCC 14028	625	1250	625	2500
GM[a] (μM)	20.6	47.0	41.4	55.6
HC_{50}[b] (μM)	22	67.1	22.4	68.6
TI[c]	1.1	1.4	0.5	1.2

[a] The observed geometric mean (GM) of the MICs of the compound against all bacterial strains.
[b] HC_{50} is the minimal concentration that caused 50% hemolysis of red blood cells.
[c] Therapeutic index is the ratio of the HC_{50} to the geometric mean of the MICs. Larger values mean greater cell selectivity.

2.3. Hemolytic activity

The *in vitro* safety of citrate compounds was evaluated using a hemolysis assay (Fig. 3). Compounds **4** and **2** were the least active at 50 μM, causing respectively 7.8 and 11% hemolysis. Although these compounds were the most hemocompatible at 10 and 50 μM, they did not effectively kill *E. coli* at these concentrations. They nevertheless showed the highest therapeutic index, 1.4 for compound 2 and 1.2 for compound 4. Both showed the highest minimal hemolytic concentration (HC_{50}), at respectively 67 and 68 μM. Compounds **1** and **3** showed similar hemolytic activity profiles. Compound **1** caused about 7% and 100% hemolysis respectively at 8 μM (MIC against Gram-positive bacteria) and 50 μM (MIC against Gram-negative bacteria). It was found previously to cause lysis of mouse erythrocytes, with an EC_{50} of 25.90 μM [13], and showed a minimal hemolytic concentration (HC_{50}) of 22 μM in the present study. Its therapeutic index was 1.1.

2.4. Mode of action

2.4.1. Induced ion efflux from listerial cells

Total K^+ and Na^+ contents of freshly prepared cells of *L. monocytogenes* dropped after treatment with compounds **1**, **2** or **3** at 1–5 times the MIC for 30 min (Fig. 2). Nisin A, used at 2 μg/mL as a positive control, induced a total K^+ release of 361.79 ± 7.03 nmol per mg of cell dry weight (Fig. 2A). Efflux of intracellular K^+ from listerial cells treated with compounds 1, 2 or 3 at the MIC was respectively 162.15 ± 2.36, 183.74 ± 33.28, and 119.03 ± 2.21 nmol per mg of CDW. In the presence of compound 2 at 5 times its MIC, the efflux reached 344 nmol/mg CDW, or 95% of the total intracellular potassium. Treatment of listerial cells with compounds **1** or **3** at 5 times the MIC led to efflux of 290.77 ± 26.97 and 183.54 ± 24.15 nmol K^+ per mg of CDW respectively. Nisin at 2 μg/mL caused a significant efflux of sodium (635.96 ± 7.03 nmol/mg CDW, Fig. 2B). A notable decline was also detected in total Na^+ release from *Listeria* treated with compound **1** at the MIC, which reached 164.26 ± 7.03 nmol/mg CDW. After 30 min of treatment with compounds **1**, **2** and **3** at 5 times the MIC, the Na^+ efflux from listerial cells was respectively about 67%, 68% and 54% of the total contents.

2.4.2. Transmission electron microscopy

The transmission electron microscopy (TEM) images of untreated cells of *L. ivanovii* revealed regular rod-shaped structure with intact cell walls and well-defined membranes (Fig. 4A1). After 5 min of exposure of *Listeria* to compounds **1**, **2**, **3** or **4** at 5 times the MIC, the cell wall appeared swollen and severely damaged and showed alterations in morphology, as well as corrugation indicating a loss of integrity (Fig. 4A2–A5). Moreover, damaged cells showed rougher and blurred membrane boundaries, a more uniform electron density of the cytoplasm and formation of vacuoles (Fig. 4A2). Cells treated with compound **4** showed very condensed and localized intracellular content (Chart 1).

Untreated cells of *E. coli* showed a normal cell shape with an undamaged structure of the inner membrane and an intact, slightly corrugated and well-defined outer membrane (Fig. 4B1). After 5 min incubation of *E. coli* cells with compounds **1**, **2**, **3** or **4** at 5 times the MIC, severe damage of the inner or outer membranes was detected (Fig. 4B2–B5). In all cases, the cells presented blurred boundaries and highly altered cell walls. In addition, cells treated with compounds **1** and **2** presented a more uniform electron density of the cytoplasm and formation of mesosome-like structures (Fig. 4B2, B4).

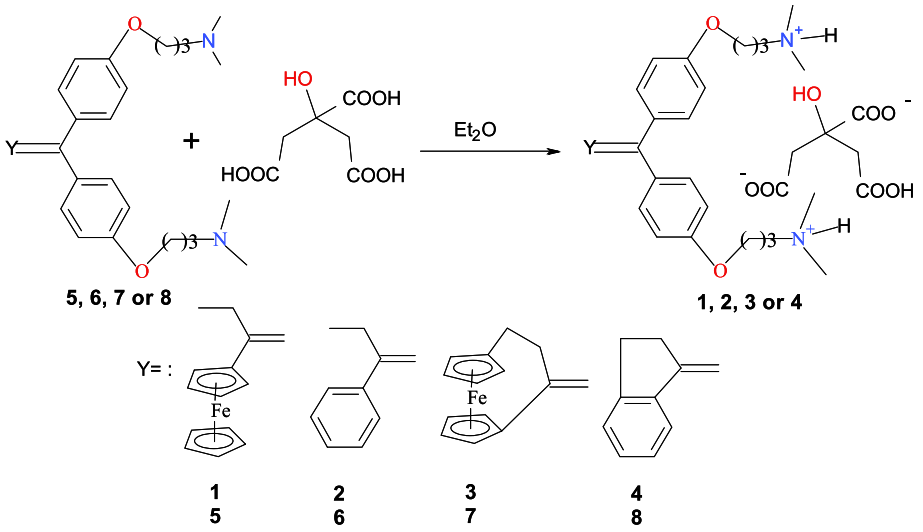

Fig. 1. General procedure for synthesis of citrates.

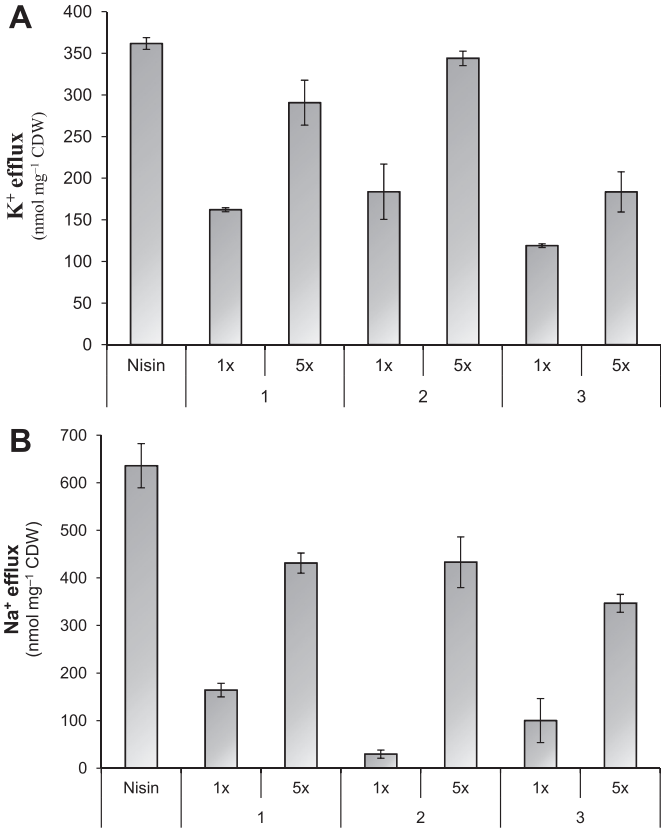

Fig. 2. Ion efflux determination.

3. Discussion

The aim of this study was to evaluate the antibacterial activity of novel synthesized triaryl butene analogues of tamoxifen. Several compounds were synthesized and transformed into citrate salts to increase hydrosolubility. Four compounds were found to have antibacterial activity, especially against Gram-positive species. While 1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-ferrocenyl-but-1-ene was shown previously to inhibit *P. aeruginosa* (MIC < 21.02 μM) [6], its conversion to 1,1-bis-[4-(3-dimethylamoniumpropoxy)phenyl]-2-ferrocenyl-but-1-ene citrate (compound **1** in this study) caused a dramatic decrease in its antibacterial activity (MIC > 5000 μM).

In addition to being antimicrobial at 50 μM, compounds **4** and **2** showed the lowest hemolytic effect. The presence of ferrocene or ferrocenophane decreased the hemolytic activity of compounds **1** and **3** (HC_{50} of 22 μM and 22.4 μM respectively). The hemolytic activity of the compounds seemed affected by the presence of amine side chains. In fact, hydroxytamoxifen, the active metabolite of tamoxifen, was not found to be hemolytic (EC_{50} = 213.7 μM) [7]. However, the ferrocene moiety made compound **1** the most antibacterial and with a therapeutic index (1.1) comparable to that of compounds **2** and **4**. Compound **3** with ferrocenophane was as active as compound **1** but had a much lower therapeutic index of 0.5. This pronounced difference in antimicrobial activity between synthesized compounds is likely due to the presence of iron atoms in the compound structures. Indeed, the cytotoxicity of ferrocenyl compounds has been attributed previously to oxidation to ferrocenium cation, which can then engage in Fenton generation of highly reactive hydroxyl radicals [14].

Tamoxifen is a membrane-active drug since it causes ultrastructural alterations [7]. However, this action is not specific to the microorganism or cell line [2,8] and can also affect blood cells, thus limiting the usable dose to the minimal hemolytic concentration. The antibacterial activity of compounds 1 to 4 in this study is likely due, as is the case for tamoxifen, to their effect on the cell membrane [9]. We therefore assessed the mode of action of the synthesized compounds on bacterial membranes. Compounds **1**, **2** or **3** were thus found to cause significant efflux of K^+ and Na^+ from bacterial cells. Such efflux causes loss of trans-membrane potential and ultimately cell death. Ultrastructural alterations were visible using transmission electron microscopy. After 5 min of incubation of bacterial cells with any of the four compounds, severe damage of the inner or outer membrane was detected and cell walls appeared swollen and corrugated. These alterations in morphology clearly indicate a loss of integrity.

The activity of numerous analogues of tamoxifen, particularly phenols, has been attributed previously to oxidative metabolism to quinone methide species [14]. It has been reported that compounds lacking free phenols, similar to those in this study, would not be expected to form such electrophilic species in the cell, that a mechanism related to metal coordination, possibly of Zn^{2+}, could

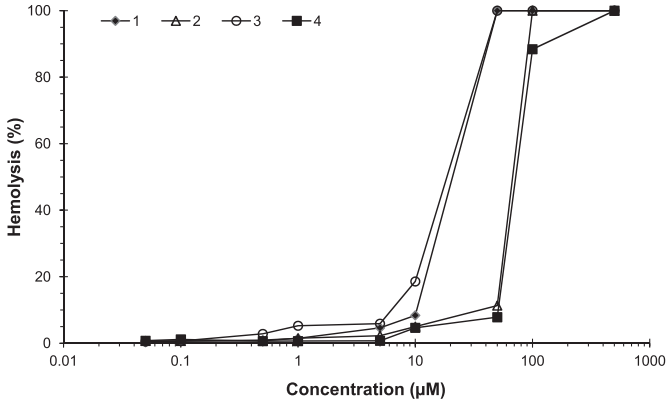

Fig. 3. Hemolytic activity of citrate compounds at various concentrations.

intervene [15]. Since this metal ion is abundant in cells and its displacement (due to basic groups) could lead to loss of molecular function, molecular modeling was used to estimate the affinities of compounds **5**, **6** and **7** (see experimental section) for Zn^{2+}. This cation binds between the two amino chains of compounds **5**, **6** and **7**, and variations in $\Delta_r H^0$ enthalpy of complex formation were calculated. It was thus confirmed that these molecules are likely good complexing agents for divalent cations such as Zn^{2+} as well as Ca^{2+} [15]. Furthermore, the lack of a phenol group may be advantageous for *in vivo* activity. For example, one of the difficulties in obtaining benefits from natural polyphenols found in the diet is their metabolism to more polar compounds and fast elimination from the body [16].

These attractive features, coupled with low hemolytic activity, suggest that compound **1** is a good candidate for the formulation of antimicrobial agents and is worthy of *in vivo* examination. Organometallic compounds may offer interesting opportunities for the design of novel classes of antimicrobial compounds.

4. Experimental section

4.1. General procedure for the synthesis of citrates

The synthesis of all compounds was performed under an argon atmosphere, using standard Schlenk techniques. Anhydrous tetrahydrofuran and diethyl ether were obtained by distillation from sodium/benzophenone. Infrared spectra were obtained on FT/IR-4100 JASCO spectrometers (http://www.jascofrance.fr). ^1H and ^{13}C NMR spectra were recorded on a 300 MHz Bruker spectrometer (http://www.bruker.com/).

One mmol of diamine compound (1,1-bis[4-{3-dimethylaminopropoxy}phenyl]-2-ferrocenyl-but-1-ene (compound **5**), 1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-phenyl-but-1-ene (compound **6**), 1-[bis(4-{3-dimethylaminopropoxy}phenyl)methylidene]-[3]ferrocenophane (compound **7**), 1-[bis(4-{3-dimethylaminopropoxy}phenyl)methylidenyl]indan (compound **8**) [17], was dissolved in 30 mL of dry diethyl ether (Fig. 1). A solution of citric acid monohydrate (0.210 g, 1 mmol) in dry tetrahydrofuran (5 mL) was added drop-wise to the first solution, which resulted immediately in the formation of a precipitate. After stirring for 20 min, the mixture was filtered under argon and the obtained solid was washed with 3 × 5 mL of diethyl ether and dried under vacuum.

4.1.1. 1,1-Bis-[4-(3-dimethylammoniumpropoxy)phenyl]-2-ferrocenyl-but-1-ene citrate (compound **1**)

The synthesis of this compound has been reported previously [6].

4.1.2. 1,1-Bis[4-(3-dimethylamoniumpropoxy)phenyl]-2-phenyl-but-1-ene citrate (compound **2**)

This synthesis was carried out using the general procedure starting from 1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-phenyl-but-1-ene (compound **6**) [15] (0.487 g, 1 mmol). The yield was 95%. ^1H NMR (DMSO D_6): δ 0.88 (t, J = 7.3 Hz, 3 H, CH_3), 1.91–2.16 (m, 4 H, 2 CH_2), 2.43 (q, J = 7.3 Hz, 2 H, CH_2), 2.52 (d, J = 9.1 Hz, 2 H, CH_2), 2.57 (d, J = 9.1 Hz, 2 H, CH_2), 2.64 (s, 6 H, NMe_2H^+), 2.69 (s, 6 H, NMe_2H^+), 2.98 (t, J = 7.6 Hz, 2 H, CH_2N), 3.05 (t, J = 7.6 Hz, 2 H, CH_2N), 3.91 (t, J = 5.9 Hz, 2 H, CH_2O), 4.08 (t, J = 5.9 Hz, 2 H, CH_2O), 6.62 (d, J = 8.7 Hz, 4 H, C_6H_4), 6.76 (d, J = 8.7 Hz, 4 H, C_6H_4), 6.97 (d, J = 8.7 Hz, 2 H, C_6H_4), 7.08–7.27 (m, 7 H, C_6H_5 + C_6H_4). ^{13}C NMR (DMSO D_6): δ 14.4 (CH_3), 25.6 (CH_2), 25.7 (CH_2), 29.6 (CH_2), 43.9 (NMe_2H^+), 44.0 (NMe_2H^+), 45.4 (2 CH_2 citrate), 55.5 (CH_2N), 55.6 (CH_2N), 65.9 (2 CH_2O), 72.4 (C citrate), 114.4 (2 CH C_6H_4), 115.2 (2 CH C_6H_4), 127.1 (CH C_6H_5), 128.9 (2 CH C_6H_4), 130.4 (2 CH C_6H_4), 131.2 (2 CH C_6H_5), 132.4 (2 CH C_6H_5), 136.5 (C), 136.7 (C), 138.5 (C), 141.5 (C), 143.0 (C), 157.2 (C), 158.1 (C), 172.6 (2 CO), 178.1 (CO). IR (KBr, ν cm^{-1}): 3431 (OH), 3035, 2964, 2928 (CH_2, CH_3), 2723 (CO). Compound **2** crystals contained traces of the solvents.

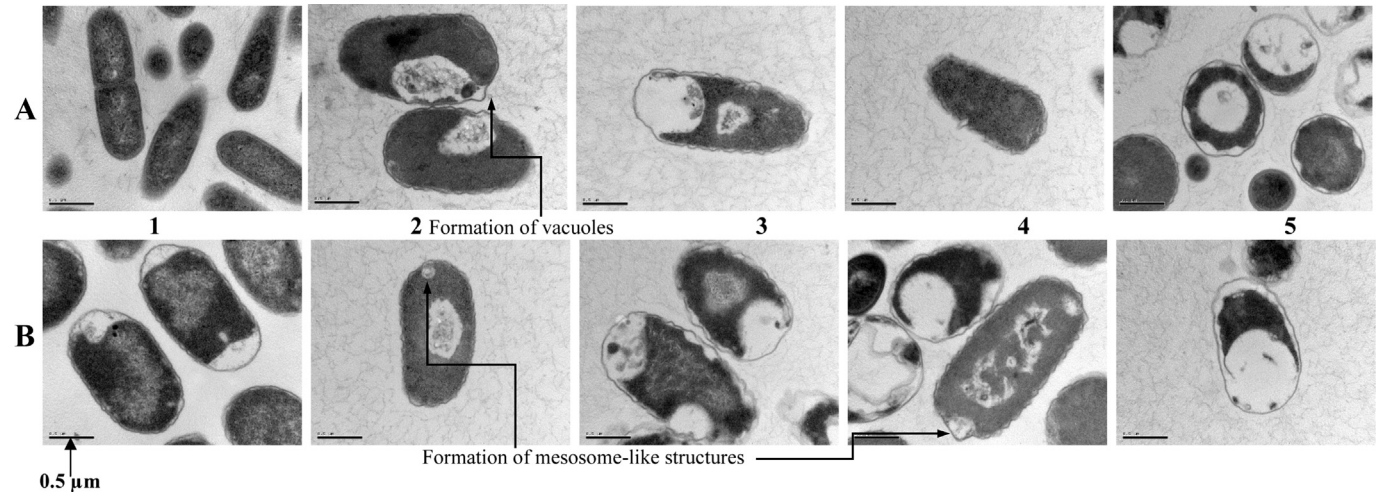

Fig. 4. Transmission electron microscopy. **A**: *Listeria ivanovii* HPB28, **B**: *Escherichia coli* MC4100, **1**: Control, **2**: Compound **2**, **3**: Compound **3**, **4**: Compound **1** and **5**: Compound **4**.

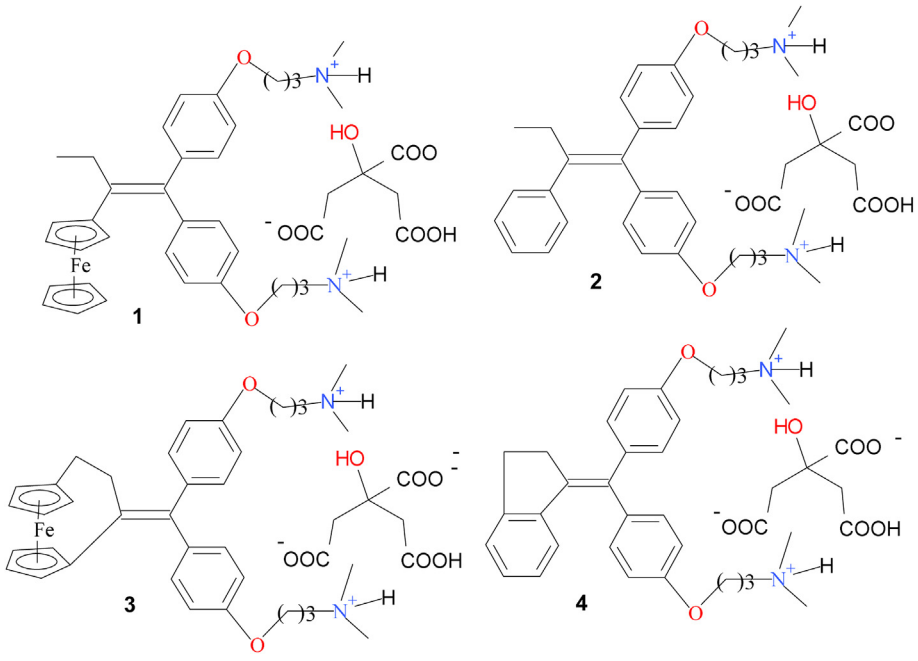

Chart 1. Chemicals tested.

*4.1.3. 1-[Bis(4-(3-dimethylamoniumpropoxy)phenyl)methylidenyl]-[3]ferrocenophane citrate (compound **3**)*

This synthesis was carried out using the general procedure starting from 1-[bis(4-(3-dimethylaminopropoxy)phenyl)methylidene]-[3]ferrocenophane (compound **7**) [15], (0.593 g, 1 mmol). The yield was 82%. ^{1}H NMR (DMSO D_6) : δ 1.92–2.15 (m, 4 H, 2 CH_2), 2.78–2.38 (m, 2 H, CH_2 cycle), 2.51 (d, J = 10.4 Hz, 2 H, CH_2), 2.52–2.56 (m, 2 H, CH_2 cycle), 2.56 (d, J = 10.4 Hz, 2 H, CH_2), 2.63 (s, 6 H, NMe_2H^+), 2.67 (s, 6 H, NMe_2H^+), 2.90–3.10 (m, 4 H, CH_2N), 3.88–3.97 (m, 2 H, CH_2O), 3.98 (s, 2 H, CH C_5H_4), 4.02 (s, 2 H, CH C_5H_4), 4.04 (s, 2 H, CH C_5H_4), 4.05–4.13 (m, 2 H, CH_2O), 4.32 (s, 2 H, CH C_5H_4), 6.68 (d, J = 8.5 Hz, 2 H, C_6H_4), 6.90 (d, J = 8.5 Hz, 2 H, C_6H_4), 6.97 (d, J = 8.5 Hz, 2 H, C_6H_4), 7.14 (d, J = 8.5 Hz, 2 H, C_6H_4). ^{13}C NMR (DMSO D_6) : δ 25.7 (2 CH_2), 28.9 (CH_2 cycle), 41.0 (CH_2 cycle), 44.0 (NMe_2H^+), 44.1 (NMe_2H^+), 45.3 (2 CH_2 citrate), 55.6 (2 CH_2N), 65.7 (CH_2O), 65.9 (CH_2O), 69.1 (2 CH C_5H_4), 69.5 (2 CH C_5H_4), 70.7 (2 CH C_5H_4), 71.0 (2 CH C_5H_4), 72.3 (C citrate), 84.4 (C C_5H_4), 87.5 (C C_5H_4), 114.2 (2 CH C_6H_4), 115.1 (2 CH C_6H_4), 131.1 (2 CH C_6H_4), 132.2 (2 CH C_6H_4), 134.2 (C), 136.7 (C), 136.8 (C), 140.3 (C), 157.4 (C), 158.0 (C), 172.4 (2 CO), 178.0 (CO). IR (KBr, ν cm^{-1}): 3429 (OH), 3034, 2931, 2702, 2520 (CH_3, CH_2), 1721 (CO). Compound **3** crystals contained traces of the solvents.

*4.1.4. 1-[Bis(4-(3-dimethylamoniumpropoxy)phenyl)methylidenyl]-indane citrate (compound **4**)*

This synthesis was carried out using the general procedure starting from 1-[bis(4-{3-dimethylaminopropoxy}phenyl)methylidenyl]indan (compound **8**) [17] (0.485 g, 1 mmol). The yield was 88%. ^{1}H NMR (DMSO D_6): δ 1.98–2.15 (m, 4 H, 2 CH_2), 2.51 (d, J = 9.4 Hz, 2 H, CH_2), 2.56 (d, J = 9.4 Hz, 2 H, CH_2), 2.64 (s, 6 H, NMe_2H^+), 2.66 (s, 6 H, NMe_2H^+), 2.90 (s, 4 H, CH_2–CH_2 indane), 2.94–3.07 (m, 4 H, CH_2N), 4.00–4.14 (m, 4 H, CH_2O), 6.35 (d, J = 7.9 Hz, 1 H, H_{indane}), 6.81–7.03 (m, 5 H, H_{arom}), 7.05–7.14 (m, 3 H, H_{arom}), 7.18 (d, J = 8.5 Hz, 2 H, C_6H_4), 7.29 (s, J = 7.5 Hz, H_{indane}). ^{13}C NMR (DMSO D_6): δ 25.8 (2 CH_2), 31.0 (CH_2 indane), 34.6 (CH_2 indane), 44.1 (2 NMe_2H^+), 45.3 (2 CH_2 citrate), 55.6 (2 CH_2N), 65.9 (2 CH_2O), 72.3 (C citrate), 114.8 (2 CH C_6H_4), 115.7 (2 CH C_6H_4), 125.1 (CH indane), 126.0 (CH indane), 126.5 (CH indane), 128.2 (CH indane), 131.0 (2 CH C_6H_4), 130.6 (2 CH C_6H_4), 134.6 (C), 135.9 (C), 136.8 (C), 139.9 (C), 142.0 (C), 148.2 (C), 157.9 (C), 158.4 (C), 172.4 (2 CO), 178.0 (CO). IR (KBr, ν cm^{-1}): 3434 (OH), 3033, 2932, 2703 (CH_2, CH_3), 1715 (CO). Compound **4** crystals contained traces of the solvents.

4.2. Bacteria and growth conditions

Microorganism growth inhibition assays were performed using cultures of *L. ivanovii* HPB28, *L. monocytogenes* LDS 532, *L. monocytogenes* LMA 1045, *E. faecalis* ATCC 27275, *S. aureus* ATCC 6538, *E. coli* MC4100, *E. coli* ATCC 11229, *P. aeruginosa* ATCC 15442 and *S. enterica* ATCC 14028. Strains were grown in tryptic soy broth (TSB; Difco Laboratories, Sparks, MD) supplemented with 0.6% (w/v) yeast extract and incubated aerobically at 30 °C. The microbial strains were propagated at least three times in TSB tubes before use.

4.3. Minimum inhibitory concentration assay

This bioassay was performed using polystyrene micro-assay plates (96-well Microtest, Becton Dickinson Labware, Sparks, MD, USA) as described by Hammami et al. [18]. The MIC was determined against indicator microorganisms. Two-fold serial dilutions were prepared from 512 μg/mL stock solution to obtain concentrations of 256 to 2 μg/mL in tryptic soy broth (TSB). To each sterile well containing 5 × 10^4 cells in 125 μl was added an equal volume of solution containing the test compound. The MIC is the lowest concentration that inhibited cell growth, based on the absence of turbidity after 24 h.

4.4. Hemolysis assay

Erythrocytes were harvested by centrifugation for 10 min at 5000 × g at 4 °C, washed two times in PBS (10 mM sodium phosphate, pH 7.4 in 150 mM NaCl) and finally suspended in PBS for immediate use. Of this suspension, 90 μl was added in triplicate to wells containing 100 μl of a two-fold serial dilution series of

compound in the same buffer in a 96-well microtiter plate. Total hemolysis was achieved with 1% Triton X-100. The plates were incubated for 1 h at 37 °C and then centrifuged for 5 min at 10 000 × *g* at 4 °C. Of the supernatant, 150 µl was transferred to a flat-bottom microplate and the absorbance was measured at 450 nm. Hemolysis was calculated as a percentage: [(A450 of the compound treated sample − A450 of buffer treated sample)/(A450 of Triton X-100 treated sample − A450 of buffer treated sample)] × 100. HC_{50} represents the compound concentration at which 50% hemolysis was observed.

4.5. Determination of ion efflux

A mid-log-phase culture of *L. ivanovii* was harvested, washed and re-suspended in the same volume of MES buffer (10 mM, pH 6, supplemented with 0.2% glucose). To estimate the total ion content of bacterial cells, intracellular K^+ and Na^+ were released by treating cells with 2 µg of nisin for 1 h. Bacterial cells were treated with 1 or 5 times the MIC for 30 min, filtered, and assayed by atomic absorption spectrometry accordingly. The ion efflux was calculated and expressed in nmol/mg.

4.6. Transmission electron microscopy (TEM)

TEM was performed in order to visualize intracellular morphological changes in *L. ivanovii* HPB20 and *E. coli* MC4100 during exposure to the compounds. An overnight culture of bacteria in TSB (1% YE) was harvested and washed three times in sodium phosphate buffer (pH 7.4). To 100 µL of *Listeria* suspension was added 900 µL of compound at 5 times the MIC. Sterile water was used as a negative control. After 5 min at 37 °C, samples were fixed in PBS containing 2.5% glutaraldehyde and left overnight at 4 °C.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.02.037.

References

[1] A.C. Jacobs, L. Didone, J. Jobson, M.K. Sofia, D. Krysan, P.M. Dunman, Adenylate kinase release as a high-throughput-screening-compatible reporter of bacterial lysis for identification of antibacterial agents, Antimicrobial Agents and Chemotherapy 57 (2013) 26–36.

[2] K. Dolan, S. Montgomery, B. Buchheit, L. DiDone, M. Wellington, D.J. Krysan, Antifungal activity of tamoxifen: in vitro and in vivo activities and mechanistic characterization, Antimicrobial Agents and Chemotherapy 53 (2009) 3337–3346.

[3] D.C. Miguel, R.C. Zauli-Nascimento, J.K.U. Yokoyama-Yasunaka, S. Katz, C.L. Barbiéri, S.R.B. Uliana, Tamoxifen as a potential antileishmanial agent: efficacy in the treatment of *Leishmania braziliensis* and *Leishmania chagasi* infections, Journal of Antimicrobial Chemotherapy 63 (2009) 365–368.

[4] G. Jaouen, S. Top, A. Vessieres, G. Leclercq, J. Quivy, L. Jin, A. Croisy, The first organometallic antioestrogens and their antiproliferative effects, Comptes Rendus de l'Académie des Sciences − Series IIC − Chemistry 3 (2000) 89–93.

[5] M. El Arbi, P. Pigeon, A.C. Rkhis, S. Top, A. Rhouma, A. Rebai, G. Jaouen, S. Aifa, Antimicrobial effect of ferrocenyl diaryl butenes against olive plantlet diseases, Journal of Plant Pathology 93 (2011) 651–657.

[6] M. El Arbi, P. Pigeon, S. Top, A. Rhouma, S. Aifa, A. Rebai, A. Vessières, M.-A. Plamont, G. Jaouen, Evaluation of bactericidal and fungicidal activity of ferrocenyl or phenyl derivatives in the diphenyl butene series, Journal of Organometallic Chemistry 696 (2011) 1038–1048.

[7] C. Luxo, A.S. Jurado, V.M.C. Madeira, M.T. Silva, Tamoxifen induces ultrastructural alterations in membranes of *Bacillus stearothermophilus*, Toxicology in Vitro 17 (2003) 623–628.

[8] M.M.C. Silva, V.M.C. Madeira, L.M. Almeida, J.B.A. Custodio, Hydroxytamoxifen interaction with human erythrocyte membrane and induction of permeabilization and subsequent hemolysis, Toxicology in Vitro 15 (2001) 615–622.

[9] M.M.C. Silva, V.M.C. Madeira, L.M. Almeida, J.B.A. Custodio, Hemolysis of human erythrocytes induced by tamoxifen is related to disruption of membrane structure, Bba-Biomembranes 1464 (2000) 49–61.

[10] C.K. Ching, P.G. Smith, R.G. Long, Tamoxifen-associated hepatocellular damage and agranulocytosis, Lancet 339 (1992) 940.

[11] A. Montes, T.J. Powles, M.E.R. Obrien, S.E. Ashley, J. Luckit, J. Treleaven, A toxic interaction between mitomycin-C and tamoxifen causing the hemolytic-uremic syndrome, European Journal of Cancer 29A (1993) 1854–1857.

[12] J.A. Hurteau, M.F. Brady, K.M. Darcy, W.P. McGuire, P. Edmonds, M.L. Pearl, I. Iyanov, K.S. Tewari, R.S. Mannel, K. Zanotti, D.M. Benbrook, Randomized phase III trial of tamoxifen versus thalidomide in women with biochemical-recurrent-only epithelial ovarian, fallopian tube or primary peritoneal carcinoma after a complete response to first-line platinum/taxane chemotherapy with an evaluation of serum vascular endothelial growth factor (VEGF): a gynecologic oncology group study, Gynecologic Oncology 119 (2010) 444–450.

[13] A.C. de Oliveira, E.A. Hillard, P. Pigeon, D.D. Rocha, F.A. Rodrigues, R.C. Montenegro, L.V. Costa-Lotufo, M.O. Goulart, G. Jaouen, Biological evaluation of twenty-eight ferrocenyl tetrasubstituted olefins: cancer cell growth inhibition, ROS production and hemolytic activity, European Journal of Medicinal Chemistry 46 (2011) 3778–3787.

[14] E.A. Hillard, A. Vessieres, S. Top, P. Pigeon, K. Kowalski, M. Huche, G. Jaouen, Organometallic diphenols: the importance of the organometallic moiety on the expression of a cytotoxic effect on breast cancer cells, Journal of Organometallic Chemistry 692 (2007) 1315–1326.

[15] P. Pigeon, S. Top, A. Vessieres, M. Huche, M. Gormen, M. El Arbi, M.-A. Plamont, M.J. McGlinchey, G. Jaouen, A new series of ferrocifen derivatives, bearing two aminoalkyl chains, with strong antiproliferative effects on breast cancer cells, New Journal of Chemistry 35 (2011) 2212–2218.

[16] Z. Liu, M. Hu, Natural polyphenol disposition via coupled metabolic pathways, Expert Opinion on Drug Metabolism & Toxicology 3 (2007) 389–406.

[17] K. Jellali, P. Pigeon, F. Trigui, S. Top, S. Aifa, G. Jaouen, M. El Arbi, Effect of the amino chain length and the transformation into citric acid salts of aryl-diphenyl-butenes and ferrocenyl-diphenyl-butenes bearing two dimethylaminoalkyl chains on their antimicrobial activities, SpringerPlus 2 (2013) 508.

[18] R. Hammami, A. Zouhir, J. Ben Hamida, M. Neffati, G. Vergoten, K. Naghmouchi, I. Fliss, Antimicrobial properties of aqueous extracts from three medicinal plants growing wild in arid regions of Tunisia, Pharmacien Biologiste 47 (2009) 452–457.