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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).