Antibacterial Properties of Phenolic Triterpenoids against *Staphylococcus epidermidis*

Manuel R. López, Leandro de León, Laila Moujir
Departamento de Microbiología y Biología Celular,
Facultad de Farmacia, Universidad de La Laguna, Tenerife,
Canary Islands, Spain

Abstract

Two phenolic triterpenoids, pristimerol (30 µg/mL) and 8-*epi*-6-deoxoblepharodol (20 µg/mL), obtained by catalytic reduction of pristimerin, exhibited bacteriostatic action against *Staphylococcus epidermidis*. This activity was not dependent on the inoculum size and the growth phase although it showed a stronger effect when cells were growing actively. Addition of phenolic triterpenoids to *S. epidermidis* cultures in the log-phase of growth led to an inhibitory effect on incorporation and uptake of radiolabeled precursors thymidine, uridine, leucine, and *N*-acetyl-glucosamine after 30 min of treatment. Furthermore, a clear release of UV-absorbing material and leakage of intracellular potassium were also detected. These findings, coupled with the high lipophilicity of these molecules, shown by high ClogP values, suggest that *8-epi* and pristimerol are able to interact within the lipid bilayer and as a consequence cause functional alterations on the cytoplasmic membrane of *S. epidermidis* cells.

Key words
pristimerol · 8-*epi*-6-deoxoblepharodol · Celastraceae · *Staphylococcus epidermidis* · antibacterial activity · membrane disruption

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The worldwide emergence of antibiotic-resistant bacteria and hitherto unknown disease-causing microbes continues to be a matter of concern for the medical community [1]. In this context, coagulase-negative staphylococci, including *Staphylococcus epidermidis*, have acquired high clinical importance since resistance to standard antibiotics and biocides is increasing [2, 3].

As part of an intensive search of biologically active metabolites from natural sources, we have evaluated the antimicrobial activity of several phenolic triterpenoids from *Maytenus blepharodes* (Celastraceae) [4, 5]. Additionally, in a previous work [6] the catalytic reduction of pristimerin, a triterpenoid quinonemethide restricted to the plant families Celastraceae and Hippocrateaceae [7], yielded three phenolic triterpenoids: pristimerol, a known synthetic compound [8], 6-deoxoblepharodol and 8-*epi*-6-deoxoblepharodol. These were active against different species of gram-positive bacteria: pristimerol and 8-*epi*-6-deoxoblepharodol showed the highest activity against *S. epidermidis* and spore-forming bacteria such as the genus *Bacillus* [6]. However, to our knowledge, the action mechanism of these compounds is still unknown.

Our purpose in this work is therefore to determine the antibacterial properties of these compounds against *S. epidermidis,* so as to aid in the development and application of new and effective substances acting on this bacterium.

Pristimerol with a double bond at C7–C8 (**Fig. 1**) has a stronger activity than 8-*epi*-6-deoxoblepharodol (8-*epi*) against *S. epidermidis*. The difference between MIC and MBC values was greater than twofold dilutions, suggesting static activity against this species. Moreover, both phenolic triterpenoids showed MICs lower than known antibiotics such us bacitracin, penicillin G, or tetracycline, and higher than ciprofloxacin, gentamicin, and rifampin (**Table 1**).

Killing kinetic assays of *S. epidermidis* (**Fig. 2**) indicate that pristimerol at 30 µg/mL (48 × MIC) and 8-*epi*- at 20 µg/mL (10 × MIC) had a bacteriostatic effect (< 3-$\log_{10}$ in CFU reduction) independently of the growth phase, although the highest killing rates were obtained when the triterpenoids were added in the log-phase of growth, which indicates a stronger activity on cells growing actively. Positive controls, vancomycin (64 × MIC) added in the log-phase and rifampin (7 × MIC) in the lag-phase of growth, had a similar effect to the phenolic triterpenoids. The anti-staphylococcal activity of the triterpenoids was not dependent on the inoculum size (data not shown).

The action of pristimerol and 8-*epi* on macromolecular synthesis of *S. epidermidis* cells was evaluated (**Fig. 3**). Although both compounds blocked all processes, pristimerol caused a more drastic effect, inhibiting > 70% [5-^{3}H] uridine and *N*-acetyl-D-[1-^{14}C] glucosamine incorporation after 30 min (**Fig. 3B–D**), while DNA and protein syntheses were inhibited up to 51% and 62%, respectively (**Fig. 3A–C**). However, inhibition of all radiolabeled precursors did not exceed 50% after treatment with 8-*epi*. Clofoctol, a cytoplasmic membrane disruptor [9], blocked more than 70% of all biosynthetic processes, to the same extent as the known specific inhibitors.

A significant decrease in the incorporation of all precursors into their respective macromolecules can be interpreted as a direct effect on macromolecular synthesis, but also as a decrease in the amount of incorporated precursor in the cytosol, due to the inhibition of transport into cells through the cytoplasmic membrane or cell wall [10]. The effect of pristimerol and 8-*epi* on solute transport was measured by cellular uptake of radiolabeled precursors in *S. epidermidis*. The uptake of solutes is defined as the

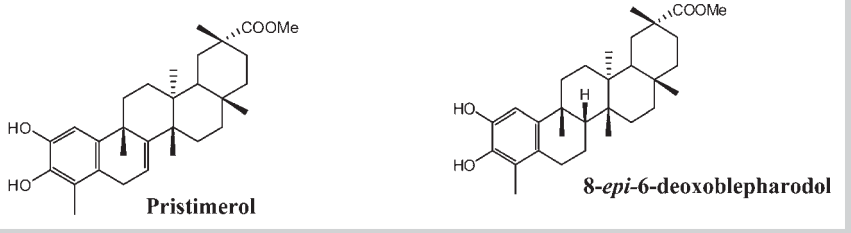

Fig. 1 Structure of the phenolic triterpenoids.

Table 1 Minimal inhibitory and bactericidal concentrations (MIC and MBC) in µg/mL against *S. epidermidis* and ClogP values of phenolic triterpenoids assessed in this work, compared with control antibiotics.

Antibacterial substances	*S. epidermidis* ATCC 14990		
	ClogP	MIC	MBC
8-*epi*-6-Deoxoblepharodol	8.06	2	10
Pristimerol	7.58	0.625	40
Bacitracin	nd	12.5	> 40
Ciprofloxacin	− 1.146	0.125	0.25
Clofoctol	8.55	1.25	12.5
Gentamicin	− 2.89	0.2	0.5
Penicillin	1.694	20	> 40
Rifampin	nd	0.003	0.012
Tetracycline	− 0.911	> 40	> 40
Vancomycin	nd	1.56	> 40

nd: not determined; positive controls (≥ 98 %, Sigma)

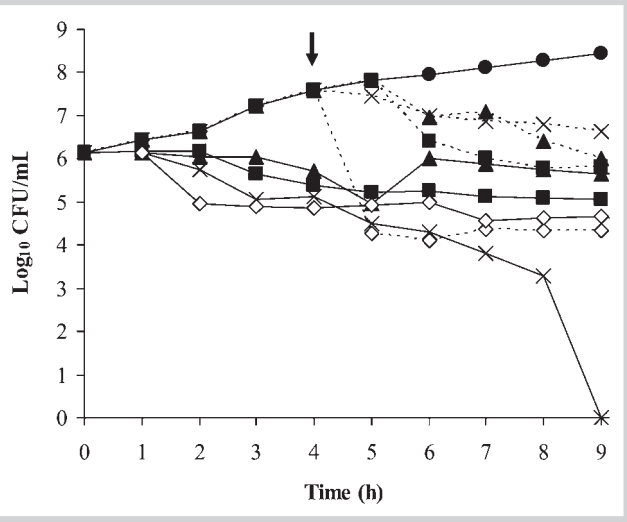

Fig. 2 Time-kill curves of *S. epidermidis* measured by CFU counts after treatment with different compounds added in the lag-phase (solid line) and log-phase (dashed line, time of addition indicated by arrow) of growth. Compounds: 8-*epi* (■) at 20 µg/mL (10 × MIC), pristimerol (▲) at 30 µg/mL (48 × MIC), rifampin (◊) at 0.02 µg/mL (7 × MIC), vancomycin (×) at 100 µg/mL (64 × MIC), and negative control without drug and with the maximum proportion of DMSO (●).

total cell-associated counts after cells are separated from the labeled solutes in the incubation medium. Pristimerol caused > 70 % inhibition of *N*-acetyl-D-glucosamine and uridine uptake (**Fig. 4B, D**), whereas thymidine and leucine uptakes were less affected (**Fig. 4A, C**). Addition of 8-*epi* at 20 µg/mL did not inhibit (< 25 % inhibition) the uptake of any radiolabeled precursors within 30 min (**Fig. 4A–D**). The differences between the two compounds in the incorporation and uptake rates of radiolabeled precursors could however be due to the different concentrations used (48 × MIC for pristimerol versus 10 × MIC for 8-*epi*) and probably not to a difference in the mechanism of action. Assays with a higher concentration of 8-*epi* were not possible due to its precipitation in culture medium.

According to these results, the inhibition of radiolabeled precursor transport into the cells could be a consequence of injuries to the cytoplasmic membrane, as observed with other phenolic triterpenoids [5, 11] and cationic peptides [12] against gram-positive bacteria. To confirm this hypothesis, the effect of pristimerol and 8-*epi* on the cytoplasmic membrane was also examined in cultures of *S. epidermidis* using the BacLight assay, loss of ultraviolet-absorbing materials, and measuring leakage of intracellular potassium. Microscopic observation revealed that the cells treated with pristimerol and 8-*epi* maintained membrane integrity as in untreated cells, showing green fluorescence after 1 h of treatment (data not shown). However, red fluorescence was exhibited by cultures treated with clofoctol, revealing injuries to the cytoplasmic membrane.

When the supernatant fluids from cells treated with pristimerol were examined, a clear leakage of intracellular potassium and a release of UV-absorbing material were evident (**Fig. 1S** and **2S**,

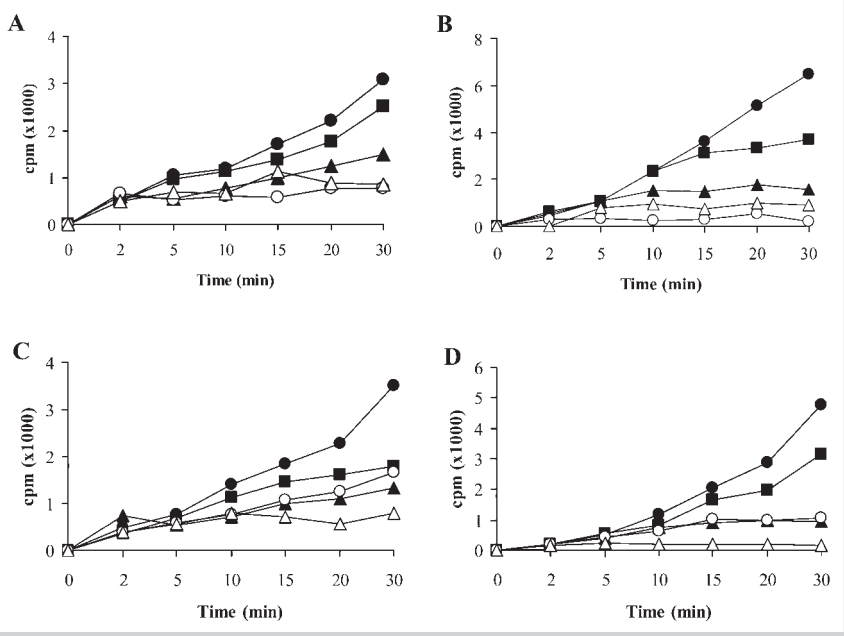

Fig. 3 Inhibition by phenolic triterpenoids of precursor incorporation into macromolecules in *S. epidermidis*. Synthesis inhibition was measured for DNA (**A**), RNA (**B**), protein (**C**), and cell wall (**D**). The products 8-*epi* (■) at 20 µg/mL (10 × MIC) and pristimerol (▲) at 30 µg/mL (48 × MIC) were added a time 0. Negative control (●) with the same proportion of DMSO and known specific inhibitors (○): ciprofloxacin at 5 µg/mL (40 × MIC), rifampin at 0.02 µg/mL (7 × MIC), gentamicin at 5 µg/mL (25 × MIC), and vancomycin at 100 µg/mL (64 × MIC), were included in each case. Clofoctol (△) at 10 µg/mL (8 × MIC), a membrane inhibitor, was included in all experiments.

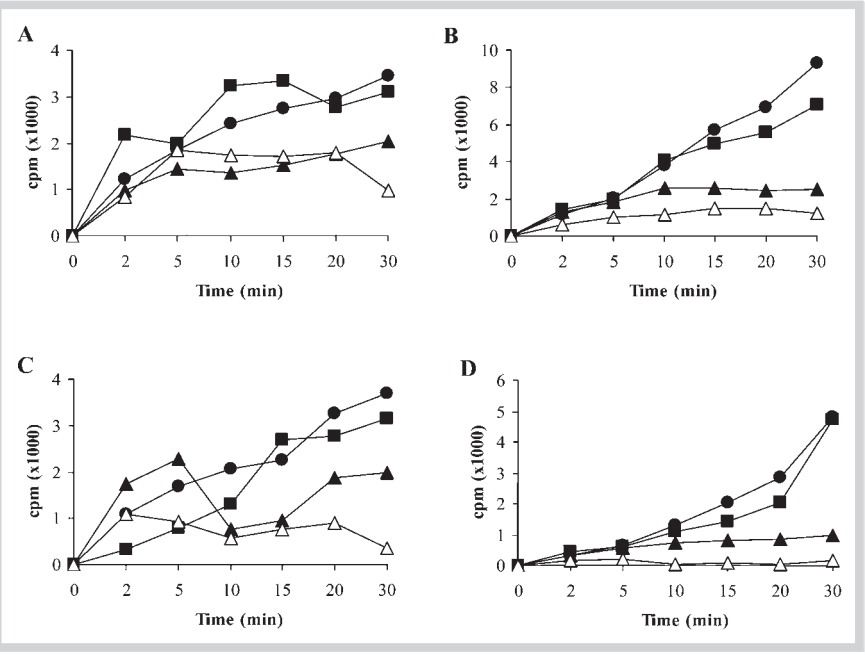

Fig. 4 Inhibition of thymidine (**A**), uridine (**B**), leucine (**C**), and *N*-acetyl-D-glucosamine (**D**) uptake in *S. epidermidis* cells after treatment with 8-*epi* (■) at 20 µg/mL (10 × MIC) and pristimerol (▲) at 30 µg/mL (48 × MIC). Negative controls (●) with the same proportion of DMSO and positive controls with clofoctol (△) at 10 µg/mL (8 × MIC) were also included in each experiment.

Supporting Information). Again, 8-*epi* had a lesser effect than pristimerol, probably due to the lower concentration used in the assays.

These findings demonstrate an effect of the phenolic triterpenoids on the cytoplasmic membrane, similar to that obtained previously by Sikkema et al. [13], who concluded that many cyclic hydrocarbons, e.g., phenolic compounds, cause disruption of membrane structure by hydrophobic interaction with the lipid bilayer due to their lipophilicity. In fact, ClogP values, calculated by Chem Draw software based on the chemical structure of pristimerol and 8-*epi*, revealed values of 7.58 and 8.06, respectively, similar to clofoctol (8.55). Such high lipophilicity indicates that they may indeed interact within the lipid bilayer, causing functional alterations in the cytoplasmic membrane of *S. epidermidis* cells.

Materials and Methods

Pristimerol and 8-*epi* (≥ 98 % purity, HPLC analyses), obtained by a catalytic reduction of pristimerin [6], were added predissolved in DMSO in all experiments and never exceeded 1 % (v/v).

Antibacterial properties of the compounds were assessed on *Staphylococcus epidermidis* ATCC 14990 strain. Cultures of the bacteria were developed in nutrient broth (NB) at 37 °C from an overnight liquid culture prepared in YP medium (yeast extract 10 g/L; peptone 10 g/L).

The MICs and MBCs were determined for each phenolic triterpenoid in triplicate by the broth microdilution method in 96-well microtiter plates. The killing curves against *S. epidermidis* in the lag-phase (10^6 CFU/mL) and log-phase of growth (OD_{550} ~ 0.2) and the effect on different inoculumn size (from 10^4 to 10^7 CFU/mL) were performed in NB medium in the presence of pristimerol at 30 µg/mL and 8-*epi* at 20 µg/mL, as previously described [4].

The incorporation of radiolabeled precursors (thymidine, uridine, leucine, and *N*-acetylglucosamine) into acid-insoluble material and solutes uptake was measured on exponentially growing cultures (OD_{550} ~ 0.2) as previously reported [11].

The effects of pristimerol at 30 µg/mL and 8-*epi* at 20 µg/mL on the cytoplasmic membrane of *S. epidermidis* in the log-phase of growth was evaluated using the LIVE/DEAD BacLight kit (catalog no. L-7012; Molecular Probes), leakage of potassium, and cellular constituents absorbing at 260 and 280 nm [4].

All assays were repeated at least three times and parallel cell cultures were treated with known inhibitors or the same proportion of DMSO, as positive and negative controls, respectively. The variations were less than 10 % and the average value was estimated.

Supporting information

Detailed information of purity criteria of the compounds, protocols for susceptibility testing, radioactivity assays, and assessment of cytoplasmic membrane damage is available as Supporting Information.

Acknowledgements

This study was supported by a grant from the Government of Canarias (SolSubC200801000049). We thank Antonio Padrón of the Departamento de Edafología (Universidad de La Laguna) for determining the potassium release.

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received July 29, 2010
revised September 15, 2010
accepted October 6, 2010

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DOI http://dx.doi.org/10.1055/s-0030-1250500
Published online November 3, 2010
Planta Med 2011; 77: 726–729

ISSN 0032-0943

Correspondence
Prof. Dr. Laila Moujir
Departamento de Microbiología y Biología Celular,
Facultad de Farmacia
Universidad de La Laguna
Avenida Astrofísico Francisco. Sánchez, s/n
38206 Tenerife
Canary Islands
Spain
Phone: + 34 9 22 31 85 13
Fax: + 34 9 22 31 84 77
lmoujir@ull.es