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Synthetic organic compounds with potential for bacterial biofilm inhibition, a path for the identification of compounds interfering with quorum sensing

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ABSTRACT

New unconventional approaches to the development of antimicrobial drugs must target inhibition of infection stages leading to host colonisation or virulence itself, rather than bacterial viability. Amongst the most promising unconventional targets for the development of new antimicrobial drugs is bacterial adherence and biofilm formation as well as their control system, the quorum-sensing (QS) system, a mechanism of communication used to co-ordinate bacterial activities. Here we describe the evaluation of synthetic organic compounds as bacterial biofilm inhibitors against a panel of clinically relevant Gram-positive and Gram-negative bacterial strains. This approach has successfully allowed the identification of five compounds (**GEt**, **GHex**, **GOctad**, **G19** and **C33**) active not only against bacterial biofilms but also displaying potential to be used as antagonists and/or inhibitors of bacterial QS.
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1. Introduction

The worldwide practice of indiscriminate and continuous use of antibiotics for the control and prophylaxis of bacterial pathogens has led to the development of bacterial resistance to most available antimicrobials [1]. Concurrently, the rapid increase in bacterial resistance to currently available antimicrobial drugs has led researchers to search for new sources of molecules active against bacterial pathogens, outlining a new generation of anti-infective drug development.

A promising approach for the development of a new generation of antimicrobial drugs has arisen from the studies of host–pathogen interactions, which prompted a shift of drug targets from bacterial survival to pathogenicity control. Examples of potential non-conventional targets for microbial control are molecules and receptors involved in bacterial adherence to biotic and abiotic surfaces as well as signal systems controlling bacterial group behaviour of populations organised in biofilms, such as quorum sensing (QS) [2]. In contrast to conventional antibiotics, antimicrobial drugs directed against such unconventional targets do not jeopardise bacterial survival, imposing a low selection pressure and thus avoiding the development of resistance [3].

One of the most promising targets is systems controlling the early steps of bacterial adhesion, essential for the establishment of infection and colonisation in general, as well as the subsequent stage of biofilm formation [4]. Studies on bacterial resistance demonstrate that when organised in biofilms bacteria are able to survive antibiotic treatments at concentrations up to a thousand times higher than those used to kill their planktonic counterparts [5]. Conventional antibiotic therapies may eliminate the symptoms of an infection by eradicating planktonic bacteria arising from adhered populations, but are ineffective against those bacteria buried in biofilms, emphasising the importance of intervening in the adhesion process to avoid biofilm formation [6,7].

Experimental data have shown that biofilm formation, like the expression of many other virulence factors, responds to regulation dependent on population density [8], e.g. QS; therefore, development of drugs interfering with QS appears to be a promising approach for the development of new antimicrobial drugs. Recent

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studies reported the identification of different classes of compounds capable of inhibiting or antagonising bacterial QS: synthetic halogenated furanones were identified as potent QS inhibitors to *Pseudomonas aeruginosa* (Fig. 1a and b) [7,9]; Smith et al. identified an antagonist of a *P. aeruginosa* autoinducer (AI) (Fig. 1c) [10]; and, soon after, Geske et al. identified the most potent inhibitor reported to date against the QS of *P. aeruginosa* (Fig. 1d) [11]. More recently, esculetin (Fig. 1e) showed good activity as a QS inhibitor against *Chromobacterium violaceum*, *Escherichia coli* and *P. aeruginosa* [12].

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2013.07.006.

Here we describe the identification of new compounds capable of inhibiting biofilm formation of a panel of clinically relevant Gram-positive and Gram-negative bacterial pathogens. These compounds, which include alkyl-esters of gallic acid, (*E*)-*N*′-benzylidene-benzohydrazides, 1,3,4-oxadiazoles and (*E*)-chalcones, were chosen for the present study on the basis of their similarity to some known QS inhibitors (Fig. 1) [7,9–12]. Quantification of their activities against biofilm formation, indicative of their potential use in the control of biofilm-related infections, allowed structure–function analysis that will guide future studies in the search for new compounds to be used for biofilm control. Analysis of cytotoxicity against human cells allowed the identification of molecules potentially suitable for the development of new antibiofilm drugs for therapeutic purposes.

2. Materials and methods

2.1. Bacterial strains

Standard reference strains *Enterococcus faecalis* ATCC 19433, *P. aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 35547 and *Streptococcus mutans* ATCC 25175 were used in this study. Isolates were kept in trypticase soy broth (TSB) supplemented with 20% (v/v) glycerol at −20 °C and were routinely cultivated in TSB. Conditions for the best biofilm production were established for each strain (data not shown) and are mentioned when necessary.

2.2. Synthesis and purification of the compounds

Reagents were obtained commercially from Sigma–Aldrich (St. Louis, MO) and solvents were from Vetec (Duque de Caxias, Brazil). Exceptions were 3,4,5-trimethoxybenzohydrazide, 2,4,5-trimethoxyacetophenone and 3-methoxy-4-(phenylmethoxy)-benzaldehyde, prepared as previously described [13–15], with yields of 80%, 81% and 88%, respectively.

All alkyl-esters of gallic acid were synthesised and characterised as previously described [16]. Gallic acid (5 mmol) and the corresponding alcohol (15 mmol) were mixed. For compounds **GMet**, **GEt**, **GProp**, **GBut** and **GHex**, the mixture was dissolved in toluene (70 mL) and concentrated sulphuric acid (0.4 mL), heated for 8–12 h in reflux using a Dean–Stark apparatus, and the solvent was removed under reduced pressure. For compounds **GOct** and **GOctad**, the mixture was dissolved in dioxane (10 mL) and *p*-toluenesulfonic acid (0.3 mL) and heated for 2–4 h in bath oil under vacuum. All products were purified by column chromatography.

The (*E*)-*N*′-benzylidene-benzohydrazides were synthesised as previously described [17,18] by condensation of the benzohydrazide (2 mmol) or 3,4,5-trimethoxy-benzohydrazide (2 mmol) with the appropriate aldehyde (2 mmol) in methanol (15 mL) and refluxing for 2 h. After cooling, the crude product was collected by filtration, washed and recrystallised from hot ethanol to give white solids. The compounds **G1**, **G8**, **G23** and **G24** have been previously described [14] and compounds **G7**, **G19** and **F41** were recently patented by our group [19].

The 1,3,4-oxadiazoles were prepared by cyclisation of the previously obtained (*E*)-*N*′-benzylidene-benzohydrazides with acetic anhydride under reflux for 3 h as described previously [20]. After cooling, the crude product was collected by filtration, washed and recrystallised from acetone/water to give white solids. The compounds **Y18**, **Z43** and **Z47** have been previously described [21] and compounds **Z5** and **Z8** were recently patented by our group [19].

The (*E*)-chalcones were prepared by aldol condensation using methanol as solvent under basic conditions (KOH 50%, w/v) at room temperature for 24 h. Distilled water and 10% hydrochloric acid were added to the reaction for total precipitation of the compounds, which were then obtained by vacuum filtration and later recrystallised in dichloromethane and hexane. All structures (**C1**, **C6**, **C7**, **C24**, **C28**, **C33**, **C37**, **L15**, **L48**, **L50**, **J4**, **J61**, **J62**, **Lou5**, **R58** and **R61**) were previously described by our group [21–23].

2.3. Bacterial growth and biofilm inhibition assays

Biofilm production and quantification assays were performed by colorimetric assay as described previously [24]. No antibiotics were added at any point during these assays. To assay for biofilm inhibition, bacterial strains were grown statically for 20 h at 37 °C in TSB. Polystyrene 96-well microtitre plates were then inoculated with 100 μL/well of bacterial suspension previously diluted to 5×10^8 CFU/mL in TSB supplemented with 4% sucrose (w/v) and 3.5% (v/v) dimethyl sulphoxide (DMSO). Then, 100 μL of the compound to be tested for antibiofilm activity, prepared in TSB supplemented with 4% sucrose (w/v) and 3.5% (v/v) DMSO, was added to the first well. After homogenisation by pipetting up and down the 200 μL content of the well, 100 μL of this mixture was transferred to the next well and the procedure was repeated until a final concentration of 0.2 μg/mL was reached, and the final volume of each well was 100 μL. Microtitre plates were incubated for 20 h at 37 °C in a humidified chamber and bacterial growth was quantified by absorbance at 630 nm to assess eventual growth inhibition. Bacterial suspensions were discarded and biofilm was stained with a 0.1% (w/v) crystal violet solution for quantification. After solubilisation of crystal violet in 1% sodium dodecyl sulphate (SDS), biofilm was quantified by measuring the absorbance at 595 nm. For *S. mutans* assays, incubation was performed in microaerophilic conditions. Eventual growth inhibition was accessed for each compound by reading the optical density at 600 nm before biofilm quantification. Growth inhibition, when identified, was considered as an indication of potential conventional antibacterial properties of the chemical compounds, and those compounds were selected for future investigation.

2.4. Cell culture and selectivity assays

Selectivity towards bacteria of molecules positive for biofilm inhibition was evaluated using the human glioma cell line A172 by the MTT (thialyl blue tetrazolium bromide) colorimetric assay as previously described [25]. Briefly, A172 cells were cultured in Dulbecco's Modified Eagle Medium: nutrient mixture F-12 supplemented with 10% foetal bovine serum in 25 cm^2 culture flasks at 37 °C in a humidified atmosphere of 5% CO_2. Cells were plated in 96-well plates and were subsequently exposed for 24 h to the compounds for cytotoxicity assays. Cell viability was quantified by MTT colorimetric assay. Absorbance was measured at 540 nm and results were expressed as percentage viability of the control samples.

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