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A new plant-derived antibacterial is an inhibitor of efflux pumps in *Staphylococcus aureus*

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ABSTRACT

An in-depth evaluation was undertaken of a new antibacterial natural product (**1**) recently isolated and characterised from the plant *Hypericum olympicum* L. cf. *uniflorum*. Minimum inhibitory concentrations (MICs) were determined for a panel of bacteria, including: meticillin-resistant and -susceptible strains of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*; vancomycin-resistant and -susceptible *Enterococcus faecalis* and *Enterococcus faecium*; penicillin-resistant and -susceptible *Streptococcus pneumoniae*; group A streptococci (*Streptococcus pyogenes*); and *Clostridium difficile*. MICs were 2–8 mg/L for most staphylococci and all enterococci, but were ≥16 mg/L for *S. haemolyticus* and were >32 mg/L for all species in the presence of blood. Compound **1** was also tested against Gram-negative bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhimurium but was inactive. The MIC for *Mycobacterium bovis* BCG was 60 mg/L, and compound **1** inhibited the ATP-dependent *Mycobacterium tuberculosis* MurE ligase [50% inhibitory concentration (IC_{50}) = 75 μM]. In a radiometric accumulation assay with a strain of *S. aureus* overexpressing the NorA multidrug efflux pump, the presence of compound **1** increased accumulation of ^{14}C-enoxacin in a concentration-dependent manner, implying inhibition of efflux. Only moderate cytotoxicity was observed, with IC_{50} values of 12.5, 10.5 and 8.9 μM against human breast, lung and fibroblast cell lines, respectively, highlighting the potential value of this chemotype as a new antibacterial agent and efflux pump inhibitor.

1. Introduction

Plants produce an array of biologically active molecules that have been used as anticancer agents [1], including Taxol®, the camptothecins, the vinca alkaloids and podophyllotoxin derivatives such as etoposide [2]. Less research has been done to evaluate plants as sources of antibacterial agents [3] and among the reports that do exist many lack cytotoxicity data and tested only a few bacterial species. Even fewer studies have attempted to unravel the mechanisms of action for plant products, notable exceptions being studies on (i) the diterpene totarol from many coniferous plants as an inhibitor of FtsZ [4] and of efflux [5] and (ii) epicatechin gallate from green tea, which potentiates β-lactam activity against meticillin-resistant *Staphylococcus aureus* (MRSA) [6].

Nevertheless, plants may be a valuable source of new antibacterials. They must protect themselves against environmental microbes, and plant extracts are widely used as systemic and topical antimicrobials in Western herbal and traditional Chinese

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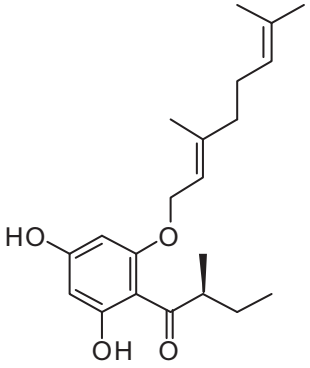

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Fig. 1. Chemical structure of compound **1**.

medicine as well as in Ayurvedic medicine [7]. Plant extracts are also marketed as 'food materials' with antibacterial properties, such as the various cranberry and bearberry preparations used in the management of urinary tract infections, with bearberry products having better understood chemistry and pharmacology [8]. Also of note are light-activated antibacterial terthiophenes, with minimum inhibitory concentrations (MICs) of 0.022 mg/L for *S. aureus* [9].

We have previously examined plant-derived antibacterials from the acetylene [10], diterpene [11], alkaloid [12] and flavonoid [13] groups. We have focused on acylphloroglucinol products [14], largely due to the considerable antibacterial activities of hyperforin from St John's wort [15], which displayed a MIC of 0.1–1 mg/L for penicillin- and meticillin-resistant *S. aureus* [16]. Acylphloroglucinols are complex natural products with an acylated aromatic-derived core and many prenyl groups, which may be cyclised or oxidised to give a class of chiral products rich in functional groups [17]. Early work on these molecules led to their isolation and characterisation from other *Hypericum* spp. such as *Hypericum drummondii*, which yielded the drummondins, some of which have potent activity towards *S. aureus* (MIC = 0.39 mg/L [18]). Other new acylphloroglucinol products with moderate antibacterial activity [14] were found in *Hypericum foliosum* and *Hypericum beanii*, whilst lipophilic extracts of the aerial parts of *Hypericum olympicum* L. cf. *uniflorum* were found to contain a new acylphloroglucinol antibacterial (**1**), given the trivial name olympicin A (Fig. 1); this was spectroscopically characterised and patented [19,20].

Here we describe the antibacterial spectrum of activity of compound **1** in the presence and absence of blood against multiple bacteria, including *Mycobacterium bovis* BCG, and provide a cytotoxicity evaluation for mammalian cell lines. We additionally examined the ability of compound **1** to inhibit ATP-dependent MurE ligase of *Mycobacterium tuberculosis* as well as to increase the accumulation of the quinolone antibiotic enoxacin by a strain of *S. aureus* overexpressing the NorA major facilitator superfamily (MFS) efflux pump. This work shows the value of simple plant natural product chemotypes as antibacterials and as modifiers of bacterial resistance.

2. Materials and methods

2.1. Isolation of compound *1*

Aerial parts of *H. olympicum* L. cf. *uniflorum* (Kew accession no. **1969-31184**) were collected from the Royal Botanic Garden Kew at Wakehurst Place (Ardingly, UK): 937 g of dried, powdered material was sequentially extracted with 3.5 L of *n*-hexane, dichloromethane (DCM) and methanol using a Soxhlet apparatus (Fisher Scientific, Loughborough, UK). The *n*-hexane and DCM extracts were active against the NorA-overexpressing *S. aureus* strain 1199B (SA-1199B) [21] at 32 mg/L and 16 mg/L, respectively, but had similar profiles by thin-layer chromatography (TLC) (Merck, Darmstadt, Germany). The hexane extract (15.2 g) was fractionated by vacuum-liquid chromatography (VLC) (silica gel $PF_{254+366}$; Merck) using a step-gradient solvent system from 100% hexane to 100% ethyl acetate with a 10% increment and a final methanol wash. VLC fractions 6–8 were active against *S. aureus* SA-1199B at 64 mg/L, had similar TLC profiles and were pooled (total of 842.0 mg). This combined fraction was separated by Sephadex LH-20 (Amersham Biosciences, Little Chalfont, UK) chromatography, giving five fractions eluted with chloroform:methanol (1:1) and one fraction eluted with methanol. The fraction eluted with methanol (80.9 mg) was active at a MIC of 1 mg/L against SA-1199B, and compound **1** (29.1 mg) was isolated from this fraction by preparative TLC [silica; toluene:ethyl acetate:acetic acid (80:18:2), retention factor (R_f) = 0.62]. The compound gave an orange colour reaction with vanillin–sulphuric acid spray on the TLC plate.

2.2. Antibiotics and media

Unless otherwise stated, all chemicals were obtained from Sigma–Aldrich (Poole, UK).

2.3. Bacteria

The bacteria were recent clinical isolates or were reference controls (Table 1). The clinical isolates represented important resistance phenotypes currently prevalent in the UK and worldwide. They comprised: (i) MRSA, including the epidemic MRSA (EMRSA)-15 and -16 strains dominant in the UK [22]; (ii) meticillin-resistant coagulase-negative staphylococci (i.e. *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*); (iii) vancomycin-resistant and -susceptible *Enterococcus faecalis* and *Enterococcus faecium*; (iv) penicillin-resistant and -susceptible *Streptococcus pneumoniae*; (v) group A streptococci (*Streptococcus pyogenes*), which remain universally susceptible to penicillin; and (vi) *Clostridium difficile*. Among the Gram-negative organisms tested were *Escherichia coli* NCTC 10418, *E. coli* STHG69 (multiresistant CMY-4 β-lactamase-producing isolate) and *Pseudomonas aeruginosa* strains NCTC 10662 and NCIB 8626. *M. bovis* BCG ATCC 35734 (Pasteur) was also used. SA-1199B was used in ^{14}C-enoxacin accumulation assays.

2.4. Susceptibility testing

MICs were determined on Iso-Sensitest agar (ISA) by the method of the British Society for Antimicrobial Chemotherapy (BSAC) (http://www.bsac.org.uk) or by microdilution in Iso-Sensitest broth (ISB). Both media were from Oxoid–Thermo Fisher (Basingstoke, UK); ISA was supplemented or not with 5% whole equine blood and ISB with 5% lysed equine blood. Plates were variously incubated at 35–37 °C in (i) air, (ii) air enriched with 5% CO_2 or (iii) under anaerobic conditions. The antimycobacterial activity of compound **1** was determined against slow-growing *M. bovis* BCG using an agar-based spot culture growth inhibition assay as described previously [23]. The front-line antitubercular drug isoniazid was used as a positive control.

2.5. Functional assay for M. tuberculosis MurE activity

The activity of MurE from *M. tuberculosis* was monitored by measuring the release of inorganic phosphate following ATP

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