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Short Communication

Antibacterial activity of the marine sponge constituent cribrostatin 6

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The antibacterial activity of the nitrogen heterocyclic sponge constituent cribrostatin 6 was examined. Cribrostatin 6 was bacteriostatic for a variety of Gram-positive species and was bactericidal for the majority of clinical isolates of *Streptococcus pneumoniae*, including penicillin-resistant strains. Minimum bactericidal concentration/MIC ratios were $\leqslant$2 for 75 % of *S. pneumoniae* clinical isolates. Kill-curve analysis confirmed the bactericidal action of cribrostatin 6. Bactericidal activity was rather slow, beginning at 2, 4 or 8 h, depending on the strain. The frequency of occurrence of bacterial spontaneous mutations to resistance was $\leqslant 10^{-7}$. The maximum tolerated dose of cribrostatin 6 in mice was 750–1000 μg kg^{-1} day^{-1}. Cribrostatin 6 is a promising lead antibiotic for Gram-positive bacteria, particularly *S. pneumoniae*, a leading cause of infection and mortality worldwide.

Introduction

Infections caused by antibiotic-resistant Gram-positive bacteria are a major cause of morbidity and mortality. Several of the more important resistance problems include vancomycin resistance in enterococci, penicillin resistance in streptococci and methicillin resistance in staphylococci. *Streptococcus pneumoniae* is the most common bacterial cause of acute respiratory infection and otitis media, and results in millions of deaths each year worldwide from pneumonia, bacteraemia and meningitis (Greenwood, 1999; Musher, 1992). Before 1990, most clinical isolates of *S. pneumoniae* in the US were susceptible to penicillin and various other antibiotics. As of 2000, 36 % of *S. pneumoniae* clinical isolates in the US were trimethoprim-sulfamethoxazole-resistant, 34 % were penicillin-resistant, 26 % were macrolide-resistant, 17 % were tetracycline-resistant, 9 % were clindamycin-resistant and 8 % were chloramphenicol-resistant (Doern *et al.*, 2001). Also as of 2000, two-thirds of penicillin-resistant isolates were high-level-resistant and multiresistant (resistant to two or more non-β-lactam antimicrobials) (Doern *et al.*, 2001). There is an urgent need for new structural classes of antibiotics active against *S. pneumoniae* and other Gram-positive genera.

The blue marine sponge *Cribrochalina* sp. contains a variety of biologically active constituents, including the antimicrobial cribrostatins 2 and 4 (Pettit *et al.*, 2000). We recently summarized the isolation and X-ray crystal structure determination of the dark-blue *Cribrochalina* constituent cribrostatin 6 (Pettit *et al.*, 2003). Against a small panel of microbes, cribrostatin 6 demonstrated activity against Gram-positive bacteria (Pettit *et al.*, 2003). Cribrostatin 6 was also weakly cytotoxic for human cancer cell lines (Pettit *et al.*, 2003). Recently, interest in developing antineoplastics and their derivatives as antimicrobials, and vice versa, has increased. The antineoplastic agents methotrexate, cyclophosphamide, vincristine, bleomycin, daunorubicin, 5-fluorouracil, mitomycin C and taxol, for example, have antifungal activity (Cardenas *et al.*, 1999). Tetracycline derivatives with human cancer and/or antifungal action are being pursued (Liu *et al.*, 2002; Lokeshwar *et al.*, 1998), and the lavage antibiotic taurolidine is currently being evaluated as a cancer chemotherapeutic in patients with glioblastoma and ovarian cancer (Calabresi *et al.*, 2001). Our interest here is the *in vitro* development of cribrostatin 6 as a lead antibiotic for Gram-positive bacteria.

Abbreviations: BMHII, Mueller–Hinton II (cation-adjusted) broth containing 3 % lysed horse blood; MBC, minimum bactericidal concentration; MTD, maximum tolerated dose.

Methods

Cribrostatin 6. Cribrostatin 6 (Fig. 1) was isolated in our laboratory as described previously (Pettit *et al.*, 2003) and stored desiccated in the dark. Prior to each experiment, cribrostatin 6 was reconstituted in a small volume of sterile DMSO and then diluted in the appropriate growth medium.

Strains. Non-duplicate clinical isolates and antibiotic resistance information were obtained from the Arizona Department of Health

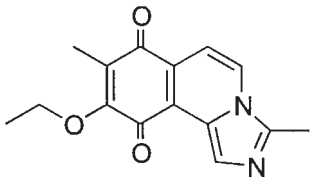

Fig. 1. Structure of cribrostatin 6.

Services. Penicillin and methicillin resistance were verified in our lab by the broth microdilution assay outlined by the NCCLS (2000). Penicillin-resistant strains required MICs $\geqslant$2 μg ml^{-1} (NCCLS, 2000). Methicillin resistance was evaluated with oxacillin and defined as an MIC $\geqslant$16 μg ml^{-1} (NCCLS, 2000). Invasive *S. pneumoniae* were cultured from sterile sites; their antibiotic resistance profiles are unknown. Reference strains were obtained from the American Type Culture Collection (Manassas, VA).

Susceptibility testing with cribrostatin 6. Susceptibility testing with cribrostatin 6 was performed by the reference broth microdilution assay outlined by the NCCLS (2000). Isolated colonies from overnight cultures were suspended and diluted as recommended to yield final inocula of approximately 5×10^5 c.f.u. ml^{-1}. Tests were performed in sterile microtitre plates containing twofold dilutions of cribrostatin 6 in Mueller–Hinton II (cation-adjusted) broth containing 3 % lysed horse blood (BMHII) (*Arcanobacterium*, *Erysipelothrix*, *Lactobacillus*, *Streptococcus*) or Mueller–Hinton II broth (all other bacteria). One well was

Table 1. Broth microdilution MICs and MBCs of cribrostatin 6 for reference strains and clinical isolates

Where more than one strain was tested, the number of strains is given in parentheses; ranges are given in these cases.

Micro-organism	MIC (μg ml^{-1})*	MBC (μg ml^{-1})†
Arcanobacterium haemolyticum	32	> 64
Bacillus cereus	8	> 64
Bacillus subtilis (4)	1–8	2–> 64
Corynebacterium diphtheriae	8	Eagle‡
Corynebacterium hoagii	2	> 64
Vancomycin-resistant *Enterococcus faecalis*	32	> 64
Vancomycin-resistant *Enterococcus* spp.	32	> 64
Erysipelothrix rhusiopathiae (2)	4–8	4–8
Lactobacillus spp.	2	> 32
Listeria monocytogenes	8	> 64
Nocardia asteroides	16	> 64
Nocardia farcinica	4	> 32
Rhodococcus bronchialis	8	16
Rhodococcus equi	16	> 64
Methicillin-resistant *Staphylococcus aureus* (7)	2–16	> 64
Staphylococcus saprophyticus	32	> 64
Streptococcus pneumoniae ATCC 6303	0·5	Eagle§
Multidrug-resistant *S. pneumoniae* ATCC 700904‖	0·25	Eagle¶
Multidrug-resistant *S. pneumoniae* ATCC 700673#	0·125	0·25
Penicillin-resistant *S. pneumoniae* (7)	0·25–4	2–16
Invasive *S. pneumoniae* (28)	0·25–4 (1; 2)	0·25–32 (1; 8)
S. pneumoniae (15)	0·5–16 (1; 4)	0·5–16 (1; 16)
Streptococcus pyogenes (32)	2–> 64 (16; 64)	16–> 64 (> 64; > 64)

*Values in parentheses are the MIC_{50} and MIC_{90}.

†Values in parentheses are the MBC_{50} and MBC_{90}.

‡Gave > 500 colonies on 8 μg ml^{-1} plate, 0 colonies on 16 and 32 μg ml^{-1} plates and 179 colonies on 64 μg ml^{-1} plate.

§Gave 0 colonies on 0·5, 1 and 2 μg ml^{-1} plates and > 250 colonies on 4 μg ml^{-1} plate.

‖Resistant to rifampicin, penicillin, clindamycin, erythromycin, tetracycline and chloramphenicol (ATCC product literature).

¶Gave > 500 colonies on 0·25 μg ml^{-1} plate, 15–24 colonies on 0·5–16 μg ml^{-1} plates (> 99·9 % kill) and 35–44 colonies on 32–64 μg ml^{-1} plates (> 99 % kill).

#Resistant to trimethoprim-sulfamethoxazole, penicillin, erythromycin, tetracycline and chloramphenicol (ATCC product literature).

left drug-free (but contained an equivalent volume of DMSO) for a turbidity control. Each well contained a total volume of 100 µl. Plates were incubated without agitation at 37 °C with 5 % CO_2 (*Arcanobacterium*, *Erysipelothrix*, *Lactobacillus*) or at 35 °C (all other organisms). MICs were determined at 16–20 h for *Bacillus*, *Corynebacterium*, *Enterococcus*, *Listeria*, *Rhodococcus equi* and *Staphylococcus*, at 24 h for *Arcanobacterium* and *Streptococcus* and at 48 h for *Erysipelothrix*, *Lactobacillus*, *Nocardia* and *Rhodococcus bronchialis*. Broth microdilution assays were also performed in BMHII prepared at pH 6, 7 and 8, in BMHII with and without 25 % normal human serum (Lampire Biological Labs) and in BMHII with and without 20 and 40 µg BSA ml^{-1} (Sigma). The MIC was defined as the lowest concentration of drug that inhibited all visible growth of the test organism (optically clear). No trailing was observed. Minimum bactericidal concentrations (MBCs) were determined by subculture of 50 µl from each negative well and from the positive growth control well of the broth microdilution series onto drug-free plates. Plates were incubated at the appropriate temperature for 24–48 h. The MBC was defined as the lowest drug concentration that resulted in a ⩾99·9 % reduction in the initial inoculum.

Frequency of spontaneous mutants. The frequency of occurrence of single-step resistant mutants was determined. Overnight cultures of three strains of *S. pneumoniae* were diluted to an OD_{625} of 0·08–3. One hundred microlitres of each preparation was spread onto agar plates containing four times the broth microdilution MIC of cribrostatin 6. The starting inoculum for each organism was also diluted and plated onto drug-free plates for determination of c.f.u. ml^{-1}. After 48 h incubation at the appropriate temperature, the number of bacterial colonies on drug-supplemented agar was counted. The frequency of occurrence of spontaneous resistant mutants was calculated by dividing the number of colonies on drug-containing plates by the number of c.f.u. in the inoculum. When no colonies were visualized on drug-containing plates, the calculation was (<)1 colony divided by the number of c.f.u. in the inoculum.

Time-kill studies. Early log cultures in BMHII were inoculated into the same medium containing multiples of the broth microdilution MIC of cribrostatin 6 or an equivalent volume of DMSO. Cultures were shaken at 35 °C and aliquots were removed aseptically at various times for dilution plating. In addition, aliquots were plated directly from drug-treated flasks at the later time-points. Thus, the detection limit in these experiments was 10 c.f.u. ml^{-1}. Standard errors of the means were calculated from at least two experiments.

Mouse toxicity evaluation. Female CD-1 mice (5 weeks old) were obtained from Charles River Laboratories and assigned randomly into groups of five mice each. Cribrostatin 6 was dissolved in methanol and diluted in sterile PBS. Vehicle controls consisted of the same diluent mixture. Mice were given i.p. injections of 100, 200, 400, 500, 750 or 1000 µg cribrostatin 6 kg^{-1} day^{-1}, 12 h apart, for 5 days. Mice were weighed daily and observed for gross signs of toxicity such as weight loss, dehydration and activity loss.

Results and Discussion

In broth microdilution assays, cribrostatin 6 inhibited the growth of all Gram-positive bacteria tested (Table 1). Cribrostatin 6 was most active against *S. pneumoniae*, including multidrug-resistant strains. MBC/MIC ratios were ⩽2 for 75 % of *S. pneumoniae* clinical isolates, consistent with a bactericidal mechanism of action. We were puzzled by the appearance of increasing numbers of survivors at concentrations higher than the MBC for some *S. pneumoniae* strains (0·1 %) and for many *Streptococcus pyogenes* strains (68 %). This so-called Eagle effect has been described for other antimicrobials, including the β-lactams, and is believed to be the result of high antimicrobial concentrations inhibiting protein synthesis to a degree that prevents the growth necessary for expression of the lethal effect of the antimicrobial (Amsterdam, 1996). There are apparently no therapeutic implications associated with this effect (Amsterdam, 1996). As no MBC could be defined for these strains, they were not

Table 2. Effect of pH or human serum on MICs of cribrostatin 6

Treatment	MIC (µg ml^{-1})
S. pneumoniae **ATCC 6303**	
pH 6	1
pH 7	4
pH 8	16
No serum	0·5
25 % Human serum	4
Invasive *S. pneumoniae* (clinical isolate 528863)	
pH 6	0·5
pH 7	0·5
pH 8	4
No serum	0·5
25 % Human serum	2
Invasive *S. pneumoniae* (clinical isolate 42549858)	
pH 6	1
pH 7	0·25
pH 8	0·5
No serum	0·5
25 % Human serum	4
S. pneumoniae **(clinical isolate 407150)**	
pH 6	0·5
pH 7	0·25
pH 8	0·5
No serum	0·5
25 % Human serum	4
S. pneumoniae **(clinical isolate 404090)**	
pH 6	0·25
pH 7	0·25
pH 8	0·5
No serum	0·5
25 % Human serum	4
S. pyogenes **(clinical isolate 46986898)**	
pH 6	ND
pH 7	4
pH 8	4
No serum	4
25 % Human serum	4
S. pyogenes **(clinical isolate 30524975)**	
pH 6	ND
pH 7	2
pH 8	8
No serum	4
25 % Human serum	4

ND, Not determined.

included in MBC_{50} (MBC at which 50 % of the strains are killed), MBC_{90} (MBC at which 90 % of the strains are killed) and MBC/MIC ratio calculations.

The effects of two host factors, pH and serum, on broth microdilution MICs were examined. For some strains, MICs increased slightly in acidic or alkaline medium (Table 2). Attempts were made to determine MICs at pH 6 for *S. pyogenes*, but these strains did not grow in acidic medium. MICs for *S. pneumoniae* increased three- to fourfold in 25 % human serum, while *S. pyogenes* MICs were unchanged (Table 2). Serum inactivation did not appear to be due to serum albumin binding, as MICs with or without 20 or 40 μg BSA ml^{-1} typically varied by no more than one, twofold dilution (data not shown).

The frequency of occurrence of single-step-resistant mutants at four times the MIC was $< 10^{-7}$ for *S. pneumoniae* ATCC 6303, $9{\cdot}3 \times 10^{-7}$ for invasive clinical isolate 19486130 and 3×10^{-7} for clinical isolate 404090. Mutation frequencies were in the range expected for a compound in preclinical development.

Time-kill curves confirmed the bactericidal mechanism of action of cribrostatin 6. Fig. 2 summarizes the time-kill curves for three *S. pneumoniae* strains, ATCC 6303 (Fig. 2a), invasive clinical isolate 19486130 (Fig. 2b) and clinical isolate 404090 (Fig. 2c), and one *S. pyogenes* clinical isolate (30524975) (Fig. 2d). We attempted to perform kill-curves with the *S. pneumoniae* clinical isolates listed in Table 2, but the untreated controls did not yield typical growth curves. Killing was not concentration-dependent, with the exception of *S. pneumoniae* 404090 (Fig. 2c), where killing was concentration-dependent between one and four times the MIC. Killing was time-dependent for the three *S. pneumoniae* strains, but not for the single *S. pyogenes* strain during the first 8 h (Fig. 2d). Killing occurred slowly, after 2 h for the two clinical isolates of *S. pneumoniae* (Fig. 2b, c) after 4 h for *S. pneumoniae* ATCC 6303 (Fig. 2a) and after 8 h for the *S. pyogenes* clinical isolate (Fig. 2d). The number of survivors in cultures of *S. pneumoniae* ATCC 6303 and 404090 treated with an intermediate dose (four times the MIC) for 24 h varied greatly, with regrowth occurring in some cases (Fig. 2a, c). *In vitro* time-kill systems lack host parameters like immune defences. Thus, clinical studies will be necessary in order to define any *in vivo* significance of the regrowth phenomena.

The MTD of cribrostatin 6 in mice was 750–1000 μg kg^{-1} day^{-1}. At all doses except 1000 μg kg^{-1} day^{-1}, mice showed no signs of gross toxicity. Mice at the highest dose exhibited toxicity through lower overall activity and weight loss. Efficacy studies in topical and systemic models of Gram-positive infection await synthesis of cribrostatin 6. Synthesis of cribrostatin 6 and derivatives is in progress in our laboratories.

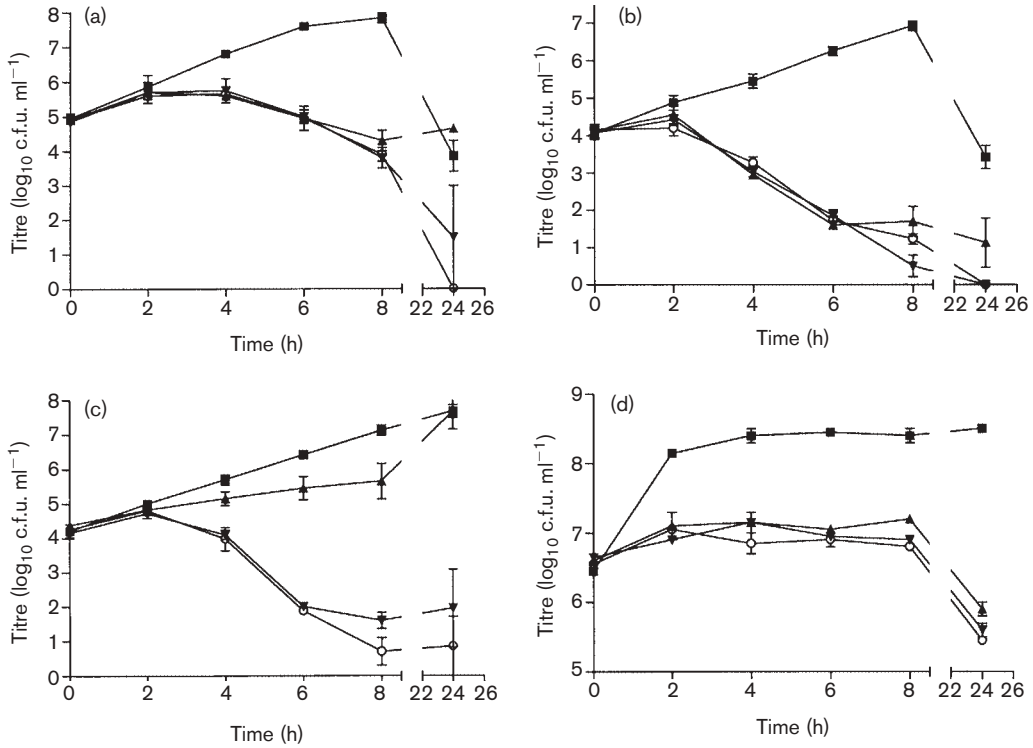

Fig. 2. Kill curves for *S. pneumoniae* ATCC 6303 (a), *S. pneumoniae* invasive clinical isolate 19486130 (b), *S. pneumoniae* clinical isolate 404090 (c) and *S. pyogenes* clinical isolate 30524975 (d). Killing was determined with DMSO alone (■) and at the MIC (▲) and at four (▼) and eight (○) times the MIC of cribrostatin 6. The results are means ± SE of at least two experiments.

There has been an alarming increase in the number of drug-resistant bacteria. The novel nitrogen heterocyclic compound cribrostatin 6 is a promising lead compound for Gram-positive infections, particularly *S. pneumoniae*. *In vivo* studies are necessary in order to validate the *in vitro* properties of cribrostatin 6. The relative potency of the cribrostatins for cancer cells versus bacterial cells will be critical when selecting candidates for further preclinical development.

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References

Amsterdam, D. (1996). Susceptibility testing of antimicrobials in liquid media. In *Antibiotics in Laboratory Medicine*, 4th edn, pp. 52–111. Edited by V. Lorian. Baltimore: Williams & Wilkins.

Calabresi, P., Goulette, F. A. & Darnowski, J. W. (2001). Taurolidine: cytotoxic and mechanistic evaluation as a novel antineoplastic agent. *Cancer Res* **61**, 6816–6821.

Cardenas, M. E., Cruz, M. C., Del Poeta, M., Chung, N., Perfect, J. R. & Heitman, J. (1999). Antifungal activities of antineoplastic agents: *Saccharomyces cerevisiae* as a model system to study drug action. *Clin Microbiol Rev* **12**, 583–611.

Doern, G. V., Heilmann, K. P., Huynh, H. K., Rhomberg, P. R., Coffman, S. L. & Brueggemann, A. B. (2001). Antimicrobial resistance among clinical isolates of *Streptococcus pneumoniae* in the United States during 1999–2000, including a comparison of resistance rates since 1994–1995. *Antimicrob Agents Chemother* **45**, 1721–1729.

Greenwood, B. (1999). The epidemiology of pneumococcal infection in children in the developing world. *Philos Trans R Soc Lond B Biol Sci* **354**, 777–785.

Liu, Y., Ryan, M. E., Lee, H. M., Simon, S., Tortora, G., Lauzon, C., Leung, M. K. & Golub, L. M. (2002). A chemically modified tetracycline (CMT-3) is a new antifungal agent. *Antimicrob Agents Chemother* **46**, 1447–1454.

Lokeshwar, B. L., Houston-Clark, H. L., Selzer, M. G., Block, N. L. & Golub, L. M. (1998). Potential application of a chemically modified non-antimicrobial tetracycline (CMT-3) against metastatic prostate cancer. *Adv Dent Res* **12**, 97–102.

Musher, D. M. (1992). Infections caused by *Streptococcus pneumoniae*: clinical spectrum, pathogenesis, immunity, and treatment. *Clin Infect Dis* **14**, 801–807.

NCCLS (2000). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*. Approved standard M7-A5. Villa Nova, PA: National Committee for Clinical Laboratory Standards.

Pettit, G. R., Knight, J. C., Collins, J. C., Herald, D. L., Pettit, R. K., Boyd, M. R. & Young, V. G. (2000). Antineoplastic agents 430. Isolation and structure of cribrostatins 3, 4, and 5 from the Republic of Maldives *Cribrochalina* species. *J Nat Prod* **63**, 793–798.

Pettit, G. R., Collins, J. C., Knight, J. C., Herald, D. L., Nieman, R. A., Williams, M. D. & Pettit, R. K. (2003). Antineoplastic agents 485. Isolation and structure of cribrostatin 6, a dark blue cancer cell growth inhibitor from the marine sponge *Cribrochalina* sp. *J Nat Prod* **66**, 544–547.