PHYTOTHERAPY RESEARCH
Phytother. Res. **14**, 139–140 (2000)

SHORT COMMUNICATION

The Effect of Reserpine, a Modulator of Multidrug Efflux Pumps, on the *in vitro* Activity of Tetracycline Against Clinical Isolates of Methicillin Resistant *Staphylococcus aureus* (MRSA) Possessing the *tet*(K) Determinant

Simon Gibbons[1]* and E. E. Udo[2]

[1]Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK
[2]Department of Microbiology, Faculty of Medicine, Kuwait University, P.O. Box 24923, Safat 13110, Kuwait

As part of a screening programme to identify modulators of multidrug efflux in methicillin resistant *Staphyloccocus aureus* (MRSA), we have validated our assays using the antihypertensive plant alkaloid reserpine. Clinical isolates of MRSA were resistant to tetracycline and shown to possess the *tet*(K) determinant which encodes for the Tet(K) efflux protein, which conferred high level resistance to tetracycline (MIC = 128 μg/mL). In the presence of reserpine, a known inhibitor of multidrug resistance (mdr) efflux pumps, this MIC was significantly reduced (MIC = 32 μg/mL).

Keywords: efflux; MRSA; Tet(K); multidrug resistance; mdr.

INTRODUCTION

The occurrence and proliferation of methicillin resistant *Staphylococcus aureus* (MRSA) is cause for great concern in the clinical environment due to the few effective therapeutic agents that can be used against this organism (Horikawa *et al.*, 1999). The last group of antibiotics available for the treatment of MRSA are the glycopeptides, typically vancomycin, but unfortunately resistance to this agent has already been encountered in Japan (Hiramatsu *et al.*, 1997) and the United States (Martin and Wilcox, 1997; Perl, 1999; Rotun *et al.*, 1999), and more recently in the United Kingdom (unpublished report on vancomycin resistance in *Staphylococcus aureus* at Glasgow Royal Infirmary, June 1999).

Efflux is an important mechanism of resistance in many clinically relevant pathogens, notably, *Streptococcus pneumoniae* (Markham, 1999), *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae* (Marshall and Piddock, 1997). In *Staphylococcus aureus*, efflux mechanisms have been demonstrated to confer resistance to macrolides, fluoroquinolones and the tetracyclines *via* the Msr(A) (Ross *et al.*, 1990), Nor(A) (Ng *et al.*, 1994) and Tet(K) (Guay *et al.*, 1993) efflux proteins respectively. The *tet*(K) gene encodes for an hydrophobic, 50.6 KDa membrane bound protein that actively effluxes tetracycline and is assumed to confer resistance to this agent (Guay *et al.*, 1993).

We have been screening elements of the Kuwaiti flora for modulators of Tet(K) mediated resistance in clinical isolates of MRSA and have validated our assays using the multidrug efflux inhibitor reserpine, which has been shown to modulate resistance in bacteria possessing the NorA and Bmr efflux mechanisms (Ng *et al.*, 1994).

* Correspondence to: Dr S. Gibbons, The School of Pharmacy, 29–39 Brunswick Square, London, WC1N 1AX, UK.

MATERIALS AND METHODS

Bacterial strains. MRSA strains IS-58 and XU212 were cultured from clinical isolates from the Ibn Sina and Adan hospitals, respectively. *S. aureus* standard strain ATCC 25923 was obtained from the American Type Culture Collection. All strains were cultured on nutrient agar (Oxoid) prior to determination of minimum inhibitory concentration.

Primer details for the *tet*(K) gene. The *tet*(K) gene in strains IS-58 and XU212 was detected in polymerase chain reaction (PCR) experiments using two 18- mer oligonucleotide primers, synthesis based on the DNA sequence of the *S. aureus* plasmid, pT181, which encodes a tetracycline efflux protein. The sequences were: primer K1, 5′-CAG CAG ATC CTA CTC CTT-3′ corresponding to nucleotides 531 to 549 and primer K2, 5′-TCG ATA GGA ACA GCA GTA-3′, which is complementary to nucleotides 682 to 700 of the *tet*(K) gene and separated by 168 base pairs.

Protocol for PCR. The PCR mixture consisted of 3 μL of template DNA, 10 pmol of K1 and K2 primers and 45 μL of PCR supermix (Gibco BRL). DNA amplification was carried out for 30 cycles in a final volume of 50 μL of reaction mixture as follows: denaturation at 94 °C (1 min),

Received 30 July 1999
Accepted 10 October 1999

Table 1. Minimum inhibitory concentrations (MICs) for test organisms

	MIC (μg/mL) of tetracycline	
Strain		+R
XU212	128	32
IS-58	128	32
ATCC 25923	0.16	0.16

+R, MIC measured in the presence of reserpine at a concentration of 10 μg/mL.

annealing at 55 °C (1 min) and extension at 72 °C for 1 min. After amplification, 15 μL of product was analysed by agarose gel 1.5% (w/v) electrophoresis in Tris buffer and stained with ethidium bromide. The amplified DNA was visualized under UV light and photographed.

Determination of Minimum Inhibitory Concentration (MIC). Mueller-Hinton broth (MHB) (Oxoid) was adjusted to contain 20 mg/L and 10 mg/L of Ca^{2+} and Mg^{2+} respectively. An inoculum density of 10^5 cfu of each of the test organisms was prepared in normal saline (0.9 g/L). MHB (125 μL) was dispensed into 10 wells of a 96-well microtitre plate (Nunc, 0.3 mL volume) and then 125 μL of tetracycline (2048 μg/mL) (Sigma Chemical Co.) was serially diluted (2 fold) into each of the wells. 125 μL of inoculum was then added to each of the wells, which resulted in a tetracycline concentration range of 512–1 μg/mL. The plate was then incubated at 36 °C for 18 h and the MIC was recorded as the lowest concentration at which no growth was observed. In the case of ATCC 25923 *Staphylococcus aureus*, a tetracycline concentration range of 10–0.02 μg/mL was used.

For the modulation of resistance experiment, reserpine (Sigma Chemical Co.) was incorporated into the MHB to give a final concentration of 10 μg/mL.

RESULTS AND DISCUSSION

MRSA strains IS-58 and XU212 were highly resistant to tetracycline (Table 1) and exhibited MICs of 128 μg/mL as a result of the presence of the *tet*(K) determinant which encodes for the Tet(K) efflux protein. When compared with the standard ATCC 25923 strain, IS-58 and XU212 exhibited an 800 fold increase in resistance to tetracycline, which renders the use of this antibiotic against these organisms as therapeutically redundant.

The incorporation of the antihypertensive plant alkaloid reserpine into the medium, clearly affected the MIC of tetracycline in the MRSA strains and lowered the MIC by a factor of 4. In contrast, the incorporation of reserpine into the medium did not effect the MIC for the standard ATCC strain.

This 4-fold decrease in MIC is in good agreement with data for MICs recorded in the presence and absence of reserpine of norfloxacin resistant *S. aureus* possessing the Nor(A) fluoroquinolone efflux protein (Ng *et al*., 1994).

This is, we believe, the first report of a reduction of MIC in clinical isolates of MRSA possessing the Tet(K) efflux protein in the presence of reserpine.

Modulators of drug resistance would clearly have benefit for the treatment of multidrug resistant (mdr) strains of bacteria for which the majority of therapeutic antibiotics are of no further clinical use. Inhibitors of drug efflux mechanisms could, in combination, greatly extend the useful lifetime of older conventional antibiotics, for example with the tetracyclines. This concept of resistance modulation could be extended to other multidrug resistant pathogens such as fluoroquinolone resistant *Streptococcus pneumoniae*, azole resistant *Candida albicans* or macrolide resistant *Escherichia coli*, all of which produce efflux proteins as part of their resistance mechanisms (Markham, 1999; Cannon *et al*., 1998, Marshall and Piddock, 1997).

REFERENCES

Cannon, R. D., Fischer, F. J., Niimi, K., Niimi, M., and Arisawa, M. (1998). Drug pumping mechanisms in *Candida albicans*. *Jpn J. Med. Mycol.* **39**, 73–78.

Guay, G. G., Khan, S. A., and Rothstein, D. M. (1993). The *tet*(K) gene of plasmid pT181 of *Staphylococcus aureus* encodes an efflux protein that contains 14 transmembrane helices. *Plasmid* **30**, 163–166.

Hirimatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., and Tenover, F. C. (1997). Methicillin-resistant *Staphylococcus aureus* clinical isolates with reduced vancomycin susceptibility. *J. Antimicrob. Chemother.* **40**, 135–136.

Horikawa, M., Noro, T., and Kamei, Y. (1999). *In vitro* anti-methicillin-resistant *Staphylococcus aureus* activity found in extracts of marine algae indigenous to the coastline of Japan. *J. Antibiot.* **52**, 186–189.

Markham, P. N. (1999). Inhibition of the emergence of ciprofloxacin resistance in *Streptococcus pneumoniae* by the multidrug efflux inhibitor reserpine. *Antimicrob. Agents. Chemother.* **43**, 988–989.

Marshall, N. J., and Piddock, L. J. V. (1997). Antibacterial efflux systems. *Microbiologia* **13**, 285–300.

Martin, R., and Wilcox, K. R. (1997). Update: *Staphylococcus aureus* with reduced susceptibility to vancomycin, United States. *MMWR* **46** (35), 813–815.

Ng, E. Y. W., Trucksis, M., and Hooper, D. C. (1994). Quinolone resistance mediated by *norA:* physiologic characterization and relationship to *flqB,* a quinolone resistance locus on the *Staphylococcus aureus* chromosome. *Antimicrob. Agents. Chemother.* **38**, 1345–1355.

Perl, T. M. (1999). The threat of vancomycin resistance. *Am. J. Med.* **106**, (5A), 26S–37S.

Ross, J. I., Eady, E. A., Cove, J. H., Cunliffe, W. J., Baumberg, S., and Wootton, J. C. (1990). Inducible erythromycin resistance in staphylococci is encoded by a member of the ATP-binding transport super-gene family. *Mol. Microbiol.* **4**, 1207–1214.

Rotun, S. S., Schoomaker, D. J., Maaupin, P. S., Tenover, F. C., Hill, B. C., and Ackman, M. (1999). *Staphylococcus aureus* with reduced susceptibility to vancomycin isolated from a patient with fatal bacteraemia. *Emerg. Infect. Dis.* **5**, 147–149.

