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Thioridazine Reduces Resistance of Methicillin-resistant *Staphylococcus aureus* by Inhibiting a Reserpine-sensitive Efflux Pump

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Abstract. *Previous studies suggested that the phenothiazine chlorpromazine (CPZ) could reverse or reduce the antibiotic resistance of bacteria. In some areas of the world, the majority of Staphylococcus aureus isolates are now resistant to methicillin, prompting this study to see whether such resistance can be altered by phenothiazine thioridazine (TZ), an agent with equal antibacterial activity, which is free of the severe side-effects associated with chronic administration of CPZ. The results indicated that, whereas methicillin-sensitive strains of Staphylococcus aureus (MSSA) were not rendered more susceptible to oxacillin, resistance to oxacillin by highly-resistant strains (MRSA) could be significantly reduced by sub-inhibitory concentrations of TZ. Reserpine, an inhibitor of efflux pumps, was also shown to reduce the resistance of MRSA strains to oxacillin in a concentration-dependent manner. The phenothiazines have been shown, by others, to inhibit the efflux pumps of bacteria and the mechanism by which MRSA are rendered more susceptible to oxacillin in the presence of TZ is believed to be due to a similar efflux pump.*

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Methicillin resistance of *Staphylococcus aureus* is now commonplace throughout the world (1). In Portugal, the majority of clinical isolates of *S. aureus* are of a methicillin-resistant (MRSA) type (2). Although MRSA are not frequently isolated from young Portuguese males and females (2), this frequency is expected to increase in the general population. Infections caused by MRSA strains are becoming more difficult to manage since resistance to the fluoroquinolones is also quite common (3, 4). Resistance to the toxic antibiotic vancomycin has also been reported (5).

Bacterial resistance to antibiotics is commonly accepted to be due to mutations of genes encoding for the targets of a given antibiotic (6), as well as by the acquisition of plasmids that carry genes for targets that are immune to the antibiotic (7). During recent decades, the resistance of bacteria to antibiotics has been shown to involve mechanisms that are distinct from those above. Thus, the transitional presence of outer membrane proteins such as porins (8) and other proteins affects the penetration of antibiotics into the bacterium, thereby obviating any potential activity of the antibiotic on genetically intact targets beyond the plasma membrane (9, 10). More recently, the resistance of bacteria to given antibiotics has been shown, in many cases, to be due to the presence of plasma membrane energy-dependent units that pump out antibiotics that penetrate the bacterial cell envelope and eventually reach the medial side of the plasma membrane (11, 12). These units are collectively known as efflux pumps

and their activity has been shown to be present in all bacteria that have been studied to date (13, 14).

The phenothiazines have been shown to have a wide gamut of *in vitro* antibacterial activity (15-17). These compounds have also been shown to enhance the activity of antibiotics against susceptible bacteria (18-22), to cause the elimination of plasmids that bestow antibiotic resistance on bacteria (23-25) and to render antibiotic-resistant cells susceptible to given antibiotics (26-28). The phenothiazines have also been shown to inhibit efflux pumps in general (29), and bacterial efflux pumps specifically (30, 31).

The *in vitro* activity of the phenothiazines has always been shown to take place at concentrations which are far greater than those that can be achieved in a patient (32).

Moreover, the phenothiazine that has been studied most frequently is chlorpromazine (CPZ), a compound that, when chronically administered, produces severe side-effects (33, 34). It is not, therefore, surprising that little consideration has been given to the use of CPZ as an antibacterial agent. However, thioridazine (TZ) has been shown to equal CPZ with respect to all of its antibacterial properties (35) and has been shown to have activity against phagocytosed bacteria when its concentration in the medium is equal to or below the plasma concentration of a patient being managed with this compound (32, 36-38). Because TZ is relatively innocuous when administered for periods representative of those employed for the management of a bacterial infection, the objections to its use as an antibacterial agent are no longer considered valid, especially in areas of the world where antibiotic-resistant infections are prevalent. Nevertheless, prior to further consideration of TZ as an antibacterial agent, it would be useful to compare its effectiveness to that of the toxic CPZ. Here, the ability of TZ to reduce the resistance of MRSA to oxacillin was investigated.

Materials and Methods

Materials. Chlorpromazine (CPZ), thioridazine (TZ), oxacillin, reserpine and verapamil were purchased from Sigma Aldrich Química SA. (Madrid, Spain). Trypticase soy broth (TSB) and Trypticase soy agar (TSA) were purchased from Difco (Detroit, MI, USA). All solutions of the phenothiazines were prepared in distilled, sterile water on the day of the experiment.

Bacterial strains. The *Staphylococcus aureus* strain ATCC25923 was employed to serve as the absolute control while three clinical strains susceptible to oxacillin (MSSA) and five clinical strains resistant to oxacillin (MRSA) were used in the experiments. For each experiment, individual colonies of each strain were obtained from TSA plates, transferred to 10 ml of TSB and, prior to the day of any given experiment, incubated at 37˚C until they reached their stationary phase (8 hours).

Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs). The individual MICs of oxacillin, CPZ, TZ and reserpine were performed in TSB by the broth dilution method, as previously described (39). The MBCs of CPZ and TZ were determined by extending the MIC concentration curves well beyond each MIC and 10-μl aliquots of the 10-ml cultures at zero time and those after 18 hours which showed no evidence of growth were subjected to colony forming unit (CFU) counts (40). The MIC and MBC determinations were repeated three times and the values obtained did not differ.

Modulation of oxacillin activity by CPZ, TZ, reserpine and verapamil against MSSA and MRSA strains. The possible alteration of the susceptibilities of the MSSA and MRSA strains to oxacillin by CPZ, TZ, reserpine and verapamil were examined, as described in the appropriate legends of the tables in the text. Appropriate microliter volumes containing freshly-prepared compounds were added to tubes containing 10 ml of TSB to yield specific final concentrations. To each tube, an inoculum of approximately $1x10^5$ cells/ml of medium was added, followed by incubation at 37˚C for 18 hours. The results of the incubation were recorded as either an "MIC" for a given combination (no evidence of growth) or, when required, decrease of the optical density or the CFU method was employed.

Results

The separate MICs and MBCs of oxacillin, and the general inhibitors of the efflux pumps CPZ, TZ, reserpine and verapamil against the MSSA and MRSA strains are summarized in Table I. Briefly, a concentration of reserpine as high as 400 mg/L or verapamil as high as 1000 mg/L did not affect the growth of the five MRSA clinical strains. Unlike oxacillin, both CPZ and TZ killed the MRSA clinical strains but only at concentrations of 60 to 80 mg/L. These concentrations are obviously far higher than those anticipated to exist in the plasma of TZ-treated patients namely, *ca.* 0.1-0.3 mg/L. The CPZ and TZ concentrations required for killing were significantly higher than those representing the individual MICs for each of the strains studied. The MBCs of CPZ and TZ were not significantly different. Reserpine and verapamil, both well known to inhibit efflux pumps of bacteria, did not alter the growth of the MRSA strains at concentrations as high as 400 and 1000 mg/L. The susceptibilities of the MRSA strains to oxacillin, in the presence of varying concentrations of CPZ and TZ, are summarized by Table II. Briefly, the susceptibility of the MSSA strains to oxacillin, including that of the ATCC strain, were not altered by the presence of either CPZ or TZ at 25% or 50% their respective MIC (data not shown). In contrast to these findings, the presence of CPZ or TZ only at 50% of their MIC significantly reduced the resistance of all MRSA strains. This reduction of resistance was most pronounced in MRSA strain 4. The resistance of this strain could not be reduced any further by the presence of TZ at 50% of the MIC, and it remained well beyond the minimal level associated with clinical resistance (*i.e.*, *ca.* 0.8 mg/L). MRSA strains 4 and 5 were also resistant to fluoroquinolone and, because such resistance may be due to the presence of an

Table I. *The MICs and MBCs of oxacillin, chlorpromazine, thioridazine, reserpine, verapamil and CCCP against MRSA strains.*

	OXA	CPZ		TZ		RES	VER	CCCP
Strains	MIC	MBC	MIC	MBC	MIC	MIC	MIC	MIC
MRSA 1	>800	>800	40	80	20	>400	>1000	2.0
MRSA 2	>800	>800	40	70	20	>400	>1000	2.0
MRSA 3	>800	>800	40	80	30	>400	>1000	2.0
MRSA 4	>800	>800	60	80	30	>400	>1000	1.0
MRSA 5	>800	>800	60	80	30	>400	>1000	2.0

OXA (oxacillin), CPZ (chlorpromazine), TZ (thioridazine), RES (reserpine), VER (verapamil). Concentrations of all compounds are in mg/L. MIC was defined when the optical density at 545 mn of the culture was equal to that of a TSB control containing the compound at each relative concentration of the range employed. MBC was defined as the minimum concentration of the agent that was bactericidal.

efflux pump that is associated with multidrug resistance (41), the effect of reserpine on the resistance of these two strains to oxacillin was evaluated. Similarly, verapamil, a known inhibitor of some efflux pumps (42), was also examined for any effect on the resistance of these two strains to oxacillin. As shown in Table III, a concentration of reserpine of 50 mg/L, that was well below its MIC (>1000 mg/L), significantly reduced the number of CFUs at concentrations of oxacillin of 200 and 100 mg/L, as compared to those present in the cultures containing 400 mg/L of oxacillin and no reserpine. Increasing the concentration range of reserpine from 50 to 400 mg/L reduced the MIC of oxacillin against MRSA 4 and MRSA 5 to below 1.0 mg/L. At the highest concentration of reserpine, a concentration of oxacillin of 1 mg/L resulted in no CFUs. It is important to note that, if this concentration of oxacillin were to be bacteriostatic, the number of CFUs would be at least equal to those initially cultured (*ca.* 10^5 /ml of medium). The presence of verapamil at concentrations as high as 1000 mg/L did not affect the number of CFUs of the MRSA cultures containing any concentration of oxacillin (data not shown). Neither reserpine or verapamil enhanced the activity of oxacillin against the MSSA strains (data not shown). Table IV presents the average effects of reserpine on the MIC of oxacillin against the five MRSA strains. The use of CCCP, an uncoupler of the electron motive force, at a concentration that was well below its MIC, reduced the average resistance of the five MRSA strains to increasing concentrations of oxacillin, as shown in Table V.

Table II. *The effects of CPZ and TZ on the susceptibility of MRSA strains to oxacillin.*

	Oxacillin MIC (mg/L)				
Strains	orig. MIC	CPZ at 25%	CPZ at 50%	TZ at 25%	TZ at 50%
MRSA 1	>800	NC	100	NC	100
MRSA 2	>800	NC	100	NC	100
MRSA 3	>800	NC	100	NC	100
MRSA 4	>800	NC	20	NC	10
MRSA 5	>800	NC	100	NC	100

Values are in mg/L. CPZ and TZ at 50% and 25% of their respective MIC for each strain. Lowest concentration of oxacillin that, in combination with CPZ or TZ at either 25% or 50% of their respective MICs, yielded no growth. NC means no change in the MIC of oxacillin. orig. MIC (original oxacillin MIC of strain).

Table III. *The effects of reserpine on the susceptibility of MRSA strains to oxacillin.*

	CFU counts					
	Oxa Cont.	Reserpine at 50 mg/L + Oxacillin at (mg/L)				
Strains	200	200	100	50	25	10
MRSA 4	$>1x10^9$	$7x10^5$	$3x10^7$	$1x10^9$	$1x10^9$	$1x10^9$
MRSA 5	$>1x10^9$	$5x10^7$	$1x10^9$	$1x10^9$	$1x10^9$	$1x10^9$

CFU (colony forming units) per ml of 16-hour cultures. Control cultures (no drugs present) yielded about 10^9 CFUs per ml of a 16-hour culture. Oxa Cont. (oxacillin at 200 mg/L and no reserpine). Higher concentrations of oxacillin alone yielded a similar number of CFUs as did the controls (no oxacillin and no reserpine) (data not shown).

Discussion

This study indicated that a sub-inhibitory concentration of thioridazine altered the susceptibility of methicillin-resistant *Staphylococcus aureus* to oxacillin, whereas it had no effect on the susceptibility of methicillin-sensitive strains to this antibiotic. With respect to the two fluoroquinolone-resistant strains, phenothiazine was able to markedly reduce the resistance to oxacillin. Reserpine, in a concentration-dependent manner, was also able to reduce the resistance of MRSA strains. The maximum reduction of resistance to oxacillin took place at relatively high concentrations of reserpine, although these concentrations alone had no effect on the replication of the bacteria. The lowest concentration

Table IV. *The effect of reserpine concentrations on the average sensitivity of MRSA strains to oxacillin.*

Reserpine (mg/L)	Range of MIC of oxacillin
No reserpine	>800
40	75-100
50	50-85
100	50-60
200	35-50
300	10-25
400	1-5

Each of the MRSA strains was assayed for the MIC of oxacillin in the absence and presence of increasing concentrations of reserpine. The MIC of oxacillin was defined as the concentration of oxacillin that yielded an optical density of 0.00 at 545 nm.

Table V. *The effect of the uncoupler of the electron motive force CCCP on the sensitivity of MRSA strains to oxacillin.*

Culture + oxacillin (mg/L)	Average of O.D. (545 nm)	% Difference from control
Oxa @ 0	0.899	0
Oxa @ 1.5	0.597	–34
Oxa @ 3.0	0.394	–56
Oxa @ 6.0	0.310	–75
Oxa @ 12.0	0.227	–79
Oxa @ 25.0	0.189	–82
Oxa @ 50.0	0.150	–85
Oxa @ 75.0	0.085	–92
Oxa @ 100	0.010	–99

Each of the MRSA strains was cultured for 16 hours at 37˚C in medium containing 0.1 mg/L of CCCP and increasing concentrations of oxacillin. The O.D. (optical density) at 545 nm was obtained for each concentration of oxacillin for each of the MRSA strains and the average O.D. determined.

of oxacillin that was effective when combined with 400 mg/L of reserpine was 1 mg/L. Because this combination yielded no CFUs, the activity of oxacillin may, in these cultures, be considered to be bactericidal. Unlike reserpine, verapamil, also an inhibitor of efflux pumps in general, did not alter the susceptibility of the MRSA strains to oxacillin, regardless of its concentration in the medium. Neither reserpine or verapamil enhanced the activity of oxacillin against MSSA strains.

Methicillin (or oxacillin) resistance has been shown to be due to a variant of penicillin-binding protein 2 (PBP2), which has been named PBP2a (43). PBPs are an integral part of the bacterial plasma membrane and exist at levels that are relatively consistent (44-46). Although the number of efflux pump units per bacterial cell is not yet known, it would be expected that the number of such units would be far lower than those for PBPs (*ca.* 2000 per bacterial cell). Given these considerations, one would normally assume that, regardless of whether an efflux pump were to be active for pumping out oxacillin, the activity of oxacillin against MSSA would be assured inasmuch as accessibility to the greater number of PBPs present in the plasma membrane would be practically assured. Therefore, it was not surprising that inhibitors of efflux pumps such as reserpine or verapamil would have an effect on the sensitivity of MSSA to oxacillin (data not shown). Furthermore, although CPZ did enhance the activity of a beta lactam against relatively insensitive Gram-negative bacteria (22), the enhanced effect was due to an increase in the penetration of the antibiotic through the cell envelope, thereby ensuring ready access to the existing PBP, and was not due to the inhibition of any efflux pump (22). This increased penetration of the beta lactam antibiotic appeared to be due to severe alterations of the outer cell wall proteins of the Gram-negative bacterium (47). Due to the absence of an outer cell membrane in Gram-positive bacteria such as *S. aureus*, the inability of TZ to enhance the activity of oxacillin against MSSA would be expected as observed.

The activity of efflux pump inhibitors on the susceptibility of MRSA to oxacillin was not anticipated inasmuch as PBP 2a should not have been affected regardless of the presence of efflux pumps. The fact that reserpine did markedly reduce the resistance of the MRSA strains to oxacillin from over 400 to 1 mg/L, in a concentration-dependent manner, and that this combination eliminated the presence of any CFUs in these cultures, may be explained by assuming that the inhibition of an efflux pump would allow the antibiotic to reach lethal targets beyond the plasma membrane inasmuch as the antibiotic at any of the concentrations tested was, at best, bacteriostatic when present alone in the culture. Nevertheless, the authors are unaware of any targets of oxacillin other than the PBPs.

The activity of TZ in combination with oxacillin against MRSA was similar to that present with combinations of reserpine. TZ appeared to be more active than reserpine, since the concentration of TZ needed to bring about a significant reduction of oxacillin resistance was 20 mg/L, as opposed to 400 mg/L for reserpine. Given the fact that TZ has a much lower molecular weight than reserpine (407 *vs.* 608), it is clear that TZ was far more effective than reserpine in reducing the oxacillin resistance of MRSA. Whenever studied, CPZ has been shown to inhibit efflux pumps (48), and we conclude that TZ has a similar activity and it is this activity which is responsible for the significant reduction in resistance to oxacillin of MRSA strains.

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