Abstract. Chlorpromazine (CPZ) is concentrated by human macrophages where it kills intracellular mycobacteria when the concentration outside the macrophage is sub-clinical. We have previously demonstrated that thioridazine (TZ), a much milder phenothiazine, has similar activity and kills intracellular methicillin-susceptible S. aureus at sub-clinical concentrations. We have extended this latter study to include methicillin-resistant S. aureus (MRSA) and show that TZ kills intracellular MRSA at clinically relevant concentrations. The ultrastructure of MRSA exposed to in vitro concentrations of TZ just below its MIC and that of MRSA phagocytosed by macrophages previously exposed to a clinically relevant concentration of TZ was also studied. TZ inhibits the replication of phagocytosed MRSA, affecting the structure of the cell envelope, resulting in lysis of the bacterium 6 hours post-phagocytosis. These ultrastructural changes are identical to those produced in vitro by a TZ concentration that is just below the MIC. Because macrophage intracellular MRSA is not killed by the macrophage and its intracellular location protects it from antibiotics that are unable to reach that site, recurrent infections which result may be successfully managed with the use of TZ.

The use and misuse of antibiotics have resulted in the worldwide development of antibiotic-resistant bacterial infections (1). The emergence of methicillin resistance of Staphylococcus aureus within a hospital setting is particularly problematic inasmuch as these nosocomial infections are now showing resistance to vancomycin, synergic or linezolid, the last resort armamentarium (2-5). The problem of methicillin-resistant S. aureus (MRSA) is more acute in Portugal than in the rest of Western Europe where its prevalence is ca. 50 to 60% of all staphylococcal infections (6, 7). Although nasal screening of the young healthy adult Portuguese population outside of a hospital setting indicates a low frequency of MRSA (8), MRSA infections in Portuguese hospitals will continue to be a problem and therefore the need for effective, non-toxic antibiotics for the management of these infections is critical.

Chlorpromazine (CPZ), a phenothiazine employed for the management of psychosis, has long been known to have in vitro activity against a wide gamut of bacteria (9-12). However, these in vitro activities require concentrations of the drug that are well beyond those clinically achievable (10). Nevertheless, sporadic reports have appeared over the years suggesting that clinical doses of CPZ are effective for the management of bacteremia (13-16). The in vivo activity is apparently the result of the drug being concentrated by macrophages that contain the phagocytosed organism (9, 11, 12, 17, 18). CPZ is concentrated by lysosomes (19, 20) to concentrations beyond those that are active in vitro and the fusion of these lysosomes with the phagosome containing the bacterium is the means by which the macrophage has been postulated to kill the bacterium (10).
The toxic side-effects of CPZ when chronically administered for the management of psychosis are well known (10, 17) and, even though the management of a bacterial infection with CPZ would take place over a much shorter length of time, its use as an antibacterial agent would meet with severe resistance. Thoridazine (TZ), the equal of CPZ as an effective neuroleptic agent, produces fewer and milder side-effects than CPZ (10, 17), with drowsiness being the most common. Of relevant importance is that TZ is the equal of CPZ with respect to its in vitro activity against the intracellular pathogens Mycobacterium tuberculosis (21, 22, 23) and S. aureus (24). However, the concentrations required are well beyond clinical relevance.

Concentrations of TZ well below those that are achieved in the plasma of TZ-treated patients have recently been shown to kill methicillin-sensitive S. aureus (MSSA) after it has been phagocytosed by macrophages that have low killing activity against this bacterium (25). This latter study has been expanded to evaluate the killing activity of TZ against MRSA that has been phagocytosed by macrophages, that have low killing activity against this bacterium, as well as to study any alterations of bacterial ultrastructure.

Materials and Methods

Materials. Chlorpromazine (CPZ), thoridazine (TZ), oxacillin (OXA) paraformaldehyde, sodium dodecylsulphate (SDS), triton X-100, sodium azide, napthol blue black, trypan blue, glutaraldehyde and osmium tetroxide were purchased from Sigma Aldrich Quimica SA, Madrid, Spain. Balanced salt solution (BSS), phosphate-buffered saline (PBS), Hank’s balanced salt solution (HBSS), RPMI medium, Ficoll and L-glutamine were purchased from Gibco, Paisley, United Kingdom. Trypticase Soy Broth (TSB) and Trypticase Soy Agar (TSA) were purchased from Difco Laboratories, Detroit, MI, USA. Microwell tissue culture plates were purchased from Nalgene, Rochester, NY, USA. All solutions of the phenothiazines were prepared in distilled, sterile water on the day of the experiment.

Bacterial strains. Three strains of oxacillin-methicillin-resistant Staphylococcus aureus (MRSA), maintained and characterised in our laboratory since that time. Human peripheral blood monocyte-derived macrophages (PBMDM) were isolated from healthy donors by the Ficoll sedimentation method previously described (25, 29).

Short-term cultures. THP-1 macrophage cell lines or PBMDM cell cultures containing 1.0 x 10^6 cells/ml were added in triplicate to 24-well or 96-well microplates, incubated overnight at 37°C with 5% CO2 and the wells washed with HBSS for the removal of non-adhered cells (25). Adhered cells were removed for counting with 1.0 ml of 0.01% SDS; the number of adherent cells of triplicate wells did not vary by more than 3%.

Toxic activity of TZ against THP-1 and PBMDM. Because CPZ has been shown to have toxicity against macrophages (30) as well as to inhibit phagocytic processes (31), TZ was tested for these in vitro activities against THP-1 and PBMDM by the Napthol Blue-Black and Trypan Blue methods and AnnexinV-binding method (Research & Development Systems, Abingdon, UK) by flow cytometry (Ortho-Clinical Diagnostics, Raritan, NJ, USA). The results obtained demonstrated that toxicity was produced by TZ when the concentration exceeded 0.5 mg/L (25).

Phagocytosis and killing activity of strains of MRSA by THP-1 and PBMDM. After 18 hours of culture of PBMDM (1.0x10^5 cells/ml) in RPMI medium containing 2mM L-glutamine and autologous human serum, approximately 10% of PBMDM that adhere to the bottom of the wells are monocytes (32). A bacterial suspension of 1.0x10^5 (1 CFU per macrophage ratio), that had been previously found to result in optimum phagocytosis (25), was added to monolayer cultures of monocyte-derived macrophages or THP-1 macrophage cell lines and incubated at 37°C for 30 minutes. Two consecutive washes with RPMI removed extra-cellular bacteria. The washings were then pooled and subjected to CFU counts in order to determine the efficiency of phagocytosis. A third wash was performed and subjected to CFU counts for the verification of the complete absence of non-phagocytosed bacteria. The adhered cells-
Phagocytosed bacteria cultures were incubated at 37°C for 0, 2, 4, 6 hours and lysed with the addition of 1.0 ml of 0.01% SDS at the end of each incubation period. Aliquots of 0.1 ml of the lysed cultures were subjected to CFU counting.

The effects of TZ on the killing activity of THP-1 and PBMDM. The addition of non-toxic concentrations of TZ to a macrophage culture prior to infection with S. aureus does not significantly affect the ability of the macrophage to phagocytose the bacteria (25). Macrophages were pre-pulsed with TZ at non-toxic concentrations of 0.01 and 0.1 mg/L for 1 hour (25). Controls received an equivalent volume of sterile water. S. aureus was then added to the macrophage cultures at 1CFU/macrohage ratio and the cultures incubated for 30 minutes at 37°C. Free, un-phagocytosed bacteria were removed by two consecutive gentle washings of the wells with HBSS and the cultures incubated for 0, 2, 4 and 6 hours at 37°C. At the end of each culture period, 0.01% SDS was added and the number of viable bacterial cells present in the supernatant of the lysed cultures was determined by CFU counts. All experiments were conducted in triplicate and performed at least twice. The data presented was obtained with the use of human PBMDM and is similar to that obtained from the use of THP-1 cells.

Figure 1. The average effect of chlorpromazine (CPZ) and thioridazine (TZ) on intracellular growth of the 3 MRSA strains tested. CPZ and TZ were separately added to cell cultures containing THP-1 or human PBMDM at concentrations of 0.01 and 0.1 mg/L. Control cultures received no drug. Control, CPZ- and TZ-containing cultures of THP-1 and human PBMDM were infected for 30 minutes with the methicillin-resistant clinical strains of S. aureus (MRSA) at concentrations of 1 CFU per macrophage (1:1). Results are expressed as the mean CFU of the 3 MRSA strains tested in 3 independent experiments. Data presented was obtained with the use of human PBMDM and is similar to that obtained from the use of THP-1 cells.
The effects of TZ on the ultrastructure of MRSA in vitro and after it has been phagocytosed by the human macrophage that had been previously exposed to TZ. The study of the effects of TZ on the ultrastructure of MRSA cultured for 18 hours in vitro, and after its phagocytosis by human macrophages pre-pulsed with a concentration of TZ of 0.1 mg/L, was conducted in parallel with those cultures described in the previous sections. The products of in vitro or macrophage-phagocytosed-containing cultures lacking or containing 0.1 mg/L of TZ, at intervals that ranged up to 6 hours, were centrifuged at 800 g for 30 minutes. The pellets were washed with phosphate-buffered saline pH 7.4 (PBS), centrifuged and re-suspended in 2.5% glutaraldehyde. After being maintained for 2 hours at 4°C, the pellets were centrifuged, washed twice with PBS, re-suspended in 2% osmium tetroxide and maintained at 4°C for 2 hours. After this time, they were centrifuged, washed with PBS, mixed with a small volume of melted 2.5% agarose and immediately chilled on ice. The solidified agarose was cut into blocks (about 1 mm³), stained en bloc for 1 hour with 1% uranyl acetate in 0.05 M sodium maleate buffer pH 5.2 at 4°C, dehydrated in a graded ethanol series, infiltrated and embedded in Spurr’s epoxy resin and polymerised for 24 hours at 70°C. For transmission electron microscopy (TEM), ultra-thin sections of 80 nm were obtained with the aid of an Ultracut S (Microtome, Leica, Vienna, Austria) using a diamond knife. The sections were post-stained with uranyl acetate and Reynolds’s lead citrate and viewed with a Zeiss EM 900 electron microscope (Carl Zeiss, Oberkochen, Germany).

Ethical permission. Blood samples from all healthy donors in Portugal were obtained after written permission had been granted by these donors. This study was conducted subsequent to approval by the ethical committee at the Instituto de Higiene e Medicina Tropical, Lisbon, Portugal.

Results

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CPZ and TZ against MRSA strains. The in vitro activity of CPZ and TZ against MRSA strains is summarised in Table I. Briefly, although CPZ and TZ have similar MICs against either strain of S. aureus, the activity of TZ is greater than that exhibited by CPZ. The MICs for CPZ and TZ against these S. aureus are consistent with those previously reported (24, 25, 27). The bactericidal activity of each of these compounds is expressed at a higher concentration of each compound and, again, the activity of TZ, as expressed by the MBC, is higher than that of CPZ although the difference is not statistically significant.

The intracellular killing activity of CPZ and TZ on MRSA strains. A minimal concentration of CPZ or TZ of 0.1 mg/L, as shown by Figure 1, after a slow initial effect (2 hours), consistently reduced the number of viable MRSA cells by the end of 4 hours after phagocytosis by human PBMDM.
Figure 3. The ultrastructure of MRSA 6 hours after it has been phagocytosed by the human macrophage (Controls). Note: Presence of normal morphology, evidence of cross-wall formation at different stages.

Figure 4. The ultrastructure of MRSA 4 hours after it has been phagocytosed by the human macrophage that had been previously exposed to a concentration of TZ of 0.1 mg/L. Note: Cross-walls are scarce and when present are bizarre (a), periphery of cell wall is eroded (b).
Enhanced intracellular killing activity continued to increase with time such that, by the end of 6 hours, killing was almost complete. Similar results were obtained with the use of the THP-1 cell line (data not shown). The lower concentration of 0.01 mg/L of either of these two phenothiazines had a marginal effect on the number of viable bacteria recovered 6 hours subsequent to phagocytosis. The concentration of 0.1 mg/L was confirmed in this study, and in previous studies, to be non-toxic to the macrophage (data not shown) and not to affect the phagocytic processes of this cell (25).

The effects of TZ on the ultrastructure of MRSA strains in vitro. The in vitro presence of a concentration of 20 mg/L of TZ, that is below that which totally inhibits the replication of MRSA, produced the typical ultrastructure of MRSA shown by Figure 2. Briefly, major alterations noted were: blebbing of the cell wall (a); thickening of the cell wall and cross-walls (b); partial separation of the internal portion of the cell wall (c); continued production of cross-walls without subsequent separation of the replicated cells (d); asymmetrical cross-wall formation (e); partial loss of cell wall (f); and lysis of the cell (g). Only (f) and (g) alterations are expected to ultimately result in cell death insomuch as even the most severe changes (a-e) are reversible when the organism is transferred to TZ-free medium (data not shown). All of the alterations noted have been previously reported for methicillin-susceptible Staphylococcus aureus exposed in vitro to sub-inhibitory concentrations of chlorpromazine (12).

The ultrastructure of MRSA that has been phagocytosed by macrophages previously exposed to a concentration of TZ of 0.1 mg/L. The typical ultrastructure of phagocytosed MRSA of a 6-hour culture control is presented in Figure 3 and is quite normal and identical to that previously shown for in vitro controls (12). There is ample evidence that cell division is taking place within the phagosome, as noted by the presence of cross-walls in different degrees of completion and almost complete separation of the two replicated cells from each other. It should be noted that, because the thickness of the section was very small (80 nm), many of the cells present in Figure 3 show only portions of the cell that are distal to the cross-wall.

In contrast to the ultrastructure of the 6-hour control population of intracellular MRSA, exposure of the human macrophage first to 0.1 mg/L of TZ followed by the phagocytosis of that same MRSA by the TZ-loaded macrophage results in the alteration of the ultrastructure of MRSA as early as 4 hours post-phagocytosis (Figure 4). Evidence of replication was scarce as noted by the absence of cross-walls in the majority of phagocytosed MRSA and, when cross-walls are present, they are thickened (a). The periphery of the cell wall appeared eroded (b). Further alteration of the ultrastructure of S. aureus was evident after 6 hours (Figure 5). It should be noted that MRSA phagocytosed by the macrophage that had been previously loaded with 0.1 mg/L of TZ showed an incomplete cell wall and, in some cases, an incomplete plasma membrane that allowed the spilling of the cytoplasm out of the bacterium. The presence of cross-walls in this population of intracellular MRSA was then quite rare suggesting that replication had been essentially inhibited.

Discussion

The main objections raised for the use of chlorpromazine as an antibacterial agent are: (i) its known toxicity when chronically employed and, (ii) its in vitro antibacterial activity which takes place at concentrations which are well beyond those achievable in the human. The results obtained in this study suggest that, as previously shown (9, 10, 17, 21-23, 26, 33), thioridazine has in vitro antibacterial activity equal to that of chlorpromazine. Furthermore, the current study also showed that a concentration of either phenothiazine corresponding to one easily achieved in patient plasma kills intracellular staphylococci regardless of their antibiotic susceptibility. Because the in vitro concentration of either phenothiazine needed to kill MRSA is of the order of 60 mg/L, and since both of these compounds are known to be concentrated by macrophages/tissues rich in macrophages (25), we hypothesise that the concentration of either drug needed to kill intracellular Staphylococcus is reached as a result of the macrophage’s ability to concentrate these compounds. This belief is further supported by the demonstration that, whereas a concentration of TZ near its MIC is needed to alter the morphology of MRSA in vitro, the altered morphology is reproduced in phagocytosed MRSA by a concentration of TZ in the cell culture medium that is 200 to 300 times lower.

Phenothiazines intercalate between nucleic bases of DNA (34) and result in inhibition of all DNA-based processes (35-37). The in vitro concentration of TZ needed to traverse the cell envelope of MRSA and reach DNA must be exceedingly greater than that present in the medium to which the macrophage-phagocytosed MRSA is exposed. We believe that the killing of intracellular staphylococci by TZ depends on the sufficiently high concentrations of drug needed for the penetration of the drug and its subsequent intercalation into the bacterium DNA. However, because of the killing activity of the macrophage, at least with respect to methicillin-resistant S. aureus, it may well be that the initial process of killing might first involve the action of macrophage-lysosomal enzymes on the cell wall of the bacterium making it more permeable to the phenothiazine. Thus, the concentrations achieved within the macrophage need not be equal to those corresponding to the in vitro MBC. Because the partial loss of the cell wall and plasma
membrane produced in vitro is identical to that present in MRSA that have been phagocytosed by the macrophage preloaded with TZ, we conclude that the TZ-concentrating effect inside the macrophage is the primary cause for the killing of MRSA.

MRSA strains account for a large number of deaths that result from nosocomial infections (1, 3, 4). The use of fluoroquinolones rapidly resulted in resistance of S. aureus (38). Furthermore, some of these quinolones were found to be toxic and subsequently removed from use (38). Vancomycin is the only really effective drug that remains and resistance to this compound is already taking place (2, 5). We believe that TZ has potential for the therapy of persistent and recurrent MRSA infections that result from the inability of the antibiotics to reach the bacterium after it has been phagocytosed (39). Because TZ has been in safe use for many decades, and its use as an anti-bacterial agent is anticipated to be for a shorter period of time than that employed for the psychotic disorder therapy, this regimen would be safe and effective for the management of persistent and recurrent intracellular infections. Since the number of lethal bacterial targets affected by the phenothiazine is large, resistance to thioridazine is anticipated to be low, if not, improbable (12, 17, 33). Nevertheless, clinical trials are strongly recommended.

Conclusion

The results presented in this study show that TZ kills intracellular MRSA at concentrations that are well within those clinically achieved for the management of psychosis. The effects of this agent on the ultrastructure of phagocytosed MRSA parallels that observed in vitro. Furthermore, because lysosomes are known to concentrate the phenothiazine and the phagocytosed organisms are clearly within cell organelles that have the characteristics of lysosome-phagosomes, it is postulated that it is in the lysosomal-phagosome structure that the microbe is killed. Support is provided for the contention that TZ has potential for the management of MRSA intracellular infections that are difficult to treat due to the privileged site offered by the macrophage, which can serve as sources for subsequent recurrent infections.

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References


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