Accepted Manuscript

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PII: S0924-8579(09)00465-8
DOI: doi:10.1016/j.ijantimicag.2009.09.027
Reference: ANTAGE 3154

To appear in: *International Journal of Antimicrobial Agents*

Received date: 29-9-2009
Accepted date: 30-9-2009

Please cite this article as: Dasgupta A, Mukherjee S, Chaki S, Dastidar SG, Hendricks O, Christensen JB, Kristiansen JE, Amaral L, Thioridazine protects the mouse from a virulent infection by *Salmonella enterica* serovar Typhimurium 74, *International Journal of Antimicrobial Agents* (2008), doi:10.1016/j.ijantimicag.2009.09.027

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Thioridazine protects the mouse from a virulent infection by *Salmonella enterica* serovar Typhimurium 74

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ARTICLE INFO

Article history:

Received 29 September 2009

Accepted 30 September 2009

Keywords:

Thioridazine

Protection

Virulent infection

Salmonella Typhimurium 74

Two-step regulon

PmrA/B

55 kDa virulence protein

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ABSTRACT

When administered to mice at doses of 100 µg/mouse and 200 µg/mouse, thioridazine (TDZ) significantly protected animals from the lethality produced by a virulent strain of *Salmonella enterica* serovar Typhimurium and reduced the number of bacteria retrieved from the spleen, liver and heart blood. The protection conferred by TDZ against a virulent *Salmonella* infection is hypothesised to be due a reduction in the 55 kDa virulence protein of the outer membrane of the organism, as this protein is almost totally absent when the organism is exposed to the phenothiazine. It is further hypothesised that the reduction in the 55 kDa virulence factor renders the organism susceptible to the action of hydrolytic enzymes of the neutrophil phagolysosome, whereas in the absence of exposure to TDZ intracellular ingestion and localisation of the phagocytosed bacterium does not result in killing owing to rapid induction of the two-step PmrA/B regulon that results in the eventual synthesis and insertion of lipid A into the nascent lipopolysaccharide layer of the outer membrane.

1. Introduction

The therapeutic effectiveness of antibiotics and antibacterial chemotherapeutics is becoming more and more limited due to increased emergence of resistance, especially in Gram-negative bacteria. The need for new antimicrobial agents is consensually accepted, however it is anticipated that regardless of how recently the agent is introduced, resistance to that agent will soon occur. The search for newer antimicrobials has prompted studies demonstrating that many medicinal compounds belonging to various pharmacological groups, such as tranquilisers, antihypertensives, antipsychotics, anti-inflammatory agents, cardiovascular drugs, proton pump inhibitors and even antispasmodic agents, have remarkable antimicrobial activities and have been named 'non-antibiotics' [1–3].

Thioridazine (TDZ) belongs to the class of phenothiazines and was originally introduced in 1959 as an antipsychotic drug. Because it has been shown to have antimicrobial activity against many bacteria, it is now recognised as a possible antimicrobial agent [2]. However, the in vitro activities of TDZ occur at concentrations that are clinically irrelevant as they exceed the maximum concentration that can be achieved in humans (0.5 mg/L). Phenothiazines such as chlorpromazine inhibit the growth of *Salmonella* during the first 8 h of in vitro exposure to the agent and during this period the 55 kDa virulence protein of the outer membrane is markedly decreased [4]. This study therefore suggests that early exposure to a phenothiazine such as TDZ may cause elimination of the

virulence factor, rendering the organism susceptible to the immune system of the infected host.

The ability of *Salmonella* to cause infection in humans is due in part to its ability to survive within the phagolysosome of the neutrophil [5]. Survival within the phagolysosomal compartment results from activation of the two-step PmrA/B regulon, an operon that involves at least nine genes resulting in the synthesis and insertion of lipid A into the nascent lipopolysaccharide layer of the cell envelope [5]. These responses render the organism resistant to enzymatic digestion, to other antimicrobial proteins as well as to many antibiotics [5].

Mycobacterium tuberculosis [6] and *Staphylococcus aureus* [7] are able to survive the phagolysosomal environment of the human non-killing macrophage, the former for decades [6]. However, whereas the mechanism of survival by intraphagosomal *Salmonella* in neutrophils is due to the aforementioned genetic responses within an acidic milieu, the mechanism by which survival of *M. tuberculosis* takes place is due to the absence of acidification of the lysosome resulting from efflux of K^+ from that structure [8]. However, the killing of intracellular mycobacteria [6] as well as that of intracellular staphylococci [7] by non-killing human macrophages can be enhanced by TDZ, supposedly by inhibition of K^+ efflux from the phagolysosomal unit containing the respective bacteria [8]. It is this mechanism that is believed to be the means by which TDZ cures mice of antibiotic-susceptible *M. tuberculosis* infection [9].

Similar to the case with chlorpromazine [4], in vitro exposure of *Salmonella* strains to TDZ also inhibits growth of the organism during the first 6–8 h as well as promoting disappearance of the 55 kDa virulence protein. Because TDZ also cures the mouse of intracellular infection such as that produced by *M. tuberculosis* [9], we have studied the question of whether administration of TDZ may affect the course of a highly virulent *Salmonella* infection in mice.

2. Materials and methods

2.1. Bacteria

Highly virulent *Salmonella enterica* serovar Typhimurium 74 was grown overnight at 37 °C in brain–heart infusion medium from Oxoid Ltd. (distributor, Fisher Scientific, Mumbai, India).

2.2. Determination of the minimum inhibitory concentration (MIC) of thioridazine

TDZ was obtained in pure dry powder from Sigma (Copenhagen, Denmark). A stock solution was prepared by dissolving 25 mg of TDZ in 5 mL of 5% dimethyl sulfoxide (DMSO), resulting in a concentration of TDZ of 5 mg/mL. This stock solution was sterilised by filtration through a G-5 sintered glass filter and stored at 4 °C. Determination of the MIC was conducted by the broth dilution method according to Clinical and Laboratory Standards Institute guidelines as described previously [10].

2.3. Animal experiments

In vivo experiments were performed with male Swiss white mice, each weighing 18–20 g. Animals were maintained under standard conditions of temperature (21 ± 1 °C) and relative humidity (50–60%) with a photoperiod of 14:10 h of light:dark. Water and a dry pellet diet were provided ad libitum.

Determination of the virulence of infection produced by *S. Typhimurium* 74 has been previously described in detail [11]. Briefly, five groups of mice consisting of five animals each were injected with increasing concentrations of *S. Typhimurium* 74 and the number of mice that died was recorded for up to 100 h.

2.4. Determination of a protective effect of thioridazine from a virulent *Salmonella* infection

Three groups of mice (40 mice in the control group and 20 mice each in the experimental groups) were injected intraperitoneally with 0.1 mL of sterile saline containing 0.0, 100 and 200 µg of TDZ, respectively. After 3 h, all the animals were infected with *S. Typhimurium* 74 as previously described in detail [11]. The protective capacity of TDZ was determined by recording the mortality of mice in the different groups up to 100 h after the challenge. Statistical analyses were performed by the χ^2 test.

2.5. Determination of the effect of thioridazine treatment on colony-forming units (CFU) of Salmonella retrieved from the liver, spleen and heart blood of mice infected with Salmonella Typhimurium 74

In another experiment, four groups of five mice each were treated as follows: Groups 1 and 2 were administered 200 µg of TDZ; and Groups 3 and 4 received 0.1 mL of sterile saline. After 3 h, all the mice were injected with the number of *Salmonella* corresponding to a 50% lethal dose (LD₅₀) as calculated from the *Salmonella*-infected control group of the first set of experiments to determine the virulence of the infection. After 2 h and 4 h, mice in each of the four groups were sacrificed by cervical dislocation and their spleens and livers were aseptically removed; heart blood was taken directly from the heart via microsyringe for determination of CFU. The livers and spleens were homogenised in 1.0 mL of sterile saline with the aid of a tissue homogeniser maintained at 4 °C and aliquots of the homogenate were processed for CFU counts. Statistical analyses of these in vivo data were carried out by Student's *t*-test.

3. Results

The MIC of TDZ against *S. Typhimurium* 74 as determined from three separate broth dilution assays was 500 µg/mL.

The virulence of the infection produced by varying CFU of *S. Typhimurium* 74 is summarised in Table 1. Briefly, as the number of CFU of *S. Typhimurium* 74

injected intraperitoneally into mice increases, the percent mortality increases, reaching 100% with a dose of 2.2×10^8 CFU.

As shown in Table 2, the protective effect conferred by TDZ against the virulence of an *S. Typhimurium* 74 infection was complete at the highest dose level of TDZ administered (200 µg/mouse), as none of the animals died during the 100 h post infection. In contrast to the protective effect provided by TDZ at either dose administered, 34 (85%) of the 40 mice that received saline in place of TDZ died within 100 h. We may conclude that since none of the mice treated with 200 µg of TDZ died, TDZ does not produce any lethal toxicity, at least with 200 µg of the agent, as also shown by others employing similar concentrations of the agent [9].

The effects of TDZ on the CFUs of *S. Typhimurium* 74 retrieved from the liver, spleen and heart blood are summarised in Fig. 1. Briefly, treatment of mice acutely infected with *S. Typhimurium* 74 significantly decreases equally the number of CFUs of the bacterium retrieved from the sources evaluated, but only after 4 h. These results show that within a few hours following infection, the bacterial load of the heart blood, spleen and liver is reduced to more than one-half of that present in the corresponding sources of the untreated mouse.

4. Discussion

The results obtained in this study show that administration of TDZ protects the mouse from the lethality produced by a virulent *Salmonella* infection. Moreover,

the effects of the agent are evident within a few hours after the agent is administered given the marked and significant reduction in the number of CFUs of the organism retrieved from acutely infected mice. TDZ was devoid of any lethal toxicity since none of the 200 μ g TDZ-treated mice died during the 100 h of the experiment.

Introduction of *Salmonella* via the intraperitoneal route should very quickly result in their phagocytosis by neutrophils [5]. However, the organism survives the activity of hydrolases owing to induction of the two-step regulon PmrA/B that leads to resistance to the action of hydrolases [5]. As shown by this study, the MIC of TDZ against the virulent *S. Typhimurium* 74 is 500 μ g/mL and, if we assume that the weight of the mouse is equivalent to the weight of water, the concentration of TDZ in a mouse treated with 200 μ g of the agent would be more or less equal to 10 μ g/mL, which is 50-fold lower than the MIC. Therefore, why does such a low concentration of TDZ not only protect the mouse for 100 h from a virulent infection by *S. Typhimurium* 74 but also markedly reduces the number of CFU retrieved from sources expected to harbour the organism? Because phenothiazines are concentrated by macrophages as much as 100-fold over the concentration of the agent in the medium in which macrophages are maintained [7,8], and because the concentration takes place in the lysosome [3], one would suggest that this concentration effect produces a level of agent within the lysosome that is equal to the concentration that is bactericidal. However, given the fact that the phenothiazine may promote loss of the 55 kDa virulence protein

[4], it is possible that what has taken place within the phagolysosome is a significant reduction of virulence of the organism and, consequently, although the presence of the infective agent continues as expected from the very high number of CFU retrieved from sources of the infected animal, the lethality associated with virulence is obviated. Nevertheless, until there is a demonstration that the virulence protein of the phagocytosed *S. Typhimurium* 74 is lost after exposure to the phenothiazine, the mechanism by which the phenothiazine, and possibly other phenothiazines, protects the infected mouse from a lethal *Salmonella* infection remains highly speculative. Lastly, although we do not recommend that TDZ be used for the therapy of *Salmonella* infections, we do suggest that this agent be exploited as a 'lead compound' for the creation of new antimicrobial agents with significant activity against virulent *Salmonella* infections.

Funding

None.

Competing interests

None declared.

Ethical approval

The guidelines of the University of Kolkata (India) for the care and use of laboratory animals were strictly followed.

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Fig. 1. Effects of thioridazine (Th) on colony-forming units (CFU) of *Salmonella enterica* serovar Typhimurium 74 in organs of acutely infected mice. * CFU counts between two groups significantly different ($P < 0.001$). No significant difference in CFU was noted after 2 h (data not shown).

Table 1

Determination of the lethal dose of *Salmonella enterica* serovar Typhimurium

NCTC 74

Group	Inoculum (CFU)	Mortality [<i>n</i> / <i>N</i> (%)]
1	2.2×10^8	5/5 (100)
2	1.9×10^7	2/5 (40)
3	3.5×10^6	1/5 (20)
4	2.6×10^5	1/5 (20)
5	1.0×10^4	0/5 (0)

CFU, colony-forming units.

Table 2

Effect of thioridazine (TDZ) on survival of mice challenged with *Salmonella enterica* serovar Typhimurium ^a

Control group (not receiving TDZ)			Test groups (receiving TDZ)		
Saline (mL/mouse)	No. of mice died (<i>N</i> = 40)	% Mortality	TDZ (μg/mouse)	No. of mice died (<i>N</i> = 20)	% Survived
0.1	34	85	100	8	60
			200 *	0	100

^a Mice received a challenge of 0.95×10^9 colony-forming units of *S. Typhimurium* NCTC 74 in 0.5 mL of brain–heart infusion medium.

* $P < 0.001$ vs. controls (χ^2 test).