A comparative analysis of In vitro and In vivo efficacies of the enantiomers of thioridazine and its racemate

Christensen, Jørn Bolstad; Hendricks, Oliver; Chaki, Shawasti; Mukherjee, Sayanti; Das, Ayan; Pal, Tapan K.; Dastidar, Sujata G.; Kristiansen, Jette E.

Published in:
P L o S One

DOI:
10.1371/journal.pone.0057493

Publication date:
2013

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
A comparative Analysis of In Vitro and In Vivo Efficacies of the Enantiomers of Thioridazine and Its Racemate

Jørn B. Christensen¹, Oliver Hendricks², Shaswati Chaki³, Sayanti Mukherjee³, Ayan Das⁴, Tapan K. Pal⁴, Sujata G. Dastidar³, Jette E. Kristiansen⁵*

¹ Department of Chemistry, University of Copenhagen, Copenhagen, Denmark, ² King Christian X Hospital for Rheumatic Diseases, University of Southern Denmark, Gråsten, Denmark, ³ Department of Microbiology, Herbicure Healthcare Bio-Herbal Research Foundation, Kolkata, India, ⁴ Bioequivalence Study Center, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India, ⁵ Memphys Center for Biomembrane Physics, Department of Physics and Chemistry Odense, Denmark

Abstract

A long list of chemotherapeutical drugs used in the treatment of the peripheral and the central nervous systems possess anti-microbial activity. Some of these neurotropic compounds are chiral, with the one stereoisomeric form exaggerating reduced neurotropism. This is the case for the levorotatory form of thioridazine. The phenothiazine thioridazine is an interesting compound, characterized by exhibiting a significant growth inhibiting activity on a wide array of microorganisms. Thioridazine is characterized by another challenging feature, because the compound is concentrated in certain human tissue cells. The present study describes a comparative study of the two enantiomers as well as the racemic form of thioridazine. The study exploits the stereochemical aspect and the in vitro and in vivo potential of these compounds, with a focus on the effects on Gram negative organism Salmonella enterica serovar Typhimurium. In summary, the results of this study yielded a significant antibacterial activity of all forms of thioridazine, indicating the levorotatory (–)-form to be superior in terms of both its in vitro and in vivo efficacies.

Introduction

An antibiotic may be defined as a product from a microorganism capable of inhibiting the growth of another microorganism at distinctly low levels. The chemotherapeutics, on the other hand, are primarily synthetic compounds that are able to act on microorganisms in a very similar manner, but at much higher concentrations. It is now known that both antibiotics and antibacterial chemotherapeutics have lost the battle to a large extent in the fight against multidrug resistant (MDR) bacterial pathogens. However, intensive studies by various groups of scientists throughout the world have revealed that there are medicinal compounds used for the therapy of non-infectious pathology possess distinct antimicrobial properties [1–13]. These compounds are termed as non-antibiotics [10]. Non-antibiotics exhibit properties that render them important for the therapy of different MDR infections. Phenothiazines being one of the most important group of non-antibiotics have been studied extensively for their antimicrobial potentiality [1–13,14]. These non-antibiotics possess most of the characteristics of antibiotics and their antibacterial action can be further potentiated by suitable combinations [15–18].

The phenothiazine thioridazine (Tz) is a unique non-antibiotic which is highly bactericidal for Gram positive bacteria and acts as a bacteriostatic agent against Gram negative organisms [19].

Thioridazine is chiral and previous studies have reported that the levorotatory form (–) thioridazine is concentrated in human tissue cells at higher levels than the dextrorotatory form (+) [20]. Furthermore the (–) form of Tz has been reported to have less challenging pharmacodynamic activity, e.g. reduced blocking activity on centrally located dopamine D2-receptors than the (+)-form [21]. Several in vitro studies have shown another feature of racemic Tz.

The compound is concentrated in human macrophages and different tissue types, such as pulmonary epithelial cells. Racemic Tz has a great potentiality for the therapy of MDR-tuberculosis since this compound is concentrated 100-fold in the human macrophages where the tubercle bacilli multiply and remain viable and where antibiotics fail to enter [22]. Furthermore racemic Tz has been shown to possess the capacity to lower the invasion ability of Gram positive and Gram negative bacteria in human epithelial cell lines [23]. Moreover racemic Tz proved to be highly efficient in disintegrating the invading cells of Salmonella enterica serovar Typhimurium in mice at rather low levels [21]. The present study aims to define the specific antibacterial properties of the enantiomeric forms of thioridazine, e.g. the racemic, the (+)- and the (–)- compounds and clarify, whether there is a difference in the efficacy of the drug based on its stereoisomeric profile. In order to achieve this goal, we performed comparative in vitro and in vivo studies with two enantiomers along with the racemic compound available commercially (Sigma Chemicals, Denmark).

Materials and Methods

Bacteria

A total of 55 different bacteria belonging to both Gram positive and Gram negative types were taken for this study (Table 1).
Drugs
Racemic thioridazine (Sigma Chemicals, Denmark). The two enantiomers of thioridazine were prepared according to the procedure of Bourquin et al [22].

Table 1. Minimum inhibitory concentration (MIC) of three optical forms of Tz with respect to different bacteria.

<table>
<thead>
<tr>
<th>BACTERIA</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Racemic (+)</td>
</tr>
<tr>
<td>S. aureus NCTC 6571, V. cholerae 569B, V. cholerae 1023</td>
<td>25</td>
</tr>
<tr>
<td>S. aureus NCTC 8530, S. aureus ATCC 25923, S. dysenteriae 7 NCTC 519, Sh. boydii NCTC 254, V.cholerae ATCC 14033, ATCC 14035, V. cholerae DN7</td>
<td>50</td>
</tr>
<tr>
<td>Sh. flexneri 4a NCTC 24, Sh. sonnei NCTC 9774, V.cholerae 713, 820</td>
<td>100</td>
</tr>
<tr>
<td>S. aureus ML 16, ML 152, ML 329, ML 358, S. typhi NCTC 59, S. choleraesuis NCTC 36, 37, L. monocytogenes NCTC 7973, NCTC 10351, NCTC 11994</td>
<td>200</td>
</tr>
<tr>
<td>B. subtilis ATCC 6633, B. pumilus NCTC 8241, S. aureus ML 266, ML 358, ML 422, E. coli K12 Row, E. coli C600, S. berta NCTC 69, S. abony NCTC 6017</td>
<td>200</td>
</tr>
<tr>
<td>B. polymyx NCTC 4747, B. licheniformis NCTC 10341, S. London NCTC 76, S. enterica serovar Typhimurium NCTC 11, NCTC 74</td>
<td>500</td>
</tr>
<tr>
<td>S. aureus ML 277, V. cholerae 137/62</td>
<td>1000</td>
</tr>
<tr>
<td>K. pneumoniae ATCC 10031, K. oxytoca ATCC 130988</td>
<td>2000</td>
</tr>
<tr>
<td>L. monocytogenes AMRI 3, A. boumannii KPC 470, 517, P. aeruginosa ATCC 27853, ATCC 25619, C/1/S, K/8/89, BVC 1,2,3,4,5, APC1.</td>
<td>&gt;2000</td>
</tr>
</tbody>
</table>

Figure 1. The spectrophotometric scanning results show that all four compounds had the same λ\text{max} (264 nm). HPLC result: The chromatogram of the four different samples having the same retention time about 4.6 mins indicating that the compounds are identical except for chirality.

doi:10.1371/journal.pone.0057493.t001

doi:10.1371/journal.pone.0057493.g001

Media
Liquid media were nutrient broth (NB; Oxoid), peptone water (PW) containing 1.0% peptone (Oxoid) and 0.5% NaCl and Mueller Hinton broth (MHB, Oxoid). Solid media were nutrient
agar (NA, Oxoid), brain heart infusion agar (BHA), Oxoid and Mueller Hinton agar (MHA), Oxoid); pH was always maintained at 7.2 to 7.4.

Inoculum
Each bacterium was grown in NA/MHA at 37°C, harvested at stationary phase and suspended in 5 ml sterile distilled water. Turbidity of each suspension was matched against 0.5 McFarland standard [14] along with a spectrophotometer at 625 nm corresponding to $2.4 \times 10^5$ colony forming unit (CFU)/ml.

Determination of Minimum Inhibitory Concentration (MIC) of the Enantiomers and the Racemate of Tz
This was carried out according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) [24] by spotting $10^5$ CFU contained in a 2 mm loop from diluted 18 h broth cultures on plates containing 0 (control), 50, 100, 200, 500, 1000 and 2000 mg/ml of a drug. The plates were incubated at 37°C, observed for appearance of growth after 24 h and again after 72 h.

Spectrophotometry
A stock solution containing 1 mg/ml of thioridazine HCl (Sigma), racemic mixture of thioridazine HCl, dextrorotatory thioridazine HCl (+) and levorotatory thioridazine HCl (−) were prepared by dissolving these separately in methanol. Each stock solution was further diluted to 10μg/ml of methanol. Aliquots of this solution were taken in a quartz cell and scanned for $\lambda_{max}$ in the range of 200–600 nm using methanol as the blank in a double beam UV spectrophotometer (Jasco-V630). The maximum absorbance was determined by using Spectra manager (Version-2.05.03).

Animal Experiments
Swiss albino male mice each weighing 18–20 gm were selected for this work. This study was approved by the Institutional Animal Ethics Committee (IAEC) of Jadavpur University and TAAB Biostudy Services. Animals were maintained under standard conditions of temperature (24±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. The animals were checked regularly for their health and diet according to the rules and guidelines set by the Ethical Committee at definite intervals of time of 12 hr at 8 A.M. in the morning and 8 P.M. in the evening. The animals which showed symptoms of illness were carefully observed, identified and were separated from the healthy ones. These animals were not included in our experiments and were given proper treatment.

The intensity of virulence of infection caused by *Salmonella enterica* serovar Typhimurium 74 and the median lethal dose (MLD or LD_{50}) of the mouse-passaged strain was as described earlier.

Figure 2. The chromatogram showing that all four different samples had the same retention time (about 4.6 min).

doi:10.1371/journal.pone.0057493.g002

HPLC Parameter
- **Name of HPLC:** Jasco.
- **Column:** C8, 250×4.6 mm, 5 μ particle size.
- **Flow Rate:** 1.0 ml/min.
- **Loop size:** 50 μ lit.
- **UV Absorption:** 264 nm.
- **Mobile Phase:** Methanol: Water containing 0.1% v/v phosphoric acid.
- **Run Time:** 8 mins.
- **Software used:** Clarity lite (Version: 2.6.4.402)

All the samples were prepared as per the above method and each sample was diluted by methanol (HPLC Grade). Water was used for the analysis was MilliQ water.
[14,25]. Protective efficacies of the forms of Tz in mice infected with virulent S. enterica were carried out as described below. Four groups of animals with 60 mice in the control group and 20 mice each in the 6 experimental groups were taken. The control group received 0.1 ml of sterile saline while for each form of Tz there were 40 animals, in which 20 mice received 100 µg and the other 20 received 200 µg of the drug. After 3 h all the animals were infected with 50 MLD of virulent S. enterica 74 as described [25]. Protective capacity of all 3 forms of Tz was determined by recording the mortality of mice in the different groups up to 100 h after the challenge. The animals which survived after 100 h of infection were euthanized with the help of cervical dislocation as suggested in the Ethical Committee. The end point for performing euthanasia was observation up to 100 h with regular monitoring at 12 h intervals. The animals which were euthanized prior to 100 h were based on the condition of their health. Generally within 72 h if they showed severe signs of illness, for example loss of appetite, weight loss, lack of movement, breathlessness, shivering etc. they were euthanased as advised by the veterinary doctor in the Ethical Committee. The number of animals euthanased prior to 100 h varied from 2–7 in each group. Assessment of the animals euthanased prior to 100 h was monitored by the veterinary doctor after every 12 h as mentioned 8 AM in the morning and 8 PM in the evening.

### Table 2. Effects of 3 forms of thioridazine (Tz) [racemic, (+), (−)] on survival of mice challenged with Salmonella enterica serovar Typhimurium NCTC 744.

<table>
<thead>
<tr>
<th>Control group (not receiving Tz)</th>
<th>Test groups (receiving Tz)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saline (ml/mouse)</strong>: (N = 60)</td>
<td><strong>No. of mice died</strong></td>
</tr>
<tr>
<td>0.1</td>
<td>52</td>
</tr>
<tr>
<td>(+)</td>
<td>100</td>
</tr>
<tr>
<td>(−)</td>
<td>100</td>
</tr>
</tbody>
</table>

*Mice received a challenge of 0.95×10⁹ colony-forming units of S. enterica NCTC 74 in 0.5 ml of brain-heart infusion medium.
*p<0.001 vs. controls (χ² test).

### Results

**Bacterial Inhibitory Spectra of 3 Different Forms of Tz**

A total of 55 different Gram positive and Gram negative bacteria when tested against the 3 forms of Tz, racemic, (+) and (−), it was found that S. aureus NCTC 6571, V. cholerae 569B, 1023 could be inhibited at 25 µg/ml of each agent (Table 1). Among others it was found that strains of S. aureus, V. cholerae and shigellae were also sensitive to these agents MIC of racemic, (+) and (−) forms of Tz produced almost identical type of inhibition in such organisms. However, (+) variety was less inhibitory than the other two. Strains of S. enterica serovar Typhimurium were inhibited at 500 µg/ml of all the compounds. L. monocytogenes NCTC 7973, NCTC 10351, NCTC 11994 were inhibited at 200 µg/ml of racemic and (+) forms and at 100 µg/ml of (−) form. The strains of

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>CFU/ml³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sera</td>
<td>Heart blood</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.1 ml of a sterile saline</td>
<td>1.8×10⁶ to 5.9×10⁶</td>
</tr>
<tr>
<td>I</td>
<td>200 µg of Racemic form</td>
<td>9.0×10⁶ to 6.6×10⁶</td>
</tr>
<tr>
<td>II</td>
<td>200 µg of (+) form</td>
<td>3.6×10⁶ to 9.6×10⁵</td>
</tr>
<tr>
<td>III</td>
<td>200 µg of (−) form</td>
<td>1.0×10⁷ to 8.0×10⁵</td>
</tr>
</tbody>
</table>

³Mice received a challenge of 0.95×10⁹ colony-forming units of S. enterica NCTC 74 in 0.5 ml of brain-heart infusion medium.
*p<0.001 vs. controls (t-test).

doi:10.1371/journal.pone.0057493.t002

doi:10.1371/journal.pone.0057493.t003
Enantiomers of Thioridazine In Vitro and In Vivo

klesbiellae, P. aeruginosa and L. monocytogenes AMRI 3 were highly resistant to all the compounds.

**UV Spectrophotometer analysis.** Maximum absorbance ($\lambda_{max}$) of the 3 forms of Tz and the reference thioridazine (Sigma) were found at 284–311 nm. All the four analytes had the same absorbance as well as identical absorption spectra (Fig. 1).

**HPLC analysis.** The chromatogram of the three forms of Tz and the reference Tz from Sigma had the same retention time (4.6 min). All the chromatograms were identical showing peaks at the same concentration (Fig. 2).

**In vivo Experiments**

Virulence of the infection produced by S. enterica NCTC 74 is being presented in Table 2. In a control group of 60 mice that received only the challenge the mortality was 86.7%. As the number of CFU of S. enterica 74 injected intraperitoneally into mice increased, the % mortality increased, becoming 100% with a dose of 0.95 x 10^6 CFU (Table 2). The protective capacity offered by the 3 different forms of Tz shows that there was 100% survival with 200µg/mouse dose with the (−) form. With the (+) variety the survival was 70% with 200 µg/mouse dose. However, racemic proved to be better than (+) as there was 95% survival with 200µg/mouse dose (Table 2). It may be mentioned here that the animals that received (−) form went to sleep within a few minutes after intraperitoneal injections. Animals of the other two forms went to sleep after 30–40 minutes of intraperitoneal injection of the compounds.

The tests on the 3 forms of Tz in mice infected with S. enterica 74 revealed that 5 animals which received only saline and challenge had >10^5 live cells in liver, spleen and blood after 18 hr infection. However, the number of CFUs in all the organs were between 10^3 and 10^4, being much less in the test batches of mice that received one of the 3 drugs along with challenge. The data were statistically significant (Table 3).

**Discussion**

Results obtained in the present study show that racemic, (+) and (−) forms of Tz did not have much difference in their in vitro action, only except that (−) form had shown slightly better inhibitory activity than the other two. Standard strains of L. monocytogenes, e.g. NCTC 7973, 10531, 11994 could be inhibited at 500 µg/ml of the 3 forms of Tz, while L. monocytogenes AMRI 3 that was isolated from an acute systemic infection in Kolkata was highly resistant to all the drugs. Both the spectrophotometric and HPLC studies carried out with the 3 forms along with standard thioridazine from Sigma Chemicals, Denmark showed that there was no difference in the $\lambda_{max}$ and that absorption spectra were identical.

This study further revealed that administration of any form of Tz successfully protected the mice infected with virulent S. enterica from lethality. The protection offered by the drugs were also statistically significant as evidenced by the reduction in the viable cell count in the organs of infected mice compared to the animals that were not administered any drug.

Intraperitoneal infection by S. enterica in mice is likely to cause phagocytosis by neutrophils [24]. According to Gunn [26] salmonellae can efficiently resist the action of hydrolases due to the action of PmrA/B regulon responsible for inactivation of hydrolases. The MIC of all the compounds with respect to S. enterica 74 was 500 µg/ml and is equivalent to weight of water. The amount of Tz forms in a mouse receiving 200 µg dose each would be equivalent to 10 µg/ml, which is one-twentieth of the actual MIC value. Such a distinct protection by Tz forms in mice may be explained by the studies of Ordway et al [27]. Since these authors could demonstrate that phenothiazines get concentrated 100 fold inside macrophages maintained in a suitable medium, it may be possible that the concentration takes place inside the lysosome leading to rupture of bacterial cell wall. Furthermore the phenothiazines are known to promote loss of 55 kD protein [28], there may have been a significant reduction of virulence of bacterial cells in the phagolysosome and hence the lethality might have diminished distinctly. In absence of a direct proof regarding the actual mechanism of action of the different forms of Tz, the protection offered by these compounds remains an assumption. Although it may not be possible to recommend Tz alone against bacterial infections on the basis of our observation in the present study, it may be suggested that structural modifications of the original Tz molecule may open up an avenue on the possibilities of producing highly potent protective antibacterial agents in course of time.

**Author Contributions**

Conceived and designed the experiments: JBC OH SGD JEK. Performed the experiments: SC SM AD TKP. Analyzed the data; SC SM AD TKP SGD. Contributed reagents/materials/analysis tools: JBC OH SGD JEK. Wrote the paper: JBC OH SGD JEK.

**References**