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In vitro and *in vivo* toxicity evaluation of non-neuroleptic phenothiazines, antitubercular drug candidates

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ABSTRACT

The phenothiazine-derived antipsychotic drugs, such as chlorpromazine and thioridazine, are bactericidal against drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis*, but produce undesirable side effects at clinically relevant doses. We have previously modified four novel phenothiazines and maintained their antimycobacterial activity. This study evaluated the pharmacological and toxicity profiles of these novel non-neuroleptic phenothiazines, PTZ3, PTZ4, PTZ31 and PTZ32, for their metabolic stability, kinetic solubility and potential cytotoxic effects *in vitro*. To further support the safe use of these drug candidates, the *in vivo* pharmacological and toxicity profiles were assessed in C57BL/6 mice via single or repeated oral gavage. In acute toxicity studies, all four modified phenothiazines showed favourable safety in mice. When treated daily with 100 mg/kg of PTZ3 and PTZ4 for 2 weeks, mice displayed no signs of toxicity. Alternatively, treatment with PTZ31 resulted in 20% mortality with no toxicity evident in biochemical or histological analysis, while exposure to PTZ32 resulted in a 45% survival with increased serum concentrations of uric acid and alkaline phosphatase. The combined non-neuroleptic and antimycobacterial effects of the novel phenothiazines PTZ3, PTZ4, PTZ31 and PTZ32 demonstrated favourable pharmacological and toxicity profiles in this study, highlight the potential of these compounds as suitable anti-tuberculosis drug candidates.

1. Introduction

Tuberculosis (TB), typically a pulmonary infection caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), remains one of the top 10 global health issues. In 2017, there were 10 million new TB cases and 1.3 million associated deaths worldwide. Of patients newly diagnosed with TB, 9% were human immunodeficiency virus (HIV) positive (World Health Organization, 2018). HIV compromises the host immune system, thereby significantly reducing the survival rate of TB patients and increasing the risk of latent TB reactivation (Pawlowski et al., 2012). The clinical treatment required for TB-HIV co-infection adds to the current challenges of TB therapy and drug development. The combined treatment of TB and HIV increases adherence problems in addition to overlapping toxicity profiles of the anti-retroviral and anti-tuberculosis drugs (van den Boogaard et al., 2009). Also, drug-drug

interactions may lead to sub-therapeutic concentrations resulting in failure to achieve optimal treatment outcomes (Koul et al., 2011).

The World Health Organization recommends treatment for drug-sensitive TB as a daily regimen of isoniazid (INH), rifampicin (RIF), pyrazinamide and ethambutol for 2 months, followed by 4 months of INH and RIF treatment. This lengthy therapy has resulted in poor patient compliance which increases the risk of developing multi-drug resistant tuberculosis (MDR-TB). The treatment for MDR-TB typically lasts for 20 months and requires more expensive and toxic drug regimens. Even so, the success rate of standard MDR-TB chemotherapy is just over 50%. The adverse effects of current first line anti-tuberculosis drugs and particularly second line therapeutics include nephrotoxicity and hepatotoxicity (Ramappa and Aithal, 2013; Schnippel et al., 2016), which are mostly inevitable and act as a deterrent for adherence. The requirement to develop more efficacious anti-tuberculosis drugs with

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superior toxicity profiles that can shorten treatment duration is a priority to improve treatment outcomes.

Phenothiazines are a class of organic compounds consisting of a tricyclic aromatic core structure that is widely known for their significant biological activities (Jaszczyszyn et al., 2012; Pluta et al., 2011). Importantly, phenothiazines have demonstrated biological activity against infective agents and are known to exert effective intracellular bactericidal killing at clinical doses after being concentrated within macrophages (Amaral et al., 2010b; Ordway et al., 2002; Salie et al., 2014). The first phenothiazine-derived drug, methylene blue, has been proven effective for malaria treatment since the 1890s. Other phenothiazines, such as chlorpromazine and thioridazine (TZ) have also shown *in vitro* activity against *Plasmodium falciparum* (Basco and Le Bras, 1992; Weisman et al., 2006). Furthermore, chlorpromazine can act against *Leishmania donovani* and TZ plays a prominent role in the redox defences of the pathogenic *Trypanosoma* species (Lo Presti et al., 2015; Pearson et al., 1984). With substantial support from animal studies, treatment of TB with phenothiazines, particularly TZ, has proven highly successful in clinical trials. Its potential to be considered as an alternative class of anti-tuberculosis compounds for further development as MDR-TB treatment has been advocated in several studies and reviews (Amaral and Viveiros, 2017; Ordway et al., 2003; Salie et al., 2014). Nonetheless, the psychotropic effects of clinically approved phenothiazines have remained an impediment for its broader use as general antimycobacterial agents.

Previously, we have reported on the modification of a novel group of phenothiazine derivatives that were essentially devoid of serotonin and dopamine receptor binding activity yet retained its ability to inhibit *M. tuberculosis* growth (Salie et al., 2014). The separation of psychotic and antimycobacterial effects, position these compounds uniquely for further exploitation and development as viable alternatives for TB therapy. In this study, we investigated the toxicity profiles of these non-neuroleptic phenothiazines both *in vitro* and *in vivo*, and reported on cell viability in escalation dose challenges together with tolerance and biochemical profiles in mice.

2. Materials and methods

2.1. Mice

Adult female C57BL/6 mice used in this study were bred and maintained under specific-pathogen-free conditions in the Research Animal Facility at the University of Cape Town. Experimental mice were housed in filter top cages in a Bio-Safety Level I animal laboratory; supplied with required bedding, nesting material, sterile water and a formulated dietary food *ad libitum*. All animal procedures were performed in accordance with the recommendations of the South African national guidelines, and experimental protocols were approved by the Animal Research Ethics Committee of the Health Sciences Faculty, University of Cape Town (Reference number: 012/053).

2.2. Drug and lead compounds

Thioridazine (TZ) was purchased from Sigma (St. Louis, MO). Non-neuroleptic phenothiazine derivatives (PTZ3, PTZ4, PTZ31 and PTZ32) were synthesized as previously described (Salie et al., 2014). PTZ3 and PTZ4 were synthesized by the Department of Chemistry at the University of Cape Town. PTZ31 and PTZ32 were synthesized by iThemba Pharmaceuticals (iThemba Pharmaceuticals (Pty) Ltd., Gauteng, South Africa). The compounds were stored at -20°C and dissolved in distilled water on the day of use.

2.3. *In vitro* cytotoxicity assays

Cytotoxicity assays were performed on bone marrow derived macrophages (BMDM) as previously described (Salie et al., 2014). Briefly,

BMDM were isolated from the femurs of adult mice and cultured in RPMI medium (Sigma) supplemented with 20% fetal calf serum (FCS) (Gibco), 30% L929 conditioned medium, 10 mM L-glutamine (Gibco), 100 U/ml Streptomycin and 100 U/ml Penicillin at 37°C with 5% CO_2 . Confluent cells were seeded overnight at a concentration of 1×10^5 cells per well in 96-well plates. Serial dilutions of the phenothiazine derivatives from 400 mg/L to 12.5 mg/L, diluted in BMDM medium (2 mM L-Glutamine, 2% FCS, 10% L929 medium in RPMI), were added to the macrophages and incubated for five days. Serial dilutions of TZ from 25 mg/L to 1.56 mg/L were also tested in the BMDM cultures for five days. To measure the cell viability, 100 μL of supernatant was removed from each well and replaced with 20 μL Cell-titer Blue[®] reagent (Promega Corporation, Madison, USA). The plate was then read on a microplate reader (VERSAmix tunable microplate reader) after 4 h of incubation with the Cell-titer Blue[®] reagent. The cell viability percentage was calculated relative to untreated macrophages and IC_{50} values were calculated from the sigmoidal curve using GraphPad Prism[™].

2.4. *In vitro* metabolic stability and kinetic solubility studies

The non-neuroleptic phenothiazine derivatives (PTZ3, PTZ4, PTZ31 and PTZ32) were evaluated for its kinetic solubility and metabolic stability as previously described with a slight modification (Abay et al., 2015; Di and Kerns, 2016). The metabolic stability of the modified phenothiazines was determined in mouse liver microsomes using a single time point method that involved 30 min incubation and measurement of the percentage of compound remaining. The kinetic solubility of modified phenothiazines was analysed using the HPLC-UV method at pH 2 and pH 6.5; the fasted state simulated intestinal fluid (FaSSIF) medium was used to simulate the small intestine juices in fasted state (Frank et al., 2012).

2.5. *In vivo* toxicity studies

For the acute toxicity studies, mice ($n = 5$ per group) were treated with a single dose of 100 mg/kg (in 200 μL) of a phenothiazine derivative via oral gavage on day 1, and then monitored daily for 14 days. For the sub-acute toxicity studies, mice ($n = 10$ per group) were treated with concentrations of 25–100 mg/kg (in 200 μL) of a phenothiazine derivative via oral gavage daily for 14 days. At experimental end points, all mice were euthanized by halothane inhalation (5% in air), followed by exsanguinations by cardiac puncture for blood collection. Organs were weighed to determine the organ-to-bodyweight ratios, then fixed in 10% formalin and stained with haematoxylin and eosin for histological analysis.

2.6. Biochemical and haematological parameter analyses

Whole blood collected via cardiac puncture from the mice was divided between K2E microtainer tubes (BD, USA) and microfuge tubes. Blood samples in the microtainer tubes were analysed using an Abacus Junior Vet Haematology Analyzer (Ameisgasse, Austria) to determine haematological parameters. Blood samples in microfuge tubes were centrifuged (10 000 rpm for 10 min) to obtain serum which was then analysed using the following assays from Abcam (Cambridge, UK): Uric Acid Assay kit (ab65344), Aspartate Assay kits (ab102512), Creatinine Assay kits (ab65340) and Alkaline Phosphatase Assay kits (ab83369). The assays were performed as per manufacturers' specifications and the optical density was measured at 570 nm on a VERSAmix microplate reader (Molecular Devices, LLC, CA) with SoftMax software.

2.7. Statistical analysis

The data is presented as the mean \pm standard deviation (SD). Statistical analysis was performed by non-parametric one way ANOVA,

Table 1

Selectivity indices (SI) for PTZ3, PTZ4, PTZ31, PTZ32 & TZ. The modified phenothiazines (PTZ3, PTZ4, PTZ31 & PTZ32) displayed a much higher SI than TZ, implying a greater selectivity toward *M. tuberculosis* than for host macrophage cells.

Phenothiazine	MIC (mg/L)	Cytotoxic IC ₅₀ (mg/L)	SI = [cytotoxic IC ₅₀]/[MIC]
PTZ3	12.5	239.0 ± 0.02	19.1
PTZ4	12.5	279.4 ± 0.009	22.4
PTZ31	25	238.9 ± 0.012	9.6
PTZ32	25	208.6 ± 0.005	8.3
TZ	6.25	5.23 ± 0.04	0.83

using Dunn's Multiple Comparison Test or the non-parametric Mann-Whitney test (two-tailed with a 95% confidence interval). * and ** indicate $p < 0.05$ and $p < 0.01$ respectively (Graphpad Prism™).

3. Results

3.1. The solubility, metabolic stability and cytotoxicity effects of phenothiazine derivatives

Four non-neuroleptic phenothiazine derivatives, PTZ3, PTZ4, PTZ31 and PTZ32, were previously reported to have antimycobacterial activity against *M. tuberculosis* with minimum inhibitory concentration (MIC) values of 12.5–25 mg/L. Moreover, both PTZ3 and PTZ4 inhibited between 40% and 60% of intracellular *M. tuberculosis* growth at 25 mg/L (Salie et al., 2014). In the present study, the cytotoxic potential of the phenothiazine derivatives was evaluated against bone marrow derived macrophages, and the selectivity index (SI) defined as the ratio of cytotoxicity to antimycobacterial activity (IC_{50}/MIC) was calculated and compared to thioridazine (Table 1). Cytotoxic concentrations of the non-neuroleptic phenothiazines were all in the range of 200–300 mg/L, but PTZ4 displayed the best SI of 22.4. Of the non-neuroleptic phenothiazines, PTZ32 had the lowest SI (SI = 8.3), yet it was still substantially higher than TZ (SI = 0.83).

Early assessment of microsomal stability provides crucial information on metabolic liabilities of potential drug candidates, and solubility is a key determinant of absorption and oral bioavailability. Therefore, *in vitro* microsomal metabolic stability and kinetic solubility studies were conducted. All modified phenothiazines had solubility values greater than 100 which were interpreted as highly soluble (Table 2). The percentage remaining phenothiazines ranged between 72.4 and 90.0. The metabolic stability of the phenothiazine derivatives was comparable to the positive control MMV390048 (Table 2). These phenothiazine derivatives do not have any apparent highly reactive groups excluding PTZ32 (Table 1 molecular structures) which might be more susceptible to sulfoxidation. This could potentially explain why PTZ32 displayed the lowest percentage remaining (72.4) and a shorter half-life (64.9).

Table 2

In vitro microsomal stability and kinetic solubility of modified phenothiazines. * FaSSIF- bio-relevant buffer that simulates the small intestine juices in fasted state; **A single time point assay (30 min) was used for prediction of half-life; Quality control standards for validation of metabolic stability include (i) Propranolol (ii) Midazolom (iii) MMV390048.

Phenothiazine	μM solubility pH 2	μM solubility pH 6.5	μM solubility FaSSIF* pH 6.5	Percentage remaining	Projected Half-life (min)**
PTZ3	184.3	167.3	172.3	88.1	> 100
PTZ4	177.0	184.1	194.7	90.0	> 100
PTZ31	167.0	158.9	187.0	87.3	> 100
PTZ32	181.0	179.2	182.4	72.4	64.9
Propranolol	–	–	–	8.1	8.3
Midazolom	–	–	–	6.9	7.8
MMV390048	–	–	–	96.1	> 100

Collectively the data illustrates that, compared to TZ, the non-neuroleptic phenothiazines have better biological activities and selectively kill *M. tuberculosis* at doses much lower than doses shown to be cytotoxic to host cells.

3.2. Mortality and morbidity rates of mice during treatment with phenothiazine derivatives

To evaluate the tolerable dose of the non-neuroleptic phenothiazines within the full complexity of an organism, PTZ3, PTZ4, PTZ31 or PTZ32 were tested in mice either with single dose (acute toxicity) or repeated administration (sub-acute toxicity).

First, to assess the *in vivo* acute toxicity, we treated the mice with a single dose of 100 mg/kg of PTZ3, PTZ4, PTZ31 or PTZ32, and found no deaths nor significant bodyweight changes in these four groups of mice (Fig. 1). In contrast, mice which received a single dose of 100 mg/kg of TZ showed loss of movement and hind leg paralysis within 4 h of treatment. All TZ treated mice displayed signs of distress and was euthanized on day one for ethical reasons.

The sub-acute toxicity study evaluates the safety profiles of a drug/compound to generate collective physiological, haematological and biochemical changes as a result of repeated exposure. Two weeks exposure to 100 mg/kg PTZ3 or PTZ4 did not cause deaths or significant bodyweight changes in mice (Fig. 2A). In contrast, a 20% and 55% mortality rate were observed in mice treated with 100 mg/kg PTZ31

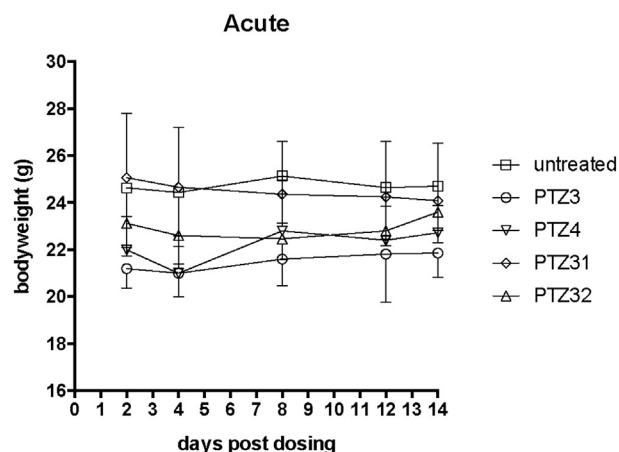


Fig. 1. Clinical parameters of mice in acute toxicity study. Mice were treated with a single dose of 100 mg/kg of either: PTZ3, PTZ4, PTZ31, PTZ32 or TZ. The bodyweights were recorded for a period of 14 days after treatment. Graphs represent pooled data of two independent experiments. (n = 5 mice/group).

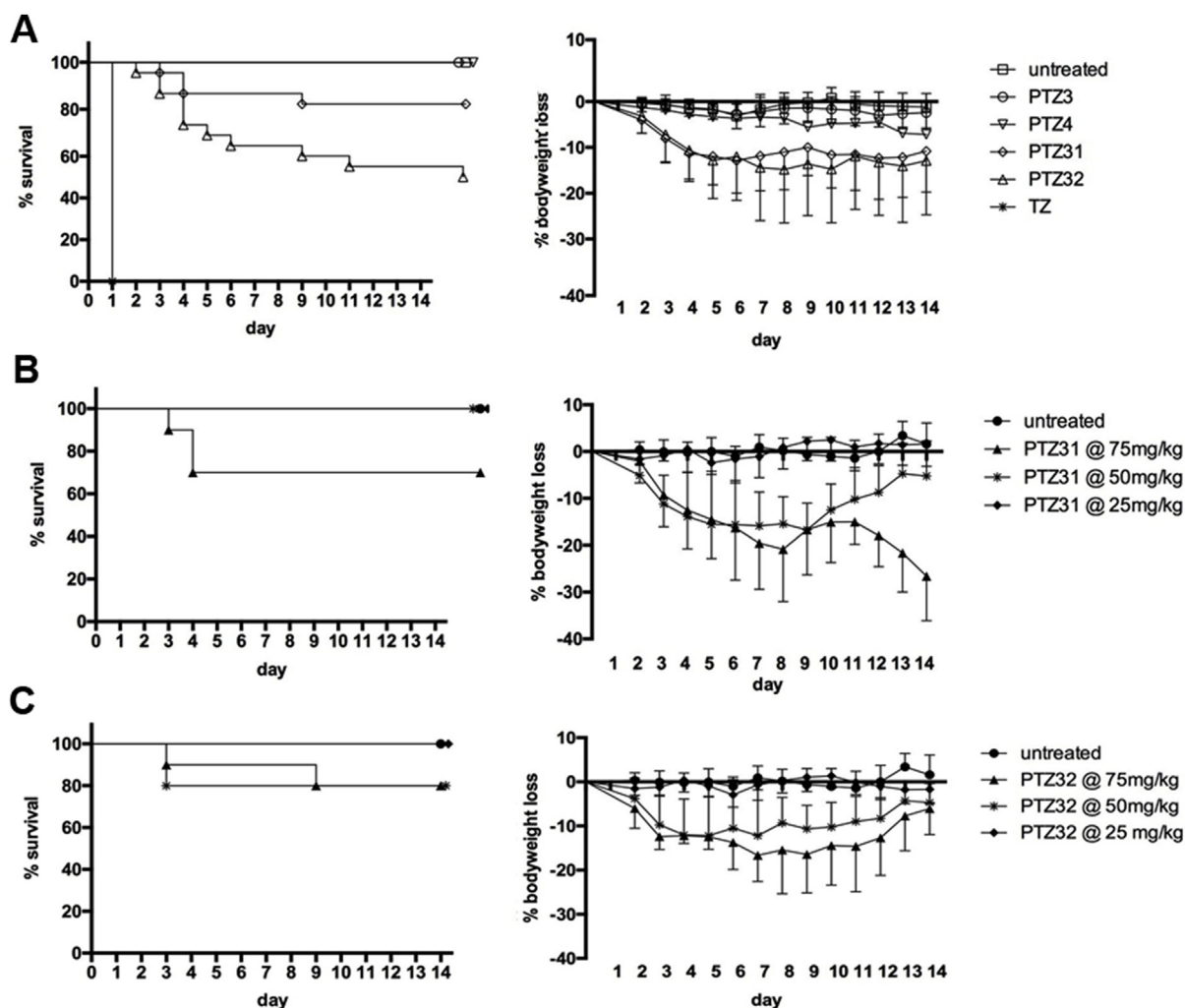


Fig. 2. Clinical parameters of mice in sub-acute toxicity study. (A) Mice were treated with 100 mg/kg daily of either: PTZ3, PTZ4, PTZ31, PTZ32 or TZ. Survivals and bodyweight changes were recorded for a period of 14 days treatment. Sub-lethal dosages of (B) PTZ31 and (C) PTZ32 were also tested at 25 mg/kg, 50 mg/kg and 75 mg/kg daily for 14 days. Graphs represent pooled data of two independent experiments. (n = 20 mice/group).

and PTZ32 respectively. Mice which survived PTZ31 and PTZ32 treatment displayed a rapid reduction in bodyweight during the first five days and remained stable for the rest of the experimental period (Fig. 2A). To further assess the maximum tolerable concentrations of PTZ31 and PTZ32, sub-acute toxicity was also tested at 25 mg/kg, 50 mg/kg and 75 mg/kg doses for 14 days. The PTZ31 treated mice displayed no mortality (100% survival) at 50 mg/kg (Fig. 2B), and PTZ32 treated mice had 100% survival at 25 mg/kg (Fig. 2C). Notably, all four modified phenothiazines (PTZ3, PTZ4, PTZ31 or PTZ32) were comparably less toxic than TZ, which induced 100% mortality within 24 h of treatment (Fig. 2A).

3.3. Pathological effects of phenothiazine derivatives treatment

Organ weight is an important indicator for toxicity and risk assessment of drugs, chemicals, biologics, food additives and medical devices (Ibrahim et al., 2016; Nirogi et al., 2014). The ratio of organ-to-bodyweight, which normalizes bodyweight fluctuations, was evaluated after 14 days of the sub-acute toxicity studies (Table 3). Mice treated at a dose of 100 mg/kg of PTZ3, PTZ4 or PTZ32 had similar organ-to-bodyweight ratios to the untreated control group (Table 3), and displayed similar outcomes for hepatic and renal histological analysis to the untreated control group (Fig. 3). Although mice receiving 100 mg/kg PTZ31 showed a significantly lower ($p < 0.01$) organ-to-

bodyweight ratio in liver weight compared to the untreated control group (Table 3), histological analysis revealed no lesions nor anomalies in the liver sections; normal architecture of the central vein, parenchyma and portal triad was displayed (Fig. 3D). Microscopic examination of the kidney tissues of the PTZ31 treated mice also exhibited normal kidney histology with intact glomeruli and renal tubules. High magnification of the medulla section showed no morphological changes such as cell degeneration in the collecting ducts and limbs of Henle's (Fig. 3I).

3.4. Haematological effects of phenothiazine derivative treatment

Haematology is sensitive to the effects of toxic substances and serves as an important index of the physiological status of the treated animal or person (Olson et al., 2000; Son et al., 2015). Therefore, in relation to possible tissue or organ injury, haematological parameters were obtained from sub-acute studies at a dose of 100 mg/kg after treatment with PTZ3, PTZ4, PTZ31 and PTZ32. Here, red blood cells (RBC), haemoglobin (Hg), haematocrit (HCT), mean cell height (MCH) and platelets (PLT) were measured. Haematological analysis of the PTZ3, PTZ4 and PTZ32 treated groups revealed outcomes that were comparable to the untreated control group (Table 4). However, PTZ31 treated mice had significantly higher ($p < 0.01$) RBCs, Hg and PLTs (Table 3). The increase in RBC and PLT parameters could imply that PTZ31 is

Table 3

Organ-to-bodyweight ratios of C57BL/6 mice during sub-acute toxicity study. Mice were treated daily with 100 mg/kg of either: PTZ3, PTZ4, PTZ31 or PTZ32. PTZ3, PTZ4 and PTZ32 organ-to-bodyweight ratios were comparable to those of the untreated control group. PTZ31 treated mice had significantly lower liver organ-to-bodyweight ratios when compared the untreated control group. This table represents data pooled from two independent experiments. (**p < 0.01).

	untreated (n = 20)	PTZ3 (n = 20)	PTZ4 (n = 20)	PTZ31 (n = 16)	PTZ32 (n = 9)
kidney	0.59 ± 0.12	0.57 ± 0.16	0.58 ± 0.11	0.59 ± 0.18	0.59 ± 0.10
liver	5.04 ± 0.58	4.72 ± 0.32	4.73 ± 0.41	3.73 ± 1.76 (**) [↓]	4.58 ± 0.55

capable of stimulating the hematopoietic system, leading to the production of PLT (thrombopoiesis) and RBCs (erythropoiesis) (Ibrahim et al., 2016). Based on the haematological data, it was concluded that PTZ3, PTZ4 and PTZ32 treatment does not result in toxicity. In contrast, dosing with 100 mg/kg PTZ31 for 14 days potentially affects the hematopoietic system.

3.5. Biochemical effects of phenothiazine derivative treatment

Biochemical indices are important indicators of toxicity. Detection of variations in enzyme concentrations present in serum can highlight tissue and/or cellular damage (Kutlu et al., 2007; Son et al., 2015). Creatinine concentrations in the sera of mice were used to determine renal function and investigate its integrity. In addition, the assessment of serum uric acid was also included due to the reported association of antituberculous drugs with hyperuricemia (Gulbay et al., 2006; Louthrenoo et al., 2015; Pokam et al., 2016). Dosed at 100 mg/kg per day for 14 days, the levels of serum uric acid and creatinine concentrations in PTZ3, PTZ4 or PTZ31 treated mice showed no significant elevations relative to the untreated control group (Fig. 4A&B). However, PTZ32 treated mice had significantly higher (p < 0.01) uric acid concentrations compared to the untreated control group (Fig. 4A). However the creatinine concentration for both the untreated control mice and PTZ32 treated mice were equivalent (Fig. 4B).

Hepatic toxicity was assessed by quantitative analysis of serum alkaline phosphatase (ALP) and aspartate aminotransferase (AST) concentrations. There was no statistical difference in the levels of ALP and AST concentrations of mice treated with 100 mg/kg PTZ3, PTZ4 or

PTZ31 compared to those of the untreated control group (Fig. 4C&D). However, PTZ32 treated mice had a significantly higher (p < 0.01) level of ALP concentration than those of the untreated control group (Fig. 4C), while no differences were measured in ASP concentration (Fig. 4D).

Overall, the sub-acute toxicity studies revealed that PTZ3, PTZ4 and PTZ31 treatment did not alter any biochemical parameters, however the PTZ32 treatment resulted in potential modifications in renal and hepatic functions.

4. Discussion

Tuberculosis remains one of the major causes of mortality worldwide, despite almost two decades of global intervention in terms of the Millennium Development Goals & Sustainable Development Goals adopted by the United Nations (World Health Organization., 2018). The numerous toxicities and side effects of phenothiazines are well documented (Thanacoody, 2007; Warman et al., 2013) and has been considered a limiting factor for the broad clinical application of thioridazine, or any other (neuroleptic) phenothiazine, as an antimycobacterial agent (Warman et al., 2013). This study reports on the toxicity profiles of a novel subset of phenothiazines which were previously found to lack psychotic potential but retained efficacy against virulent *M. tuberculosis* (Salie et al., 2014).

A favourable cytotoxicity profile is important as potential drugs should not negatively affect cell viability or produce bacterial toxins that may affect cellular function (Ordway et al., 2002). This may be essential for phenothiazines due to the accumulation of drugs within

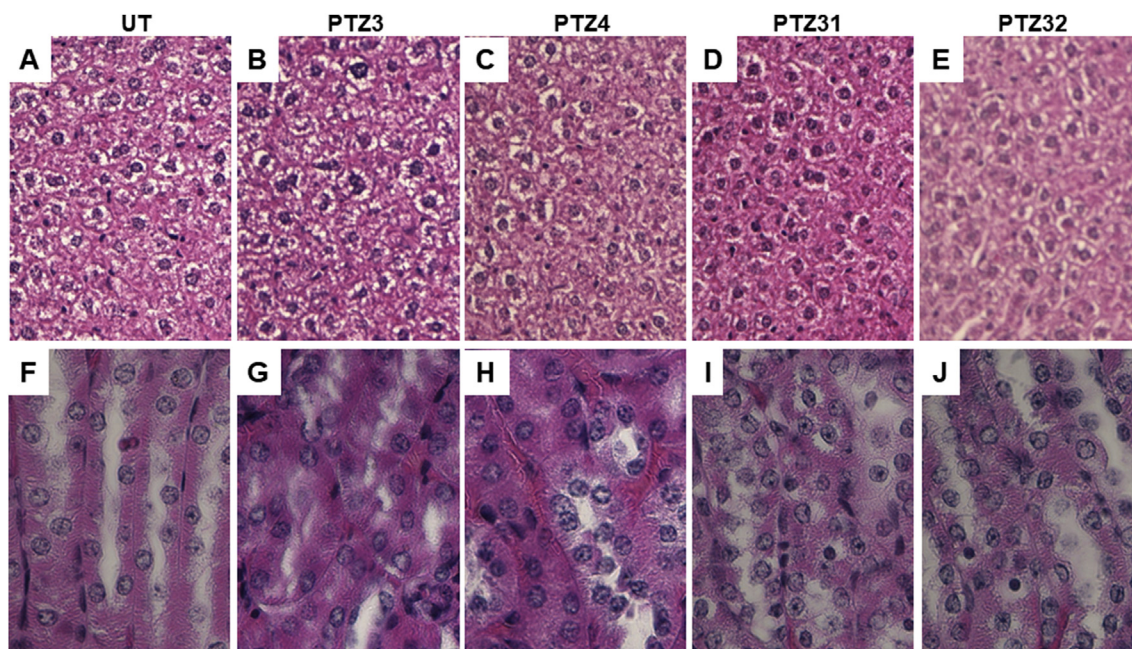


Fig. 3. Histopathological examinations of mice in the sub-acute toxicity study. Mice were dosed for 14 days with 100 mg/kg daily of either: water/untreated (UT), PTZ3, PTZ4, PTZ31 or PTZ32. (A–E) The sections of liver from the control and treated mice revealed normal architecture. Figure shows a representative image (200X) from each group of two independent experiments. (F–J) The sections of kidney from the control and treated mice revealed normal architecture. Figure shows a representative image (400X) from each group of two independent experiments.

Table 4

Hematological parameters of C57BL/6 mice during Repeat dose toxicity challenge study. Mice were treated daily with 100 mg/kg of either: PTZ3, PTZ4, PTZ31 or PTZ32. Data for PTZ3, PTZ4 and PTZ32 dosed mice were comparable to those of the untreated control group. Dosing with PTZ31 resulted in differences in the RBC, Hg and PLT parameters. This table represents data pooled from two independent experiments. (**p < 0.01).

	Untreated (n = 20)	PTZ3 (n = 20)	PTZ4 (n = 20)	PTZ31 (n = 16)	PTZ32 (n = 9)
RBC (10 ⁶ /μL)	9.27 ± 0.55	9.53 ± 0.42	9.55 ± 0.42	9.92 ± 0.26 (**) [↑]	9.63 ± 0.29
Hg (g/dL)	13.97 ± 0.86	14.18 ± 0.78	14.28 ± 0.81	15.13 ± 0.57 (**) [↑]	14.37 ± 0.44
HCT (%)	47.85 ± 3.95	48.54 ± 3.61	48.61 ± 3.81	46.46 ± 1.76	45.84 ± 1.51
MCH (pg/cell)	15.03 ± 0.48	14.88 ± 0.49	14.96 ± 0.53	15.23 ± 0.40	14.94 ± 0.20
PLT (10 ³ /μL)	1419 ± 272	1511 ± 276	1496 ± 286	1797 ± 231 (**) [↑]	1614 ± 313

[RBC – red blood cells, Hg – hemoglobin, HCT – hematocrit, MCH – mean cell height, PLT – platelets].

macrophages (Amaral et al., 2010b; Ordway et al., 2002). Analyses of the cytotoxic data indicated that PTZ3, PTZ4, PTZ31 and PTZ32 displayed a much greater selectivity index (SI = 8.3–22.4) than thioridazine (SI = 0.83), supporting its potential use at higher therapeutic doses against mycobacterial infection. The order of relative cytotoxicity can be arranged from most toxic to least toxic as follows: PTZ32 > PTZ31 > PTZ3 > PTZ4, whereas the cytotoxicity effects of the parent compounds on rat liver cells are reported to be: thioridazine (analogous to PTZ32) > fluphenazine (analogous to PTZ31) > chlorpromazine (analogous to PTZ4) > promazine (analogous to PTZ3) (de Faria et al., 2015; Salhab and Dujovne, 1986). Interestingly, these parent compounds have been tested for their potential anti-tumour potential and the cytotoxic effects on the human glioblastoma cells are reported in the same order of cytotoxicity, as follows: thioridazine > fluphenazine > chlorpromazine > promazine (Cheng et al., 2015). PTZ32 and its parent compound (thioridazine) displayed the most cytotoxic effects. Many have advocated for the repurposing of thioridazine as an antimycobacterial compound (Amaral et al., 2010a; Thanacoody, 2007) and its application reported in clinical trials (Abbate et al., 2012), highlighted the severe nature of its toxicity. When viewed against these findings PTZ3, PTZ4, PTZ31 and PTZ32 have a significant advantage and potential to be developed as anti-

mycobacterial drugs.

Currently the standard regimen for drug-susceptible TB consists of four first-line anti-TB drugs i.e. INH, RIF, pyrazinamide, and ethambutol. Although TB disease is curable through a prolonged period of drug treatment, hepatotoxicity is not an uncommon association for TB drugs as INH, RIF and pyrazinamide usage (three out of the four first line anti-TB drugs) occurs in 3–40% of TB patients (Arbex et al., 2010; Steele et al., 1991). To address this challenge, outcomes obtained in pre-clinical animal studies can provide a concordance of more than 50% with human toxicity profiles for most essential target organs (Olson et al., 2000). In the present study, PTZ3 and PTZ4 treatment revealed no discernible renal or hepatotoxicity during challenge studies. Although the results showed the potential for an improved safety profile for the non-phenothiazine derivatives tested, the results from this 14-day study *in vivo* in mice should be extended to confirm safety with longer-term exposures (90 or 120 days). Considering the lack of renal or hepatotoxicity of PTZ3 and PTZ4, it is possible that PTZ3 and PTZ4 may be less toxic compared to most of the first line anti-TB drugs. The potential improved safety profile of PTZ3 and PTZ4 positions these compounds well to proceed to *in vivo* efficacy verification studies.

Interestingly, PTZ31 treatment did not cause significant changes in organ microstructure and biochemical indices, except in organ-to-

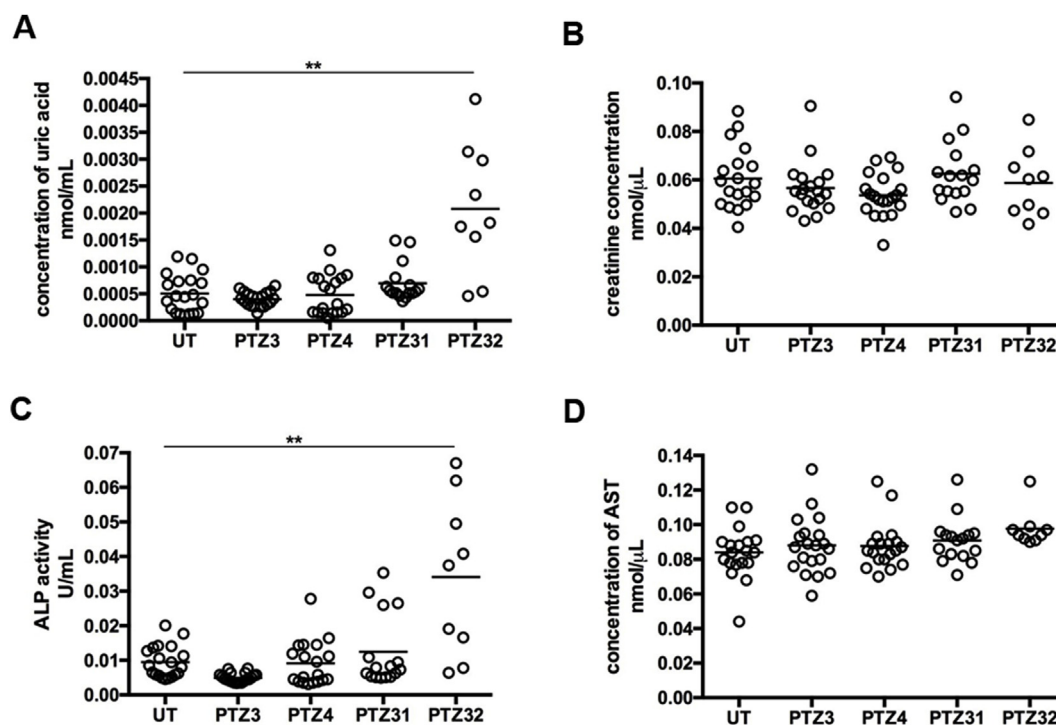


Fig. 4. Biochemical parameters of mice in sub-acute toxicity study. Mice were treated with 100 mg/kg daily of either: PTZ3, PTZ4, PTZ31 or PTZ32. After 14 days of treatment, blood was collected via cardiac puncture. (A) Sera uric acid concentration (nmol/mL), (B) sera creatinine concentration (nmol/μL), (C) sera alkaline phosphatase activity (U/mL) and (D) sera aspartate concentration (nmol/μL) were determined. Each graph represents the pooled data of two independent experiments. (**p < 0.01) [UT – untreated].

bodyweight ratio analysis where the liver was significantly reduced. This suggests that chronic exposure of the mice to PTZ31 at a dose of 100 mg/kg may induce toxic effects on liver. In contrast, oral dosing with PTZ32 (100 mg/kg/day) resulted in increased renal and hepatic biochemical indices, highlighting possible kidney and liver toxicity. This is consistent with higher metabolic processing, due to oxidation of the C-2 sulfide substituent which is analogous to thioridazine. Liver microsomal testing indicated faster metabolic clearance that may result in hepatotoxic intermediates. Generally, tests of liver damage include serum alanine aminotransferase (ALT), AST, ALP and γ -glutamyl transferase. Among the liver injury markers, ALT and AST are probably the most commonly used in both clinical diagnosis and research involving liver damage. Studies have reported elevated serum AST in rats after the treatment of phenothiazine derivatives, but no significant change in ALT levels (Engwa et al., 2016). However, we have observed no changes in AST levels after treatment with the modified phenothiazines in our study. Elevated ALP levels could indicate possible alteration or change of hepatocyte function or metabolism due to treatment with PTZ32, and are specifically linked to conditions such as hepatobiliary disorders, bone formation and hepatitis (Duan et al., 2016). Chlorpromazine is extensively metabolized by the liver via sulfoxidation and oxidation, increased serum ALP and aminotransferase levels were also reported in patients treated with chlorpromazine which is known to cause of acute cholestatic liver injury (Moradpour et al., 1994; Russell et al., 1973). Fluphenazine was also found to induce lipid peroxidation in the kidney of Wistar rats (Corte et al., 2009). Isoniazid is known to increase liver transaminase concentration, thus it is important that ideally, any potential anti-TB agent should not increase liver transaminase levels, as the effect thereon could be additive (Giannini et al., 2005; Giboney, 2005).

The haematological results of PTZ31 and PTZ32 showed an increase in some of the RBC indices, indicating possible RBC lysis or stimulation of the hematopoietic system (Ibrahim et al., 2016; Pluta et al., 2011). Pluta et al. reported that synthesized phenothiazines, very similar in structure to PTZ31 and PTZ32, resulted in alteration of the RBC membrane which lead to stomatocytosis ('leaking' of sodium and potassium ions) (Pluta et al., 2011). A different study using rat hepatocyte monolayer cultures, found that amphiphilic cationic psychotropic drugs (such as phenothiazines) have the ability to induce haemolysis in RBCs (Boelsterli et al., 1987). As changes in the haematological and biochemical parameters in animals have a high predictive value for human toxicity (Olson et al., 2000), these results indicate a necessity for further testing to fully understand the PTZ31 and PTZ32 treatment related effects noted.

Current treatment of MDR-TB consists of drugs which induce frequent and severe toxic effects (Arbex et al., 2010; Koul et al., 2011), that are directly linked to patient non-compliance and may result in changes to the therapeutic regimen, usually with less effective drug options. Accumulating evidence from *in vitro* and *in vivo* studies show the efficacy of thioridazine against drug-resistance strains of *M. tuberculosis*, and the combination of thioridazine with antibiotic regimens suggest the potential use of thioridazine as an adjunct to standard chemotherapy of MDR-TB (Amaral et al., 2010a; Amaral and Viveiros, 2017; Dutta et al., 2014; Ordway et al., 2003; Viveiros and Amaral, 2001). Although long-term administration of thioridazine is limited by its toxicity, thioridazine has been successfully used in clinical trials. Therefore, this class of modified phenothiazines with limited indicators of toxicity should be considered in the development of therapeutic strategies or the potential use in combination treatment for MDR-TB.

5. Conclusion

The results of the *in vitro* cytotoxicity and metabolism and *in vivo* toxicity studies confirm that PTZ3, PTZ4, PTZ31 and PTZ32 have a superior safety profile to thioridazine. Taken together our previous

study that demonstrates the abolishment of psychotropic effects of the modified phenothiazines, whilst maintaining antimycobacterial inhibitory potential and the findings in this study would potentially allow higher doses to be administered in contrast to current phenothiazine compounds such as thioridazine. The combination of the lack of psychotropic activity and safe toxicological profiles of the non-neuroleptic phenothiazines support their further evaluation in *in vivo* efficacy models and human trials to establish clinical benefits as effective antimycobacterial drugs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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6 Abbreviations

ALP	serum alkaline phosphatase
AST	aspartate aminotransferase
FCS	foetal calf serum
HCT	haematocrit
Hg	haemoglobin
HIV	human immunodeficiency virus
INH	isoniazid
PTZ, MCH	mean cell height
MDR-TB	multi-drug resistant tuberculosis
MIC	minimum inhibitory concentration
PLT	platelets
PTZ	phenothiazine derivatives
RBC	red blood cell
RIF	rifampicin
SI	selectivity index
TB	tuberculosis
TZ	thioridazine

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