Activity of 5-chloro-pyrazinamide in mice infected with *Mycobacterium tuberculosis* or *Mycobacterium bovis*

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Background & objectives: Pyrazinamide is an essential component of first line anti-tuberculosis regimen as well as most of the second line regimens. This drug has a unique sterilizing activity against *Mycobacterium tuberculosis*. Its unique role in tuberculosis treatment has lead to the search and development of its structural analogues. One such analogue is 5-chloro-pyrazinamide (5-Cl-PZA) that has been tested under *in vitro* conditions against *M. tuberculosis*. The present study was designed with an aim to assess the activity of 5-Cl-PZA, alone and in combination with first-line drugs, against murine tuberculosis.

Methods: The minimum inhibitory concentration (MIC) of 5-Cl-PZA in Middlebrook 7H9 broth (neutral *p*H) and the inhibitory titre of serum from mice that received a 300 mg/kg oral dose of 5-Cl-PZA 30 min before cardiac puncture were determined. To test the tolerability of orally administered 5-Cl-PZA, uninfected mice received doses up to 300 mg/kg for 2 wk. Four weeks after low-dose aerosol infection either with *M. tuberculosis* or *M. bovis*, mice were treated 5 days/wk with 5-Cl-PZA, at doses ranging from 37.5 to 150 mg/kg, either alone or in combination with isoniazid and rifampicin. Antimicrobial activity was assessed by colony-forming unit counts in lungs after 4 and 8 wk of treatment.

Results: The MIC of 5-Cl-PZA against *M. tuberculosis* was between 12.5 and 25 μg/ml and the serum inhibitory titre was 1:4. Under the same experimental conditions, the MIC of pyrazinamide was >100 μg/ml and mouse serum had no inhibitory activity after a 300 mg/kg dose; 5-Cl-PZA was well tolerated in uninfected and infected mice up to 300 and 150 mg/kg, respectively. While PZA alone and in combination exhibited its usual antimicrobial activity in mice infected with *M. tuberculosis* and no activity in mice infected with *M. bovis*, 5-Cl-PZA exhibited antimicrobial activity neither in mice infected with *M. tuberculosis* nor in mice infected with *M. bovis*.

Interpretation & conclusion: Our findings showed that 5-Cl-PZA at doses up to 150 mg/kg was not active in chronic murine TB model. Further studies need to be done to understand the mechanism and mode of inactivation in murine model of tuberculosis.

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Pyrazinamide (PZA), an analog of nicotinamide, is a first-line anti-tuberculosis drug with a unique sterilizing activity[1,2]. Its addition to the combined anti-tuberculosis regimen including isoniazid, rifampin, and ethambutol contributed to the reduction of treatment duration and thus led to the modern 6-month short-course therapy for tuberculosis[1,3]. PZA is a pro-drug that undergoes amide hydrolysis by the mycobacterial pyrazinamidase (a nicotinamidase) to pyrazinoic acid (POA)[4]. POA acts against *Mycobacterium tuberculosis*, but the exact biochemical basis of action has not been fully established. It has been hypothesized that POA either disrupts *M. tuberculosis* membrane transport and energetics, or inhibits fatty acid synthetase I (FAS I)[5-7]. Pyrazinamidase is encoded by the *pncA* gene and mutations in *pncA* that abolish the amidase activity confer PZA resistance in strains of *M. tuberculosis*[8-10]. PZA-susceptible and resistant isolates are generally susceptible to POA *in vitro*, but POA is not active *in vivo*[11].

A series of esters of POA and 5-substituted POA derivatives have been reported to enhance *in vitro* activity against both PZA-susceptible and PZA-resistant *M. tuberculosis*, as well as against the naturally PZA-resistant *M. bovis*, *M. kansasii*, and *M. avium* isolates[12,13]. One such derivative, 5-chloropyrazinamide (5-Cl-PZA), has a minimum inhibitory concentration (MIC) of 25 µg/ml against *M. smegmatis*, which is far lower than that of PZA (4000 µg/ml). Both 5-Cl-PZA, along with its acid form, 5-chloropyrazinoic acid (5-Cl-POA), were evaluated for their *in vitro* activity against *M. tuberculosis*, *M. bovis* (inherently resistant to PZA owing to a lack of nicotinamidase)[4], and several non-tuberculous mycobacteria by a broth dilution method, and 5-Cl-PZA was more active than PZA against all organisms tested[14]. At neutral *p*H, MICs of PZA and 5-Cl-PZA against *M. tuberculosis* range from 32 to 2048 µg/ml and from 8 to 32 µg/ml, respectively[12-14]. MICs of POA and 5-Cl-POA ranged from 16-64 mg/ml and from 64-256 µg/ml, respectively[12-14]. Thus MICs of 5-Cl-PZA and POA for *M. tuberculosis* are more favourable than those of PZA and 5-Cl-POA. In addition, PZA-resistant isolates retain susceptibility *in vitro* to 5-Cl-PZA, POA, and 5-Cl-POA, suggesting that 5-Cl-PZA does not require activation by mycobacterial pyrazinamidase. This is also supported by the observation that 5-Cl-PZA, unlike PZA, is active against *M. bovis* with an MIC of 8 µg/ml under *in vitro* conditions and, unlike PZA, is active even at a neutral *p*H[12-14].

The present study was planned to evaluate the dose-dependent activity of 5-Cl-PZA alone and in combination with isoniazid and rifampicin in mice infected with *M. tuberculosis* or *M. bovis* and compare it with that of pyrazinamide.

Material & Methods

The study has been conducted at the Center for Tuberculosis Research, Johns Hopkins University, Baltimore, Maryland, USA.

Bacterial strains: *M. tuberculosis* H37Rv and *M. bovis* (Ravenel) were passaged in mice, frozen in 1 ml aliquots, and stored at -80°C before use. For infection, aliquots of each species were thawed and sub-cultured in Middlebrook 7H9 broth (Fisher, USA) supplemented with 10 per cent oleic acid-albumin-dextrose-catalase (OADC; Difco, USA) and 0.05 per cent Tween 80 (Sigma, USA).

Animals: Female BALB/c mice aged 4 to 6 wk were purchased from Charles River (Wilmington, MA, USA). The study protocol and procedures were approved by the Institutional Animal Care and Use Committee at Johns Hopkins.

Antimicrobials: PZA was purchased from Acros Organics (Morris Plains, NJ, USA). Isoniazid (INH) and rifampicin (RIF) were purchased from Sigma. 5-Cl-PZA was synthesized by one of the authors (JTW)[14]. For *in vitro* studies, 5-Cl-PZA was solubilised in DMSO and diluted with distilled water. For administration to mice, homogeneous suspensions of 5-Cl-PZA were prepared in a 0.25 per cent carboxy methylcellulose formulation (CMC). Stock solutions/suspensions of all drugs were prepared weekly in distilled water and stored at 4 °C as described previously[15].

Determination of MIC: The MIC was determined by the broth dilution method[16]. Middlebrook 7H9 broth supplemented with 10 per cent OADC and containing serial two-fold concentrations of 5-Cl-PZA ranging from 0.625 to 100 µg/ml were inoculated with 0.1 ml of the 10^{-3} dilution of a log phase broth culture of *M. tuberculosis* H37Rv with an optical density corresponding to approximately 10^8 cfu/ml. Isoniazid at two-fold concentrations ranging from 0.015 to 0.6 µg/ml served as positive control while drug free broth and PZA at two-fold concentrations ranging from 0.625 to 100 µg/ml served as negative controls. The MIC was defined as the lowest concentration at which no visible growth was observed after 14 days incubation at 37°C.

Determination of the serum inhibitory titre (SIT): SIT was determined by the broth dilution method. Briefly, mice were orally administered 5-Cl-PZA at 300 mg/kg and, after anaesthesia, bled via cardiac puncture 30 min later. Two-fold dilutions of serum to a maximum of 1:32 were performed in Middlebrook 7H9 broth + OADC without Tween 80. All vials were inoculated with 0.1 ml of the 10^{-3} dilution of the same broth culture of *M. tuberculosis* $H_{37}Rv$ that was used for MIC determination. Serum from mice administered INH at 10 mg/kg or PZA at 300 mg/kg and blood collected 30 min after dosing were used as positive and negative controls, respectively, while serum from untreated mice was taken as a standard control for growth. After incubation at 37ºC for 14 days the SIT was defined as the highest dilution of serum that prevented visible growth.

Multi-doses tolerability of 5-Cl-PZA: To test whether multiple doses of 5-Cl-PZA would be toxic during chemotherapy, uninfected mice were orally given 5-Cl-PZA at two-fold doses ranging from 37.5 to 600 mg/kg/day for two weeks (five days/week), monitored for survival, behaviour and body weight changes in comparison with untreated controls. No haematology or serum chemistry parameters were studied.

Aerosol infection: Female BALB/c mice (Charles River) aged 4 to 6 wk were aerosol-infected using the Inhalation Exposure System (Glas-col Inc., Terre Haute, IN) with either *M. tuberculosis* or *M. bovis*. A log-phase broth culture at an optical density (at 600 nm) of approximately 1.0 was diluted 50-fold and used for infection. After infection, mice were randomly distributed into treatment groups (five mice per group per time point). A subgroup of 5 untreated mice were euthanized on the day after infection to determine the number of cfu implanted in the lungs and on the day of treatment initiation to determine baseline cfu counts. Quantitative lung cultures were performed on selective 7H11 plates (Becton Dickinson, USA), as described previously[17].

Drug treatment: All drugs were administered once daily 5 days a week in 0.2 ml of phosphate buffered saline (PBS) by oesophageal cannula. RIF was given 1 h prior to other drugs to avoid an adverse pharmacokinetic interaction[15-17]. The drug doses were INH 25 mg/kg, RIF 10 mg/kg, and PZA 150 mg/kg. The rationale for the use of these drug doses has been described previously[17].

Dose-ranging activities of 5-Cl-PZA alone and in combination: Control animals received one of the following drug regimens: RIF-INH-PZA, RIF-PZA, RIF-INH, RIF alone, and PZA alone (Table I). PZA was used at two doses, 150 and 75 mg/kg to compare with 5-Cl-PZA (Table I). Test mice received 5-Cl-PZA alone at two-fold doses ranging from 37.5 to 150 mg/kg to determine the minimum effective dose (MED) and the minimum bactericidal dose (MBD) of 5-Cl-PZA (Table I). In the combination regimens, 5-Cl-PZA was substituted for PZA in RIF-INH-PZA and RIF-PZA regimens, but only at the 150 mg/kg dose (Table I). Negative controls went untreated. The mice were euthanized after 4 and 8 wk of treatment for assessment of lung cfu counts.

To confirm that 5-Cl-PZA does not require activation by mycobacterial pyrazinamidase like PZA, the activities of 5-Cl-PZA and PZA alone and in combination with either RIF or RIF-INH were compared in mice infected with *M. tuberculosis* and *M. bovis*. Because *M. bovis* lacks pyrazinamidase, a similar activity of 5-Cl-PZA against *M. tuberculosis* and *M. bovis* infected mice would mean that the 5-Cl-PZA activity is independent of pyrazinamidase activity. PZA served as a negative control for this experiment as it has no activity against *M. bovis* owing to lack of activation.

Results

Each vial of broth was inoculated with 6.2×10^5 cfu. After 7 days of incubation, no visible growth was observed in tubes containing 0.06 and 12.5 µg/ml for INH and 5-Cl-PZA, respectively. After 14 days of incubation, the MIC remained the same for INH but, in the case of 5-Cl-PZA, doubtful growth was observed at 12.5 µg/ml and no growth at 25 µg/ml. Therefore, the MIC of INH was 0.06 µg/ml and the MIC of 5-Cl-PZA was between 12.5 and 25 µg/ml. In the case of PZA, visible growth was observed at all concentrations tested, up to 100 µg/ml, both at days 7 and 14, confirming the lack of *in vitro* activity of PZA at neutral *p*H.

Each tube of serially-diluted serum for SIT received the same inoculum as did those for the MIC experiment, *i.e.*, 6.2×10^5 cfu. After 14 days incubation at 37ºC, there was doubtful growth in the tube containing the 1:32 dilution and no growth in the tube containing the 1:16 dilution of serum from mice administered 10 mg/kg of INH. There was no growth in the tube containing the 1:4 dilution of serum from mice administered 300 mg/kg of 5-Cl-PZA. Therefore, the SITs of INH (after a

10 mg/kg dose) and 5-Cl-PZA (after a 300 mg/kg dose) were 1:16 and 1:4, respectively, demonstrating that 5-Cl-PZA was not only bioavailable but also achieved active serum concentrations. Keeping in view that the MICs of INH and 5-Cl-PZA were 0.06 and 12.5-25 µg/ml, respectively, one may extrapolate from the SIT data that serum concentrations of about 1 µg/ml of INH and 50 to 100 µg/ml of 5-Cl-PZA were present at the time of sampling. Broth containing either no drug or serum from mice administered 300 mg/kg of PZA showed growth at all dilutions, hence the SIT was nil for PZA.

The multi-dose tolerability of 5-Cl-PZA in BALB/C mice was carried out only up to a 300 mg/kg daily dose and not, as planned, up to a dose of 600 mg/kg because the suspension of 5-Cl-PZA in 0.25 per cent CMC was homogenous only up to 300 mg/kg. All animals survived the 2-week treatment well, with no observable adverse effects or changes in body weight.

The day after aerosol infection, the mean lung cfu counts was 2.18 ± 0.05 $\log_{10}$. Treatment was initiated 28 days later, when the mean lung cfu count was 7.38 ± 0.15 $\log_{10}$. No spontaneous mortality was recorded during the course of the study. As shown in Table I, during the treatment period, untreated control mice experienced a spontaneous decrease in lung cfu counts to 6.89 ± 0.28 and 6.40 ± 0.01 $\log_{10}$ units at weeks 4 and 8, respectively. On average, the standard regimen of RIF-INH-PZA reduced the lung cfu counts to 3.30 and 2.18 $\log_{10}$ after 4 and 8 wk of treatment, respectively. As usual, the 2-drug combination RIF-PZA reduced the mean lung cfu counts by about one $\log_{10}$ more than the RIF-INH-PZA combination[18]. Conversely, the reduction in cfu counts induced by RIF-INH was one $\log_{10}$ less than the reduction induced by RIF-INH-PZA after 4 and 8 wk of treatment. A limited response was observed with RIF alone, as the mean lung cfu counts were reduced by approximately 2 $\log_{10}$ less than by RIF-INH-PZA combination. PZA alone at 150 mg/kg was one $\log_{10}$ more active than RIF alone at week 4 and as active as RIF alone at week 8. Finally, the activity of PZA alone at 75 mg/kg was, at week 4, similar to that of RIF alone but no further killing was observed between week 4 and 8 (Table I).

Activity of 5-Cl-PZA alone and in combination: Treatment with 5-Cl-PZA alone at any dose between 37.5 and 150 mg/kg did not result in any decrease of lung cfu counts, even after 8 wk of treatment (Table I).

Table I. Activity of 5-Cl-pyrazinamide (5-Cl-PZA) against *M. tuberculosis* in mice				
Regimens	Lung Log $_{10}$ cfu			
	D-27	D-0	Wk 4	Wk 8
Controls				
1. Untreated	2.18 ± 0.05	7.38 ± 0.15	6.89 ± 0.28	6.39 ± 0.01
2. RIF-INH-PZA 150			3.34 ± 0.25	2.12 ± 0.15
3. RIF-PZA 150			2.53 ± 0.17	1.61 ± 0.51
4. PZA 150			4.05 ± 0.26	3.60 ± 0.20
5. PZA 75			5.29 ± 0.12	6.04 ± 0.24
6. RIF-INH			4.74 ± 0.27	3.39 ± 0.42
7. RIF			5.33 ± 0.14	3.93 ± 0.34
Tests				
8. RIF-INH+ 5-Cl-PZA 150			5.02 ± 0.16	3.78 ± 0.15
9. RIF+ 5-Cl-PZA 150			6.06 ± 0.47	4.81 ± 0.31
10. 5-Cl-PZA 150			7.03 ± 0.23	6.05 ± 0.08
11. 5-Cl-PZA 75			6.8 ± 0.28	5.97 ± 0.21
12. 5-Cl-PZA 37.5			6.9 ± 0.39	5.98 ± 0.15

RIF, rifampicin 10 mg/kg; INH, isoniazid 25 mg/kg; PZA, pyrazinamide at 150 and 75 mg/kg. 5-Cl-PZA, 5-chloro-pyrazinamide at 150, 75, and 37.5 mg/kg. All drugs were given orally 5 days a week. Values are mean ± SD (n=5)
D-27, D-0, and wk 4 and wk 8 refer to the day after infection, the day of treatment initiation, the days after four and eight weeks of treatment were completed, respectively

Table II. Activity of 5-Cl-pyrazinamide (5-Cl-PZA) alone or in combination with first line drugs in mice infected with *M. bovis*

Regimens	Log_{10} lungs cfu counts at the following time-points			
	D-27	D0	Wk4	Wk8
Untreated	1.29 ± 0.16	6.76 ± 0.20	5.95 ± 0.25	6.33 ± 0.40
RIF-INH-PZA			3.63 ± 0.15	1.60 ± 0.16
RIF-PZA			4.37 ± 0.26	4.27 ± 0.16
RIF-INH			2.65 ± 0.38	1.83 ± 0.34
RIF			4.66 ± 0.32	3.69 ± 0.19
PZA			6.28 ± 0.16	5.67 ± 0.15
5-Cl-PZA			6.20 ± 0.40	6.30 ± 0.36
RIF+5-Cl-PZA			5.40 ± 0.25	4.82 ± 0.21
RIF-INH+5-Cl-PZA			2.96 ± 0.28	2.92 ± 0.18

RIF, rifampicin 10 mg/kg; IHH, isoniazid 25 mg/kg; PZA, pyrazinamide at 150 mg/kg; 5-Cl-Z, 5-chloro-pyrazinamide at 150 mg/kg. All drugs were given orally 5 days a week. Values are mean ± SD (n=5)

D-27, D-0, and wk 4 and wk 8 refer to the day after infection, the day of treatment initiation, the days after four and eight weeks of treatment were completed, respectively

The addition of 5-Cl-PZA to RIF, or to RIF+INH, did not demonstrate any benefit *i.e.*, the combination of RIF + 5-Cl-PZA was not more active than RIF alone, and the 3-drug combination RIF+INH+5-Cl-PZA was not more active than RIF+INH (Table I). Overall, 5-Cl-PZA did not demonstrate activity in *M. tuberculosis*-infected mice either alone or in combination with the first-line drugs, RIF and INH. There was even a trend towards a negative impact of the 5-Cl-PZA on the antimicrobial activity of the combined drugs.

Comparative activity of PZA and 5-Cl-PZA in M. bovis infected mice: The aerosol infection of mice resulted in the lung implantation of 1.29 ± 0.16 log_{10} cfu of *M. bovis*. At treatment initiation, 28 days later, the mean lung cfu count was 6.76 ± 0.20 log_{10}. All mice survived throughout the study. In untreated controls, the cfu counts spontaneously decreased to nearly 5.95 ± 0.25 log_{10} at week 4 and remained at the same level at week 8 (Table II). In mice treated with PZA alone or 5-Cl-PZA alone, the lung cfu counts at weeks 4 and 8, were not different than those in untreated controls, indicating lack of activity for PZA and 5-Cl-PZA in mice infected with *M. bovis* (Table II). Similar conclusions may be drawn for the activity of both drugs given in combination with RIF or RIF+INH. Addition of either PZA or 5-Cl-PZA to any combination did not significantly increase the bactericidal activity of RIF or RIF+INH.

Discussion

Despite the relatively promising *in vitro* and *ex vivo* activity at neutral *p*H of 5-Cl-PZA against *M.*

tuberculosis, the present study showed lack of activity of 5-Cl-PZA in mice infected with *M. tuberculosis* or *M. bovis*. MIC was found to be between 12.5 and 25 µg/ml, in agreement with the reported MICs in the range of 8-32 µg/ml[4,5]. In addition, the *ex vivo* data provided by the SIT demonstrated that serum concentrations between 50 and 100 µg/ml were likely achieved in mice administered 300 mg/kg of 5-Cl-PZA. If the blood levels were proportional to the dose, one might expect blood levels to be in the range of 25-50 µg/ml in mice administered 150 mg/kg of 5-Cl-PZA. Such levels would be at or above the MIC and, therefore, may be associated with some *in vivo* activity. Actually, no antimicrobial activity was observed in mice infected either with *M. tuberculosis* or with *M. bovis*. It is curious, however, that at both 4 and 8 wk, in the 5-Cl-PZA+RIF regimens the addition of 5-Cl-PZA appeared antagonistic to the action of RIF against *M. bovis*. The same antagonistic effect was observed on treatment of *M. tuberculosis* with 5-Cl-PZA+RIF at week 8 but not, with PZA-RIF. This effect suggests, with regard to both mycobacterial species, either that 5-Cl-PZA is influencing the population of organisms normally susceptible to RIF or that 5-Cl-PZA has a negative pharmacological interaction with RIF.

Among all possible speculations about the overall lack of *in vivo* efficacy, the simplest and the most likely is that the ratio of C_{max}/MIC obtained with 150 mg/kg of 5-Cl-PZA is not high enough to result in antimicrobial activity *in vivo* in adequate drug exposure. Another possibility is that 5-Cl-PZA unlike PZA lacks

bactericidal activity and is just a bacteriostatic drug. It is emphasized here that this model does not permit discrimination of bacteriostatic activity because one cannot demonstrate growth inhibition against non-actively multiplying bacteria. However, activity in this model appears to indicate the potential for "sterilizing" activity, evidenced by activities of RIF and PZA. At the 150 mg/kg dose, the activity of 5-Cl-PZA might be insufficient to inhibit fatty acid synthase 1 (FAS1), especially when the C_{max} is close to the MIC. Another possible explanation might be the *in vivo* instability of 5-Cl-PZA[5].

Our experiments were performed in the mouse model of chronic TB disease. Mice were infected with a limited inoculum of $2.18 \pm 0.05 \log_{10}$ cfu and were kept without treatment for 4 wk in order to establish in the lungs a stable population that is contained by the specific immunity. Evidence of the efficacious immune containment is provided by the fact that at 28 days after infection, the size of the bacillary population was $7.38 \pm 0.15 \log_{10}$ *M. tuberculosis* cfu and was followed with small but spontaneous decline in the cfu count in untreated controls. Similar experimental conditions were realized in mice infected with *M. bovis*. Thus the activity of PZA and 5-Cl-PZA alone and in combination was tested against a non-actively-multiplying bacillary population, particularly favourable to reveal the sterilizing activity of a drug regimen. Under these experimental conditions, RIF and PZA alone were quite active against *M. tuberculosis* and, as usual[18-20] the combination RIF+PZA was the most potent of all combinations against *M. tuberculosis*. Thus, the lack of any effect of 5-Cl-PZA alone and the lack of any additive effect of the combination of 5-Cl-PZA with RIF did not result from an experimental artifact. It rather demonstrated a lack of 5-Cl-PZA activity in a mouse at the tested dose of 150 mg/kg. It is possible that the compound would be active at a higher dose than 150 mg/kg, but interest in a PZA derivative that would be given at a higher dose than PZA is unlikely because of the risks of potential toxicity.

It is well-known that anti-tuberculosis chemo-therapy is strongly dependent upon interactions between the host, the pathogen, and the chemotherapeutic agent. The unique role of PZA in chemotherapy is a consequence of a precise balance in these interactions. The contradiction between the *ex vivo* antimycobacterial activity of 5-Cl-PZA and the absence of activity in the mouse model of infection suggests the importance of identifying the mode of inactivation in the murine model, whether it be metabolic instability or poor pharmacokinetics. The development of more potent analogues logically depends on structural modifications that lead to better tolerance by the diseased host yet yield compounds that still retain the desired antibacterial properties.

Acknowledgment

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