In vitro and in vivo activity of three oxazolidinones against non-replicating Mycobacterium tuberculosis

Ming Zhang†, Claudia Sala¹, Neeraj Dhar¹, Anthony Vocat¹, Vasan K Sambandamurthy², Sreevalli Sharma², Gwendolyn Marriner³, V. Balasubramanian²#, and Stewart T. Cole¹*

¹ Global Health Institute, Ecole Polytechnique Fédérale de Lausanne, Station 19, 1015 Lausanne, Switzerland
² AstraZeneca India Pvt Ltd, Hebbal, Bangalore, India
³ Tuberculosis Research Section, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892, USA
† Present Address: Department of Biochemistry, Center of Immunity and Infection, University of Lausanne, Epalinges, Switzerland
# Present Address: Cellworks Research India Ltd., Bangalore, India.

Running title: Oxazolidinones for tuberculosis treatment

Keywords: oxazolidinones, tuberculosis, latency

Supplemental material for this article may be found at http://aac.asm.org/

* Corresponding author:

Prof. S.T. Cole
Ecole Polytechnique Fédérale de Lausanne, Global Health Institute
Station 19, 1015 Lausanne, Switzerland
Tel. +41 21 693 1851
Fax +41 21 693 1790
Email: stewart.cole@epfl.ch
Oxazolidinones represent a new class of anti-tuberculosis drugs that exert their function by inhibiting protein synthesis. Here we compared the activities of three oxazolidinones, linezolid, PNU-100480 and AZD5847, against latent tuberculosis using a simple model employing the streptomycin-starved *Mycobacterium tuberculosis* strain 18b. The *in vitro* drug susceptibility results showed that the three oxazolidinones had a bacteriostatic effect against actively growing bacilli, but potent bactericidal activity against non-replicating cells. In the murine model of latent infection with *M. tuberculosis* 18b, the efficacy of the three compounds varied greatly. Indeed, AZD5847 and its pro-drug exhibited no activity or only modest activity after 2 months of treatment, respectively, whereas linezolid and PNU-100480 were both effective against latent bacilli in mice and showed promising outcomes in combination therapy with rifampicin. Moreover, the potency of PNU-100480 was significantly greater than that of linezolid, making it an attractive drug candidate in the development of new combination therapies for latent tuberculosis.

Despite the availability of effective chemotherapy, tuberculosis (TB) is still a major global health problem and >8.6 million cases of active disease and 1.4 million deaths were reported in 2012 (1). Moreover, approximately one-third of the world’s population is latently infected with *Mycobacterium tuberculosis* and at risk of reactivation (1). The slow growth and dormancy of *M. tuberculosis* contribute to the chronic nature of the infection and effective treatment of active disease requires lengthy multidrug regimens. This situation is now worsened by the increasing emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR) and even totally drug-resistant *M. tuberculosis* strains (1), thus underscoring the urgent need for new molecules with potent activity against latent *M. tuberculosis* and no cross-resistance with existing therapeutic agents.

Oxazolidinones are one of the only three new classes of antibiotics that
have been approved by the Food and Drug Administration (FDA) in the last 35 years (2). Oxazolidinones inhibit bacterial protein synthesis via competitive binding to the 23S ribosomal RNA (rRNA) in the catalytic site of the bacterial 50S ribosomes (3, 4). Linezolid (LZD), the first oxazolidinone that entered clinical use, exhibits bacteriostatic activity against *M. tuberculosis* with an MIC of less than 1 μg/ml (5) and has been used against MDR/XDR-TB cases (4, 6, 7). However, long-term administration of LZD may cause severe side effects such as anemia, thrombocytopenia and peripheral neuropathy (8). PNU-100480 (sutezolid, PNU) is an oxazolidinone analogue that has been reported to have an improved safety profile as compared to LZD (2). PNU has an MIC similar to that of LZD for *M. tuberculosis in vitro*, but has more potent bactericidal activity in *ex vivo* whole blood cultures and in a murine model of TB (9-11). In mice, addition of PNU not only enhanced the bactericidal activity during the initial phase of treatment (9), but also increased the sterilizing activity of the standard first-line regimen in long term relapse based combination studies (12). In whole blood assays, PNU showed cumulative activity with TMC207 and SQ109 (13). LZD and PNU were both developed by companies subsequently acquired by Pfizer, and PNU has more recently been transferred to Sequella. Another antitubercular oxazolidinone in clinical development is AZD5847 (AZD), a candidate drug from AstraZeneca (14). AZD has an MIC that equals those of LZD and PNU, and its bactericidal activity has been demonstrated by 14-day daily oral dosing in the murine model of TB (2). Other second-generation oxazolidinones currently in clinical development, such as tedizolid (15) and radezolid (16), are being optimized for the treatment of infections caused by Gram-positive bacteria.

The results of a 14-day Phase II clinical trial showed a modest early bactericidal activity of LZD but no effect during the subsequent 5 days (17), whereas a recent clinical trial of LZD in patients with chronic XDR-TB suggested that LZD had sterilizing ability and was effective for treating MDR/XDR-TB (18). However, the study had limitations due to the small number of patients enrolled. Therefore, the *in vivo* potency of LZD against latent TB remains subject to question. As PNU and AZD have now advanced to phase 2 trials in pulmonary TB patients, they deserve further investigation.
to evaluate their efficacy and to determine whether they are superior to LZD in the case of latent TB.

As a novel class of anti-tuberculosis agents, oxazolidinones are gaining considerable interest and more derivatives will be developed because of their great potential of bactericidal activity on latent TB. A simple, rapid and reliable model is needed for evaluating the sterilizing activities of this class of compounds for future development of new combination regimens. We recently developed such a model for latent TB that uses the streptomycin-dependent \textit{M. tuberculosis} 18b strain; in the absence of streptomycin 18b (SS18b) enters a non-replicating state hypothesized to be that of the latent tubercle bacilli (19, 20). A panel of antitubercular compounds was compared using this model and LZD was one of the drugs capable of killing SS18b \textit{in vitro} (20). In this study we compared the activities of some previously established protein synthesis inhibitors and the three oxazolidinones against latent TB using the SS18b model both \textit{in vitro} and \textit{in vivo}.

\section*{MATERIALS AND METHODS}

\textbf{Bacterial strains and culture conditions.} \textit{M. tuberculosis} strains H37Rv and 18b were grown at 37°C with shaking in Middlebrook 7H9 (Difco) broth supplemented with 10% albumin-dextrose-catalase (ADC) enrichment, 0.2% glycerol, 0.05% Tween 80 and, in the case of 18b, 50 μg/ml streptomycin (STR), or on solid Middlebrook 7H10 medium (Difco) supplemented with 0.5% glycerol, 10% oleic acid-albumin-dextrose-catalase (OADC), and 50 μg/ml STR. Non-replicating SS18b was generated as follows: 18b was grown to mid-logarithmic phase in STR-containing medium and washed three times in phosphate buffered saline containing 0.05% Tween 80 (PBST). The bacterial pellets were re-suspended in medium without STR. SS18b cultures were maintained at an OD\textsubscript{600} between 0.2 and 0.5 for 2 weeks (with the addition of fresh medium if necessary) by which time they have stopped replicating.
Antimicrobials. STR, isoniazid (INH), rifampicin (RIF), kanamycin, amikacin, hygromycin, chloramphenicol, clarithromycin and fusidic acid were purchased from Sigma-Aldrich. Other drugs were from AstraZeneca (LZD, AZD5847, AZD5847 phosphate pro-drug (pAZD)), Janssen Pharmaceuticals (TMC207), and NIAID (PNU). Drugs for in vivo experiments were either dissolved or suspended before gavage as follows: RIF in water (21); LZD and PNU in 5% polyethylene glycol-200 and 95% methylcellulose (0.5%) (12); AZD in 0.5% hydroxypropyl methylcellulose (HPMC) plus 0.1% Tween 80; pAZD in dextrose-NaCl solution. Drug solutions for mouse treatment were prepared weekly and stored at 4°C.

Resazurin reduction microplate assay (REMA). To determine the in vitro efficacy of compounds, a two-week-old SS18b culture (OD$_{600}$ = 0.1) and an H37Rv culture (OD$_{600}$ = 0.0002) were used in REMA. Two (or three)-fold serial dilutions of each test compound were prepared in 96-well plates containing tubercle bacilli in a total volume of 100 μl and then incubated for seven days at 37°C before addition of 10 μl of 0.025% resazurin. After overnight incubation, fluorescence of the resazurin metabolite resorufin was determined (excitation at 560 nm and emission at 590 nm, Gain 80) by using a TECAN Infinite M200 microplate reader.

Drug susceptibility testing. Drug treatment was performed both in actively growing M. tuberculosis H37Rv cells and dormant SS18b cells at 37°C with shaking. Mid-logarithmic phase M. tuberculosis cultures were diluted to an OD of 0.05, split into 10-ml samples, and drugs were added at the concentrations indicated in the legend to Fig. 2, with an untreated sample serving as a control. Serial dilutions of the cultures were plated on 7H10 supplemented with glycerol, OADC, and STR (in the case of SS18b) at day 7 after addition of drugs. The STR-dependent phenotype was checked by plating the same dilutions on 7H10 without STR. Colony forming units (CFU) were counted after 4 to 5 weeks of incubation at 37°C.

Hypoxia model. Antimicrobial activity against hypoxic non-replicating M. tuberculosis H37Rv cultures was measured as described earlier (22) with minor modifications. Briefly, H37Rv cells were grown in Dubos Tween broth in
McCartney bottles with a magnetic bead using a defined head-space ratio (HSR) of 0.5. Methylene blue was added as a redox indicator (final concentration of 1.5 μg/mL) to all bottles to monitor oxygen depletion. The McCartney bottles are placed on a magnetic stirrer set at 180 rpm, inside a 37°C incubator. The methylene blue indicator started to fade by day 8 and was completely decolorized by 12 days. The antimicrobial activity of various compounds against non-replicating cells was determined in 96-well microtiter plates using a 14-day old hypoxia adapted culture. The entire assay was performed in a hypoxic chamber (DuPoy) by exposing hypoxic cells to varying concentrations of compounds for 7 days at 37°C. An anaerobic indicator strip was placed inside the chamber to visually confirm the removal of oxygen during the entire process. Bacterial enumeration was performed on Middlebrook 7H11 agar plates after incubation of plates for 21-28 days at 37°C, 5% CO₂ in a humidified atmosphere. INH was used as a control to check for hypoxic activity.

**Mice and infection.** Female BALB/c mice, aged 5 to 6 weeks, were obtained from Charles River Laboratories. For infections, logarithmic-phase 18b cells were suspended in PBST with 50 μg/ml STR, and delivered intravenously (i.v.) in the lateral tail vein at 10⁷ CFU per mouse. STR was given subcutaneously (s.c.) at a dose of 100 mg/kg of body weight once daily, 5 times/week for 3 weeks. Experiments were approved by the Swiss Cantonal Veterinary Authority (authorization n° 2218).

**Treatment.** Drug treatment was initiated 4 weeks after infection. Drugs were administered by gavage at the following doses (mg/kg): RIF, 10; LZD, 100; PNU, 100; AZD, 125; pAZD, 500. AZD and pAZD were given 6 times a week for 8 weeks. When combined with RIF, oxazolidinones were administered 1 hour after RIF. Treatment efficacy was assessed by counting the number of CFU in lungs at the end of treatment. Control and treated mice were sacrificed, the lungs were homogenized, and dilutions were plated on 7H10 agar enriched with 10% OADC and supplemented with STR (50 μg/ml), cycloheximide (10 μg/ml), and ampicillin (50 μg/ml). The plates were incubated for 28 days at 37°C before CFU were enumerated. In a different
mouse study, LZD and PNU were administered 5 times a week for 4 weeks. Treatment efficacy was assessed by lung and spleen CFU counts at the end of treatment.

Statistical analysis. CFU counts were log_{10} transformed before analysis, expressed as mean log_{10} CFU ± standard deviation (SD), and compared using unpaired Student’s t tests in Prism GraphPad version 6.0.

RESULTS

In vitro activities against SS18b determined by REMA. In our initial work, we showed that fusidic acid, a weak protein synthesis inhibitor, was slightly active against SS18b (19, 20) and this prompted us to assess the behavior of other translation inhibitors in this model. We therefore tested the old drugs, amikacin, chloramphenicol, clarithromycin, fusidic acid and hygromycin using the REMA and CFU assays. The results showed that these compounds, especially amikacin and hygromycin, were active against non-replicating 18b cells (Supplementary Figure 1). Encouraged by these findings, we then tested the activities of three oxazolidinones (LZD, PNU and AZD) and control drugs against SS18b using the REMA assay.

Fig. 1 shows the results obtained after 7 days of incubation of SS18b cultures with the compounds. The positive control drugs, RIF and TMC207, whose activity against non-replicating bacilli has been well demonstrated (19, 20), consistently caused a concentration-dependent decrease in fluorescence. INH was used as a negative control showing no activity against dormant cells but rather an increase in fluorescence, a common signature of cell wall inhibitors (20). The three oxazolidinones were all effective against SS18b. For both LZD and PNU, the activity of the drug was already seen by fluorescence readout at 50 ng/ml with maximum activity at 400 ng/ml, whereas for AZD activity was only observed at 150 ng/ml or at higher concentrations. In all cases, M. tuberculosis H37Rv served as a reference strain to determine the MICs of all compounds, and the results indicated that all three oxazolidinones were active against growing bacteria with a similar
MIC about 400 ng/ml (Fig. 1).

Validation of in vitro activities against SS18b by CFU levels. To validate the drug susceptibility results obtained by REMA, we then measured the bactericidal activities of the three oxazolidinones by CFU determination. Fig. 2 shows the results of the experiments carried out on actively growing M. tuberculosis H37Rv (Fig. 2A) and non-replicating SS18b bacilli (Fig. 2B). The untreated H37Rv culture showed more than 1 log increase in the number of CFU over 7 days of incubation. Both INH and RIF were active, leading to decreases in CFU of 1.7 log \((p = 0.0026)\) and 1.3 log \((p = 0.0041)\), respectively. All three oxazolidinones were able to prevent the growth of the bacteria at the concentrations tested. However, LZD and AZD did not cause a significant decrease in terms of CFU numbers, indicating that they are bacteriostatic drugs. On the other hand, PNU was the only bactericidal oxazolidinone compound, although it only caused a 0.3-log \((p = 0.03)\) reduction in the number of CFU.

The above-mentioned drugs were then tested against SS18b. No growth was seen in the absence of STR in the untreated sample, confirming that 18b cells were arrested in the non-growing state. Addition of INH did not cause a significant decrease in CFU level \((p = 0.1012)\), whereas RIF had potent activity against non-replicating 18b cells with a 1.8 log decrease \((p = 0.0034)\) in the number of CFU, in agreement with previous reports \((19, 20)\). Contrary to what was observed when cultures were actively multiplying, the three oxazolidinones exhibited strong killing activity against dormant cells and caused a 1.8-log CFU reduction \((p < 0.01)\), similar to that obtained with RIF. Taken together, these data confirmed the results obtained by REMA.

Efficacy of the oxazolidinones against non-replicating bacteria was also assessed using the Wayne model of hypoxia \((22)\). Interestingly, when tested against hypoxic M. tuberculosis H37Rv cells, both PNU and AZD displayed good bactericidal activity, mirroring the results obtained with SS18b, whereas LZD performed less well (Supplementary Figure 2).
**In vivo activities of AZD5847.** Interestingly, oxazolidinones displayed greater bactericidal activity against non-replicating than vs. growing cells *in vitro*, which prompted us to investigate their efficacy against SS18b *in vivo*. After infection with *M. tuberculosis* 18b, BALB/c mice received STR for 3 weeks to allow bacterial replication in the lungs. Treatment began 10 days after STR withdrawal, when the mice had established a chronic infection. In the first experiment, AZD alone and in combination with RIF was tested. After 2 months of treatment (Fig. 3), a reduction of more than 3 logs in the number of CFU (*p* < 0.0001) was observed in the lungs of mice treated with RIF in comparison with the lungs of untreated mice. On the contrary, AZD treatment did not cause any decrease in lung CFU numbers, suggesting that it had no bactericidal activity against non-replicating bacilli in mice. Furthermore, the combination of AZD and RIF was as potent as RIF alone (*p* = 0.7533), indicating no synergistic, additive or antagonistic effects.

The discrepancy between the *in vivo* and *in vitro* potency of AZD was further investigated in another mouse study that examined the efficacy of pAZD, the phosphate pro-drug of AZD5847 that provides better pharmacokinetic and pharmacodynamic parameters (AstraZeneca, unpublished results). pAZD alone and in combination with RIF was tested in the same SS18b mouse model of chronic infection. After 1 month of treatment (Fig. 4A), RIF caused a 2 log reduction in the bacterial burden in the lung, while pAZD showed no activity against SS18b *in vivo*. In addition, the potency of RIF combined with pAZD was equivalent to that of RIF alone. At the end of the experiment, after 2 months of treatment (Fig. 4B), pAZD only reduced the bacterial burden by 0.6 log (*p* = 0.0027) in mouse lungs, a considerably poorer performance compared to RIF (3 log killing, *p* < 0.0001). Moreover, combination of pAZD and RIF showed neither synergistic nor additive effects.

Overall, these results indicated that AZD was not effective at killing latent SS18b *in vivo*, or the activity was minimal in the case of its pro-drug.

**In vivo potency of linezolid and PNU-100480.** The efficacy of LZD and PNU was tested in the same mouse model of SS18b chronic infection. Fig. 5 shows the CFU results from mouse lungs (Fig. 5A) and spleens (Fig. 5B) after 1 month of treatment. RIF reduced the bacterial burden by 2.1 logs...
(p < 0.0001) and 1.6 logs (p < 0.0001) in the lungs and spleens, respectively. LZD was effective against dormant bacilli and had better activity in the spleens (1 log CFU reduction, p = 0.0002) than in the lungs (0.6-log CFU reduction, p = 0.0026). In contrast, PNU exhibited greater efficacy than LZD as it decreased the infection level by 2.5 (p < 0.0001) and 1.7 (p < 0.0001) logs in the lungs and spleens, respectively. When combined with RIF, LZD caused an additional 0.6 log reduction in both mouse lungs (p = 0.0095) and spleens (p = 0.029), while PNU further reduced the number of CFU by 2.1 logs in both lungs (p = 0.0011) and spleens (p = 0.0014). These data confirmed that PNU has stronger bactericidal activity against non-replicating \textit{M. tuberculosis} than LZD at the doses tested, in accordance with the findings from other \textit{in vivo} studies (9, 12). Moreover, no antagonism was found between RIF and LZD or PNU, suggesting that the two oxazolidinones could be included in the development of a more potent combination therapy with rifamycins.

**DISCUSSION**

We compared the bactericidal activity of various old and new protein synthesis inhibitors, including three oxazolidinones, in the non-replicating SS18b model of latent TB. Of the previously examined drugs, amikacin and hygromycin showed modest activity and were able to reduce CFU levels \textit{in vitro} by about 40% compared to the activity of RIF. By contrast, all three oxazolidinones were equally potent to RIF against non-growing bacilli. However, their \textit{in vivo} efficacy varied extensively. PNU had significantly greater activity than LZD at a dose of 100 mg/kg. The enhanced activity of PNU compared to LZD is not consistent with their similar MICs nor can it be explained by a more favorable pharmacokinetic profile since the steady state area under the concentration-time curve (AUC) for PNU and its metabolites is three times lower than the AUC for LZD (9). Other factors must therefore play a role, including permeability through the eukaryotic cell membrane and intracellular accumulation. Indeed, in a whole blood assay it has been shown that PNU accumulated in macrophages, which could partially explain the superior activity of PNU over LZD (10, 11). Importantly, our study...
demonstrated that PNU has the strongest bactericidal activity against non-replicating SS18b in the murine model and, in combination with RIF, resulted in at least an additive effect that caused significant reduction of the bacterial burden in the animals. It is worth pointing out that the results obtained here in the SS18b model, in terms of CFU log reduction over time upon treatment with PNU, match what has been reported in BALB/c mice chronically infected with H37Rv (9, 12). This validated potent activity of PNU in both chronic and non-replicating mouse models of TB shows promise for the compound in the clinic. Together with its low toxicity profile in humans (10, 11), PNU therefore deserves further attention in the context of developing new combination therapies for active and latent tuberculosis.

The most recent of the three oxazolidinones, AZD, unfortunately showed no detectable activity against 18b in vivo. However, the phosphorylated pro-drug form, pAZD, which has improved pharmacokinetic properties, did display modest activity at the highest concentration tested after 2 months of treatment. Contrary to the findings obtained with all the other drugs tested here and in previous studies (19, 20), the in vitro potency of AZD was not mirrored by in vivo efficacy in the SS18b model. Previous investigations from AstraZeneca have shown that AZD is active in an ex vivo model (14) and in an in vivo model using H37Rv (23) indicating that, like PNU, this oxazolidinone can cross the eukaryotic cell membrane and thus reach the bacteria engulfed in the macrophages. PNU, the active metabolite of PNU and LZD are 52%, 89% and 69% free in human plasma, respectively, as compared to AZD, which is 20% free (24, 25). Therefore, relatively poor efficacy of AZD in the SS18b model as compared to LZD or PNU could be due to differences in exposure to free drug at the target site. Nevertheless, further studies have to be undertaken in order to explain these differences.

Taken together our results showed that oxazolidinones, and some other ribosome inhibitors, especially amikacin and hygromycin, were active against SS18b cells but to different extents. Notably, oxazolidinones were more active on non-multiplying cells than rapidly growing bacteria. This suggests that translation might still occur at a very low level in the absence of
streptomycin and that de novo protein synthesis is required during latency. Thus, in our experiments, the oxazolidinones probably shutdown the residual protein synthesis thereby killing the non-replicating bacilli. Alternatively, oxazolidinones may irreversibly damage ribosomes leading to insufficient translational capacity remaining to support growth when the SS18b cells reactivate.

One can also not exclude the possibility that there may be a second oxazolidinone target in latent *M. tuberculosis* and therefore SS18b is effectively killed via inhibition of both this target and residual translation. Genetic studies confirmed that mutations in the peptidyl transferase center of 23S rRNA or in genes encoding ribosomal proteins (*rplV*, *rplD* and *rplC*) are associated with oxazolidinone resistance, thereby defining the main target (26). In addition, Sander and co-workers identified two classes of LZD-resistant mutants and described a non-ribosomal mechanism of resistance in *M. smegmatis*, which was verified by the *in vitro* peptidyl trasferase assay (27). This finding was supported by another study where mutations leading to low-level LZD resistance occurred in unidentified but non-ribosomal genes in *M. tuberculosis* (28).

Overall, the data reported here indicate that the translation machinery is a vulnerable target during latent tuberculosis infection, similarly to transcription, ATP biosynthesis and DNA metabolism (19, 20). Due to their great potency, oxazolidinones represent a class of translation inhibitors that offers tremendous potential for developing more powerful anti-tuberculosis regimens in general as well as better regimens for the treatment of MDR/XDR cases.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Koen Andries and C. E. Barry III for kindly providing drugs. The research leading to these results received funding from the European Community’s Seventh Framework Programme (Grant...
REFERENCES


FIGURE LEGENDS

FIG. 1. Resazurin reduction microplate assays (REMA). Serial 3-fold dilutions of each drug were prepared in a 96-well plate, and their activities against \textit{M. tuberculosis} strains H37Rv and SS18b evaluated by REMA. Graphs show the correlation between drug concentration on the x-axes and fluorescence levels on the y-axes. Bars represent the means ± standard deviation of two experiments.

FIG. 2. \textit{In vitro} drug susceptibility test against actively growing \textit{M. tuberculosis} H37Rv and non-growing SS18b. (A) \textit{M. tuberculosis} H37Rv culture was split into several samples and incubated with the different drugs. Serial dilutions were made and plated 7 days after addition of drugs. (B) A SS18b culture was divided into several samples and processed as described for the experiment shown in panel A. Bars represent the means ± standard deviation of two experiments. Statistically significant differences relative to untreated control (No Drug) were calculated using the Student’s t-test. *, \( p < 0.05 \); **, \( p < 0.005 \); ***, \( p < 0.0005 \); ns, no significant difference. Drug concentrations: INH, 0.25 μg/ml; RIF, 0.5 μg/ml; LZD, 2.5 μg/ml; PNU, 2.5 μg/ml; AZD, 2.5 μg/ml.

FIG. 3. \textit{In vivo} efficacy of AZD5847 against non-replicating \textit{M. tuberculosis} 18b. \textit{M. tuberculosis} 18b-infected mice were treated for 2 months with RIF, AZD or their combination, starting 1 month post-infection. Untreated controls (NT) and treated animals were sacrificed at the end of treatment, and CFU were determined by plating lung homogenates on 7H10 plates with STR. Bars represent the means ± standard deviation of groups of 4 mice. Statistically significant differences relative to NT (for AZD and RIF) or to RIF (for RIF+AZD) were calculated using the Student’s t-test. ***, \( p < 0.0005 \); ns, no significant difference.

FIG. 4. \textit{In vivo} efficacy of pAZD against non-replicating \textit{M. tuberculosis} 18b. \textit{M. tuberculosis} 18b-infected mice were treated for 2 months with RIF, pAZD or their combination, starting 1 month post-infection. Untreated controls (NT) and treated animals were sacrificed after 1 month (A) and 2 months (B) of treatment.
treatment, and CFU were determined by plating lung homogenates on 7H10 plates with STR. Bars represent the means ± standard deviation of groups of 4 mice. Statistically significant differences relative to NT (for pAZD and RIF) or to RIF (for RIF+pAZD) were calculated using the Student's t-test. **, p < 0.005; ***, p < 0.0005; ns, no significant difference.

FIG. 5. In vivo efficacy of LZD and PNU against non-replicating M. tuberculosis 18b. Mice infected with M. tuberculosis 18b were treated for 1 month with the indicated drugs or drug combinations, starting 1 month post-infection. Untreated controls (NT) and treated animals were sacrificed at the end of treatment, and CFU were determined by plating lung (A) and spleen (B) homogenates on 7H10 plates with STR. Bars represent the means ± standard deviation of groups of 4 mice. Statistically significant differences relative to NT (for RIF, LZD or PNU alone) or to RIF (for combinations) were calculated using the Student's t-test. * p < 0.05; **, p < 0.005; ***, p < 0.0005; ****, p < 0.0001.