Microbes and Infection 26 (2024) 105376

Contents lists available at ScienceDirect

Microbes and Infection

journal homepage: www.elsevier.com/locate/micinf

Original article

Efficacy of macozinone in mice with genetically diverse susceptibility to *Mycobacterium tuberculosis* infection

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ARTICLE INFO

Article history:
Received 4 April 2024
Accepted 4 June 2024

Keywords:
Antitubercular agents
Benzothiazinones
Comparative analysis
Tuberculosis

ABSTRACT

Host heterogeneity in pulmonary tuberculosis leads to varied responses to infection and drug treatment. The present portfolio of anti-TB drugs needs to be boosted with new drugs and drug regimens. Macozinone, a clinical-stage molecule targeting the essential enzyme, DprE1, represents an attractive option. Mice (I/St, B6, (AKRxI/St)F1, B6.I-100 and B6.I-139) genetically diverse susceptibility to *Mycobacterium tuberculosis* (*Mtb*) H37Rv infection were subjected to aerosol- or intravenous infection to determine the efficacy of macozinone (MCZ). They were treated with macozinone or reference drugs (isoniazid, rifampicin). Lung and spleen bacterial burdens were measured at four and eight weeks post-infection. Lung histology was evaluated at four weeks of treatment. Treatment with macozinone resulted in a statistically significant reduction in the bacterial load in the lungs and spleen as early as four weeks after treatment initiation in mice susceptible or resistant to *Mtb* infection. In the TB hypoxic granuloma model, macozinone was more potent than rifampicin in reducing the CFU counts. However, histopathological analysis revealed significant lung changes in I/St mice after eight weeks of treatment initiation. Macozinone demonstrated efficacy to varying degrees across all mouse models of *Mtb* infection used. These results should facilitate its further development and potential introduction into clinical practice.

1. Introduction

Tuberculosis (TB) remains one of the biggest public health threats worldwide [1]. The current drug pipeline for TB management [2] includes well-established options, but may lead to the development of resistance. Thus, there is an urgent need for anti-TB drugs that affect completely different targets [3] in *Mycobacterium tuberculosis*, as well as improved drug regimens. The treatment outcomes of tuberculosis infection also depend on a myriad of factors, including genetic variation in the host response to the infection [4,5]. Moreover, the present TB drug discovery process primarily relies on one or two animal models to prove the efficacy of TB compounds. Yet, it is poorly understood how TB preclinical candidates exhibit efficacy in genetically diverse animal models that mimic the heterogeneous human population.

The most popular model of *Mtb* infection in mice, which is considered to be as close as possible to infection in humans is the Kramnik model. The Kramnik group identified the *sst1* locus (susceptibility tuberculosis 1) on chromosome 1 [6,7] using contrasting lines of mice C57BL/6J and C3HeB/FeJ. Mice of both inbred C57BL/6J and C3HeBFe/J can be infected with *Mtb*, but there are some differences in the course, nature and severity of the infection. These include the level of mycobacterial load in organs, the survival time of infected animals, and the pathological process in the lungs. By all these measures, C3HeBFe/J mice are significantly less defenseless and defective than C57BL/6J mice. In addition, hypoxic/necrotic granulomas are formed in the lungs of C3HeBFe/J mice during *Mtb* infection. Mice segregating at the *sst1* locus show marked

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https://doi.org/10.1016/j.micinf.2024.105376

differences in the growth rate of virulent tubercle bacilli in the lungs. Lung lesions in susceptible mice are characterized by extensive necrosis and unrestricted extracellular proliferation of virulent mycobacteria. The gene *Ipr1* (intracellular pathogen resistance 1) was further localized within the *sst1* locus by positional cloning methods [7,8].

Macozinone (MCZ, PBTZ169·HCl) is an advanced benzothiazinone that inhibits mycobacterial cell wall synthesis by covalently targeting the crucial DprE1 enzyme [9–12]. Being a next-in-class agent, macozinone is more potent *in vitro* against *M. tuberculosis* (*Mtb*) strains, including multidrug- (MDR) and extensively drug-resistant (XDR) isolates than other derivatives of this chemical class [13–16]. Its safety profile and potent bactericidal activity have been confirmed in preclinical and clinical trials (clinicaltrials.gov, Phase Ia, NCT03423030 and NCT03036163; Phase Ib, NCT03776500 and NCT04150224; Phase IIb, NCT03334734). Thus, the molecule represents a drug candidate with great potential for use in treatment regimens for tuberculosis.

To answer whether host genetic susceptibility to *Mtb* infection affects the efficacy of macozinone, we have compared the efficacy of the molecule on a genetically diverse panel of inbred mice with various susceptibility to *Mtb* H37Rv infection ranging from highly susceptible I/St mice to highly resistant (AKRxI/St)F1 hybrids (Table 1). In addition, we evaluated its efficacy in an I/St mouse model of *Mtb* lung hypoxic granuloma to investigate how the presence of hypoxic granuloma might influence the potency of macozinone. The goal of this work was to investigate and confirm the efficacy of macozinone in various mouse models differing in susceptibility or resistance to *Mtb* infection, as observed in the human population.

2. Materials and methods

2.1. Ethics and animals

Animal work was conducted according to the protocols approved by the Animal Care and Use Committee of the Central Institute for Tuberculosis (CIT). Six-eight week-old female I/St, B6, and (AKRxI/St)F1 mice weighing 21.0 ± 0.8 g were raised in the laboratory animal nursery of the CIT. Two-and-half-three-month old female B6.I.100 and B6.I.139 mice weighing 21.0 ± 0.9 g were bred in the CIT [17].

2.2. M. tuberculosis *strain and drugs*

The *M. tuberculosis* reference strain H37Rv was a gift from Gilles Marchal (Institut Pasteur, France) and was prepared as described in the online data supplement.

Macozinone (MCZ), manufactured in accordance with GMP guidelines, was kindly provided by Nearmedic Pharma (Russia). Rifampicin (RIF) and isoniazid (INH) were purchased from Sigma-Aldrich.

2.3. I/St, B6, B6.I-100 and B6.I-139 mouse models of Mtb infection

Infection. I/St, B6, B6.I-100 and B6.I-139 mice were infected with 1×10^2 CFU/lung of *Mtb* strain H37Rv by aerosol route using a Glas-Col® whole-body inhalation exposure system (Glas-Col) as previously described [18]. *Treatment*. Infected mice were randomized into 22 groups ($n = 5$). Treatment was initiated 3 weeks following *Mtb* infection with MCZ at a daily dose of 50 mg/kg *per os* or INH at a daily dose of 25 mg/kg *per os*. Mice receiving INH served as a positive control and mice receiving distilled water (0.2 mL) *per os* served as a negative control (untreated). The pre-treatment animal group (start or 0) represent infected and untreated mice sacrificed prior to the start of drug treatment to control the infection. Treatment was continued for four weeks (B6.I-100 and B6.I-139) and eight weeks (I/St and B6). Mice were euthanized by exposure to carbon dioxide at the selected time point, and the lungs and spleen were removed for histopathological examination and CFU measurement. *Histopathology*. Right lung tissue samples were frozen under a temperature gradient from −20 °C to −60 °C in a Cryotome® E electronic cryostat (ThermoShandon), and serial 6-8-mm-thick sections were made through the widest area of the lobe. Lung cryosections were fixed with acetone and stained with hematoxylin-eosin. The slides were examined by a professional pathologist and photographed using an Axioskop 40 microscope and an AxioCamMRc 5 camera (Carl Zeiss).

Colony Forming Unit (CFU) Counts. The spleen was homogenized in 2.0 mL of sterile saline using a Heidolph SilentCrusher M homogenizer at 15,000 rpm for 1 min. The left lung was sliced into 1–2 mm^3 pieces and incubated at 37 °C for 90 min in RPMI-1640 medium (Thermo Fischer Scientific) supplemented with 5% fetal calf serum, 200 U/mL collagenase and 50 U/mL DNase-I (Sigma-Aldrich), followed by vigorous pipetting to obtain a single-cell suspension. 10-Fold serial dilutions of 0.2 mL samples were plated on Dubos agar (Difco™, BD), and incubated at 37 °C for 21 days before CFUs were counted.

2.4. I/St and (AKRxI/St)F1 mouse models of Mtb infection

Infection. I/St and (AKRxI/St)F1 mice were inoculated with 0.2 mL of a suspension containing $5 \cdot 10^5$ and $5 \cdot 10^6$ CFU, respectively, of H37Rv in PBS with 0.05% Tween 80 per mouse intravenously in the lateral tail vein using a disposable sterile INEKTA syringe with a 26G needle. *Treatment*. Infected mice were randomized into 8 groups ($n = 5$). Treatment was initiated 4 weeks following *Mtb* infection with MCZ at a daily dose of 50 mg/kg *per os*. Mice that received water for 4 weeks served as the untreated control. The pre-treatment group represented infected and untreated mice. Treatment was continued for eight weeks. Mice were euthanized at the studied time point, and the lungs and spleen were removed for further analysis as described above.

Table 1
Mouse strains used in this study.

Strain Name	Abbreviated Name	*H2* Haplotype	Susceptibility to *Mtb* infection	Comments
I/StSnEgY/Cit	I/St	$H2A^jE^j$	Highly susceptible [29]	Also used as a model of TB hypoxic granuloma
C57BL/6J/Cit	B6	$H2A^bE^0$	Resistant [35]	
B6.I-249.1.15.100	B6.I-100	$H2A^jE^j$	Relatively susceptible [17]	Recombinant congenic strain between C57BL/6 recipient and I/St donor
B6.I-249.1.15.139	B6.I-139	$H2A^bE^j$	Relatively resistant [17]	
(AKRxI/St)F1	(AKRxI/St)F1	$H2^{kj}$	Highly resistant	Hybrid F1 inbred strain derived from a cross of AKR x I/St

2.5. I/St mouse Model of TB hypoxic granuloma

Infection. I/St and B6 (was used as a control) mice were infected with 1×10^2 CFU/lung of strain H37Rv via aerosol using a Glas-Col® system as described above. *Treatment*. Infected mice were randomized into 6 groups ($n = 5$). Treatment was started 8 weeks after *Mtb* infection to ensure hypoxic granuloma formation in the lungs of I/St mice. Three animal groups received MCZ at a daily dose of 50 mg/kg *per os* and three groups received RIF at a daily dose of 10 mg/kg *per os*. The pre-treatment group represented infected and untreated mice. Treatment was continued for eight weeks. Mice were then euthanized, and the lungs and spleen were removed for CFU measurement as described above and histopathological examination as described below. *Pimonidazole Immunohistochemical Analysis*. The MCZ animal groups at the studied time point (4 and 8 weeks) were injected with 60 mg/kg body weight of pimonidazole hydrochloride (Hypoxyprobe™-1 Kit, Chemicon International) and sacrificed 3 h later. Lung cryosections were obtained and prepared for indirect peroxidase staining to detect tissue hypoxia according to the manufacturer's protocol. The slides were examined by a professional pathologist and photographed using a microscope and camera.

2.6. Statistical analysis

Excel and GraphPad Prism 9 software were used to perform all statistical data analysis as described in the online data supplement.

3. Results

3.1. Efficacy study in mice with genetically diverse susceptibility to Mtb infection

In the lungs, the MCZ treatment groups demonstrated a statistically significant reduction in CFUs in all mice genetically susceptible or resistant to *Mtb* infection compared to the pre-treatment control group after the 4-week treatment (Fig. 1A, Table S1). After 8 weeks of treatment, a notable decrease in the lung bacterial load, comparable to the INH treatment group, was also achieved in mouse models independently of their genetic susceptibility to infection ($p < 0.0001$ compared to pre-treatment and untreated controls for all cases).

The bacterial load in the spleen of infected untreated mice susceptible to TB infection – I/St and B6.I-100 mice – increased significantly over time, as expected (Fig. 1B). Two I/St mice died at 8 weeks after the TB aerosol challenge due to an extremely high mycobacterial load, so this group was excluded from the study. In contrast, the bacterial burden in the spleen of infected untreated mice that were more resistant to infection – B6 and B6.I-139 mice – predictably showed a smaller increase (Fig. 1B). Following 4 weeks of treatment, the MCZ treatment group showed efficacy superior to the pre-treatment group and comparable to the INH treatment group in all mice, regardless of their genetic susceptibility or resistance to *Mtb* infection (Fig. 1B). The reduction in CFU after MCZ therapy was 0.76-$\log_{10}$ and 2.20-$\log_{10}$ ($p = 0.0052$ and < 0.0001, respectively, Table S1) in susceptible I/St and B6.I-100 mice and 0.83-$\log_{10}$ CFU and 1.89-$\log_{10}$ CFU ($p = 0.0117$ and < 0.0001, respectively, Table S1) in resistant B6 and B6.I-139 mice. After 8 weeks, the MCZ treatment group also exhibited a notable CFU reduction in the spleen of I/St and B6 mice compared to the pre-treatment and untreated groups ($p < 0.0001$ for all cases, Table S1).

Using two-way analysis of variance with Tukey's multiple comparison tests, the tissue bacterial loads after MCZ treatment were compared for differences based on mouse models. A significant difference in the spleen bacterial burden was observed only between I/St mice, susceptible to infection, and B6 mice, resistant to infection, after 4 weeks of treatment ($p < 0.0001$), but no statistically significant difference was seen between MCZ treatment groups in these mouse models after 8 weeks ($p = 0.4857$). B6.I-100 and B6.I-139 мice, which are relatively susceptible or resistant to *Mtb* infection, exhibited similar treatment outcomes, and no statistically significant difference in CFU reduction was observed between these mouse models after the 4-week MCZ treatment ($p = 0.9996$; $p = 0.8884$ and $p = 0.2415$ compared to B6 mice, respectively). When comparing the efficacy of MCZ treatment groups in the lungs following a 4-week treatment course, a statistically significant difference was observed between *Mtb*-genetically susceptible or resistant mouse models ($p < 0.0001$ for all cases). There is no statistical difference in the lung CFUs only between B6.I-100 and B6.I-139 mice after MCZ treatment (Table S1).

Histopathology analysis was performed on slides of fixed lung cryosections after 4-week treatment with macozinone (Figs. 2 and 3). In I/St mice, pulmonary lesions are characterized by multiple foci of specific inflammation (Fig. 2A). In areas beyond these foci, multiple small hemorrhages in the alveolar lumens, clusters of macrophages, and areas of decreased ventilation (atelectasis) were observed. The pulmonary vessels became engorged, and the alveolar walls were significantly thickened due to infiltration by activated macrophages. Pulmonary lesions are surrounded by a cellular cuff, composed of large macrophages, mononuclear and epithelioid cells, and some lymphocytes. In the lungs of B6 mice, lung tissue appears to be normal, and only minor disease-related changes were observed (Fig. 2B). A small number of lesions were surrounded by foamy macrophages, represented mainly by monocytes with slight differentiation into binuclear macrophages.

Evaluation of lung histopathology in B6.I-100 and B6.I-139 mice showed that the morphological features of tuberculosis were more pronounced in B6.I-100 mice, relatively susceptible to *Mtb* infection, than in relatively resistant B6.I-139 mice (Fig. 3). Untreated B6.I-100 mice displayed severe granulomatous inflammation whereas B6.I-139 mice showed non-granulomatous chronic pulmonary inflammation. Following a 4-week course of MCZ treatment, the severity of lung histopathology decreased in B6.I-100 mice compared to the treatment with INH (Fig. 3). Although granulomatous inflammation was still present in the lung tissue, the granulomas became smaller and did not form clusters. In contrast, pronounced chronic pulmonary inflammation in B6.I-139 mice diminished after INH treatment.

To investigate the impact of macozinone treatment on pulmonary immune responses of B6.I-100 and B6.I-139 mice, we assessed the levels of common immune cell populations and their phenotypes (Figures S1-3). As expected, untreated B6.I-100 mice developed a more robust inflammatory response compared with B6.I-139 mice after 8 weeks of infection, with significantly elevated levels of $CD4^+$ and $CD8^+$ T cells ($p \leq 0.0001$), B cells ($p = 0.0006$), neutrophils ($p = 0.0133$) and macrophages ($p = 0.0019$; Figure S1) recruited to the lungs. MCZ treatment led to a remarkable decrease in lung cellularity ($p < 0.0001$ and $= 0.0170$ for B6.I-100 and B6.I-139 mice) as well as several immune cells, including $CD4^+$ ($p = 0.0132$ and 0.0237, respectively) and $CD8^+$ ($p = 0.0115$ and 0.0028, respectively) T cells and their activation (Figure S1, S2). The decline in inflammation was also accompanied by a decrease in the production of pro-inflammatory cytokines IFN-γ and TNF-α (Figure S3). A 4-week INH treatment was equally effective in B6.I-100 and B6.I-139 mice: no statistically significant differences were detected in the levels of the immune cell populations studied. At the same time, an influx of immune cells persisted in the lungs of

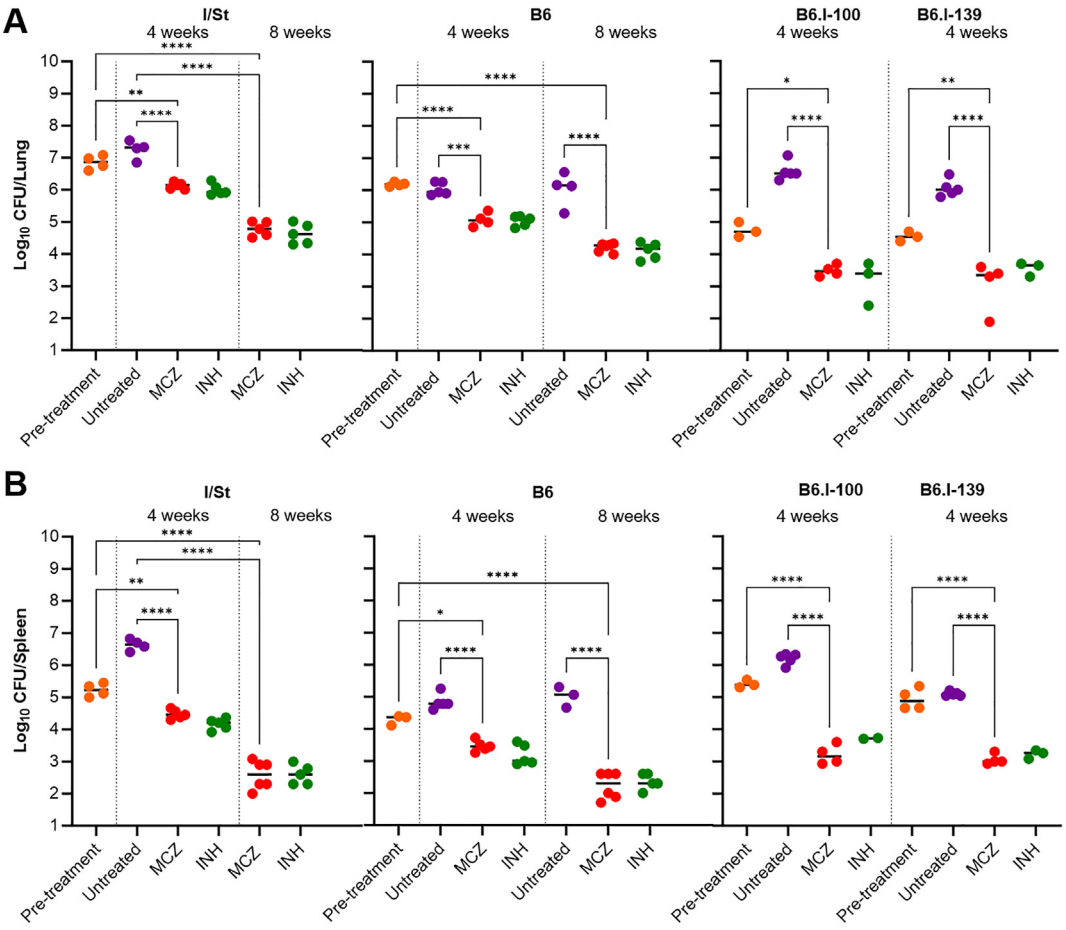

Fig. 1. Efficacy of macozinone in mice with genetically different susceptibility to *M. tuberculosis* infection. Three weeks following a 1×10^2 CFU of *M. tuberculosis* H37Rv by aerosol infection, mice were administered macozinone at a daily oral dose of 50 mg/kg. (A) Determination of Log_{10} CFU in the lungs of I/St and B6 mice after four and eight weeks of treatment, B6.I-100 and B6.I-139 mice after four weeks of treatment. (B) Determination of log_{10} CFU in the spleen of I/St and B6 mice after four and eight weeks of treatment, B6.I-100 and B6.I-139 mice after four weeks of treatment. Two I/St mice from the untreated control group died, so this group was excluded. The pre-treatment animal group represent mice infected within three weeks. The untreated animal group represent mice receiving water for four or eight weeks. The isoniazid-receiving (25 mg/kg) animal group served as a positive treatment group. Each dot represents a CFU value for each animal in the groups, and the line is the median. The lower limit of CFU detection in the lungs and spleens was 1.0 log. Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison test: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations used: CFU, colony forming unit; MCZ, macozinone; INH, isoniazid.

B6.I-100 mice compared to B6.I-139 mice after MCZ treatment, suggesting the need for a longer treatment course.

3.2. *Efficacy study in mice with directly opposite genetic susceptibility to Mtb infection*

We previously demonstrated that (AKRxI/St)F1 hybrids exhibit the highest resistance to *Mtb* infection among all inbreds or hybrids examined [19,20]. Building on this, we conducted a comparative analysis of macozinone efficacy in mice belonging to directly opposite genetic susceptibility to infection – highly susceptible I/St vs highly resistant (AKRxI/St)F1 mice – after intravenous inoculation with H37Rv. To standardize the *Mtb* infection process in the models studied, we used different doses of *Mtb* for inoculation, as described previously [21].

The lung pathology for both untreated animal groups was comparable after 4 weeks of infection (Fig. 4A and B). However, tighter aggregations of inflammatory mononuclear infiltrates were observed in the lungs of I/St mice compared to F1 mice. The (AKRxI/St)F1 hybrids suppressed mycobacterial growth of in the lungs ($p = 0.0001$, Table S2) even in the absence of drug therapy. The spleen CFU reduction in I/St and (AKRxI/St)F1 mice was markedly greater compared to pre-treatment controls after 4 and 8 weeks of treatment ($p < 0.0001$ for both cases) (Fig. 4C). High levels of CFU reduction were also achieved 4 and 8 weeks after MCZ treatment ($p < 0.0001$ for all cases) in the lungs of highly resistant (AKRxI/St) F1 mice (Fig. 4D).

3.3. *Efficacy study in an I/St mouse Model of TB hypoxic granuloma*

Necrosis and hypoxia, key features of human tuberculosis lesions, are difficult to reproduce in most mouse models, but hypoxic granulomas and necrotic lesions are known to develop in I/St mice in response to *Mtb* infection [22]. To mirror tuberculosis as it occurs in humans, we used low-dose aerosol *Mtb* infection of I/St mice. Hypoxic lung granulomas in these mice develop approximately eight weeks after infection.

In the 4th week of treatment, MCZ demonstrated efficacy superior to the pre-treatment control group and the RIF group ($p < 0.001$ for both cases) (Fig. 5A). The lung bacterial burden was reduced by 2.63 logs with MCZ treatment, whereas only a slight decrease was observed in the RIF-treated animal group (Table S3). The CFU reduction in the lungs of B6 mice was 1.53 log after MCZ therapy and 0.42 log after RIF therapy (Fig. 5B). After 8 weeks, the

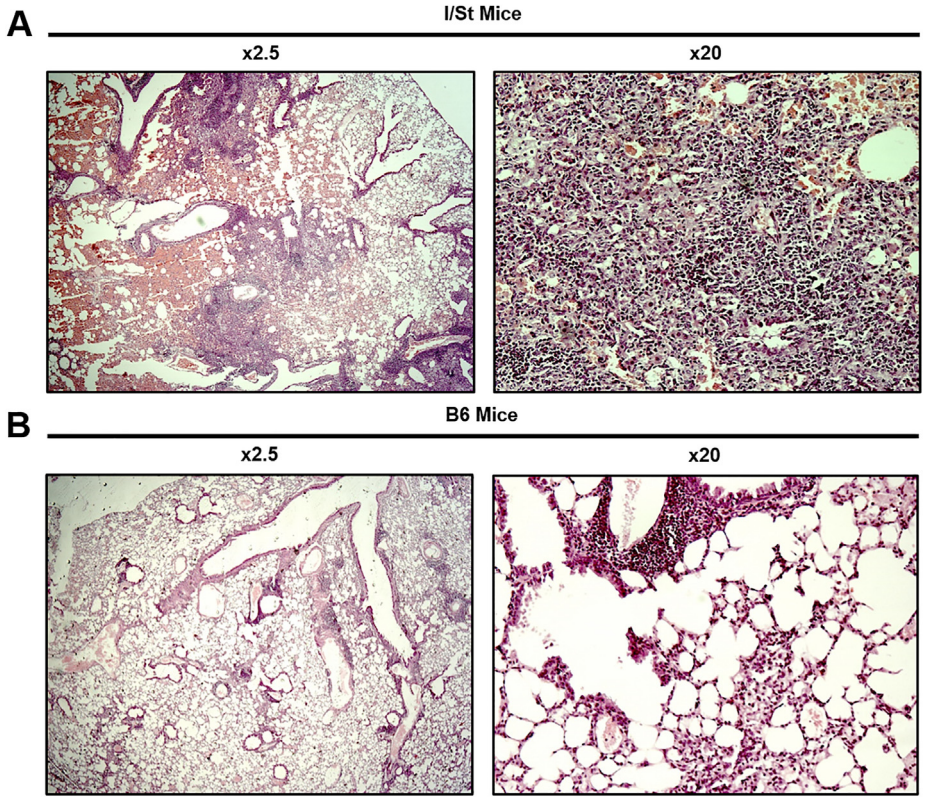

Fig. 2. Histopathology analysis of lung sections of I/St (A) and B6 (B) mice after 4 weeks of treatment with macozinone. Lung cryosections were stained with hematoxylin-eosin. The slides were photographed using an Axioskop 40 microscope and an AxioCamMRc 5 camera, magnifications are × 2.5 or 20, as indicated.

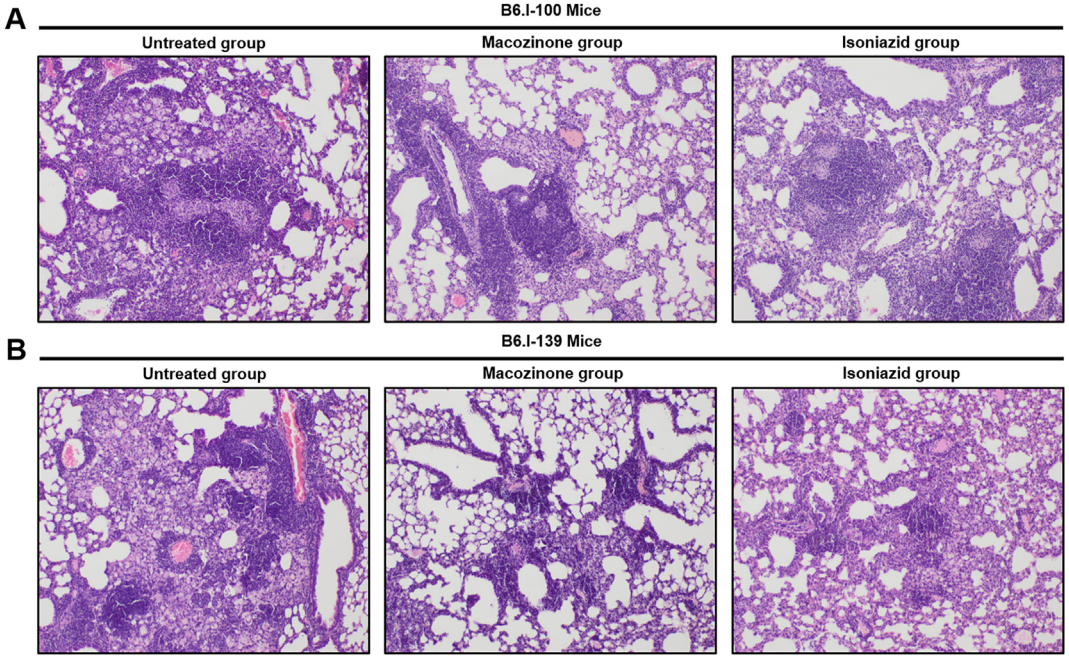

Fig. 3. Histopathology analysis of lung sections of B6.I-100 (A) and B6.I-139 (B) mice after 4 weeks of treatment with macozinone, or isoniazid, or untreated (animals receiving water). Histological preparations of lungs were fixed with 10% formaldehyde and embedded in paraffin. Hematoxylin and eosin staining was performed according to standard techniques. Photography was carried out on a Leica DM4000 B-M light microscope (Germany) using an N PLAN 100x/1.25 Oil lens and a Leica camera.

MCZ group also achieved a significant reduction in pulmonary CFUs superior to that in the RIF group ($p < 0.0001$) in the I/St mouse model of TB hypoxic granuloma (Fig. 5A). In the B6 non-granulomatous mouse model, RIF was more effective compared to MCZ ($p < 0.001$), reducing the bacterial load in the lungs by 3.41 log (Fig. 5B).

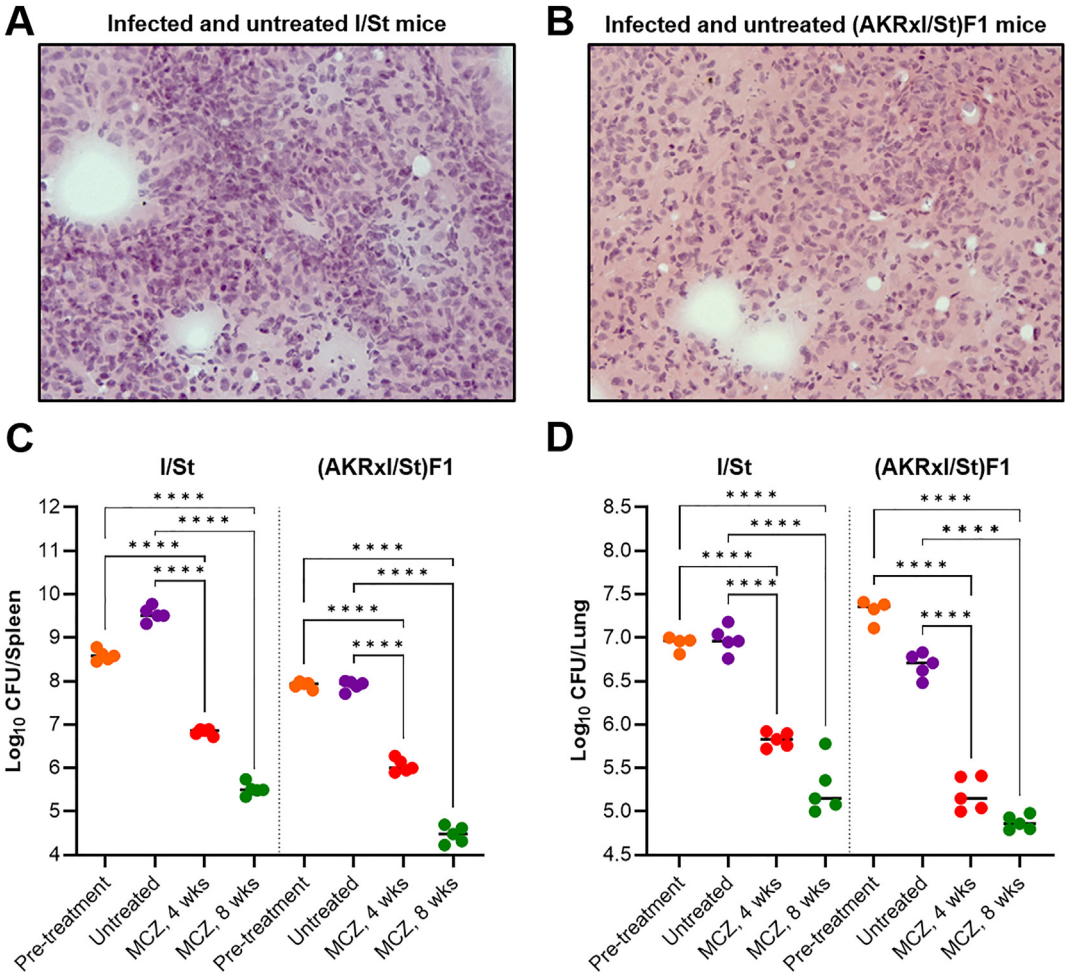

Fig. 4. Efficacy of macozinone in mice of directly opposite genetic susceptibility to *M. tuberculosis* infection. (**A**) Lung pathology of untreated I/St and (AKRxI/St)F1 mice after 4 weeks post intravenous *M. tuberculosis* H37Rv inoculation. Magnifications are × 200. (**B**) Three weeks following a $5{\cdot}10^5$ (I/St) or $5{\cdot}10^6$ (F1) CFU of *M. tuberculosis* by intravenous inoculation, mice were administered macozinone at a daily oral dose of 50 mg/kg. The pre-treatment animal group represent mice infected within three weeks. The untreated animal group represent mice receiving water for four or eight weeks. Each dot represents a CFU value for each animal in the groups, and the line is the median. The lower limit of CFU detection in the lungs and spleens was 1.0 log. Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison test: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

4. Discussion

Host heterogeneity in pulmonary tuberculosis might impact the potency of anti-TB medicines, and addressing heterogeneity is important to improve TB control and elimination [23,24]. Our TB modelling study, reflecting the diverse response of a human population to infection by employing a panel of different mouse models, provides evidence for the efficacy of the novel anti-TB drug candidate macozinone. Our findings complement the work by Smith et al. [25], in which they demonstrate that genetically diverse mice, in their response to *Mtb* infection, were not fully protected by BCG vaccination, and extend our previous work showing the role of genetic diversity in treatment with TB drugs [21]. Although host diversity in humans may arise through different mechanisms than in mice, the end result is the creation of various populations of *Mtb* in diverse physiological conditions that relate to the diversity of populations seen in human populations.

We used mice with different genetic backgrounds — B6, I/St, (AKRxI/St)F1 — but the same genetic background [17] — B6.I-100 and B6.I-139 — differing only in one gene encoding the β-chain of the H2-A class II heterodimer (Table 1). This approach allows us to examine how various genes and host mechanisms regulate interactions with mycobacteria. Mice susceptible to *Mtb* infection are not able to prevent mycobacterial growth, leading to faster development of severe lung damage. In contrast, the immune system of resistant mice is able to reduce the physiological activity of *M. tuberculosis* and control its growth. The results from our comparative analysis revealed that macozinone exhibited greater efficacy in mice susceptible to *Mtb* infection (I/St, B6.I-100) than in resistant mice (B6, B6.I-139) (Fig. 1, Table S1). This finding could be related to the specific mechanism of action of DprE1 inhibitors in general, and macozinone in particular. Being involved in cell wall synthesis, the DprE1 enzyme is essential for mycobacterial growth and survival [26,27]. Thus, the covalent DprE1 inhibitor, macozinone, demonstrated enhanced efficacy in I/St and B6.I-100 mice where mycobacterial growth is more rapid. Our results are consistent with those obtained by Lenaerts et al. [28], where they used B6 mice knocked out for the gamma interferon gene known to be responsible for resistance to *Mtb* infection. These mice lack immune protection, leading to a fast-paced development of tuberculosis. However, treatment with isoniazid and other standard anti-TB drugs resulted in significant lung CFU reduction in these gamma interferon gene-disrupted mice [28]. Surprisingly, a great decrease in the lung bacterial load was observed in (AKRxI/St)

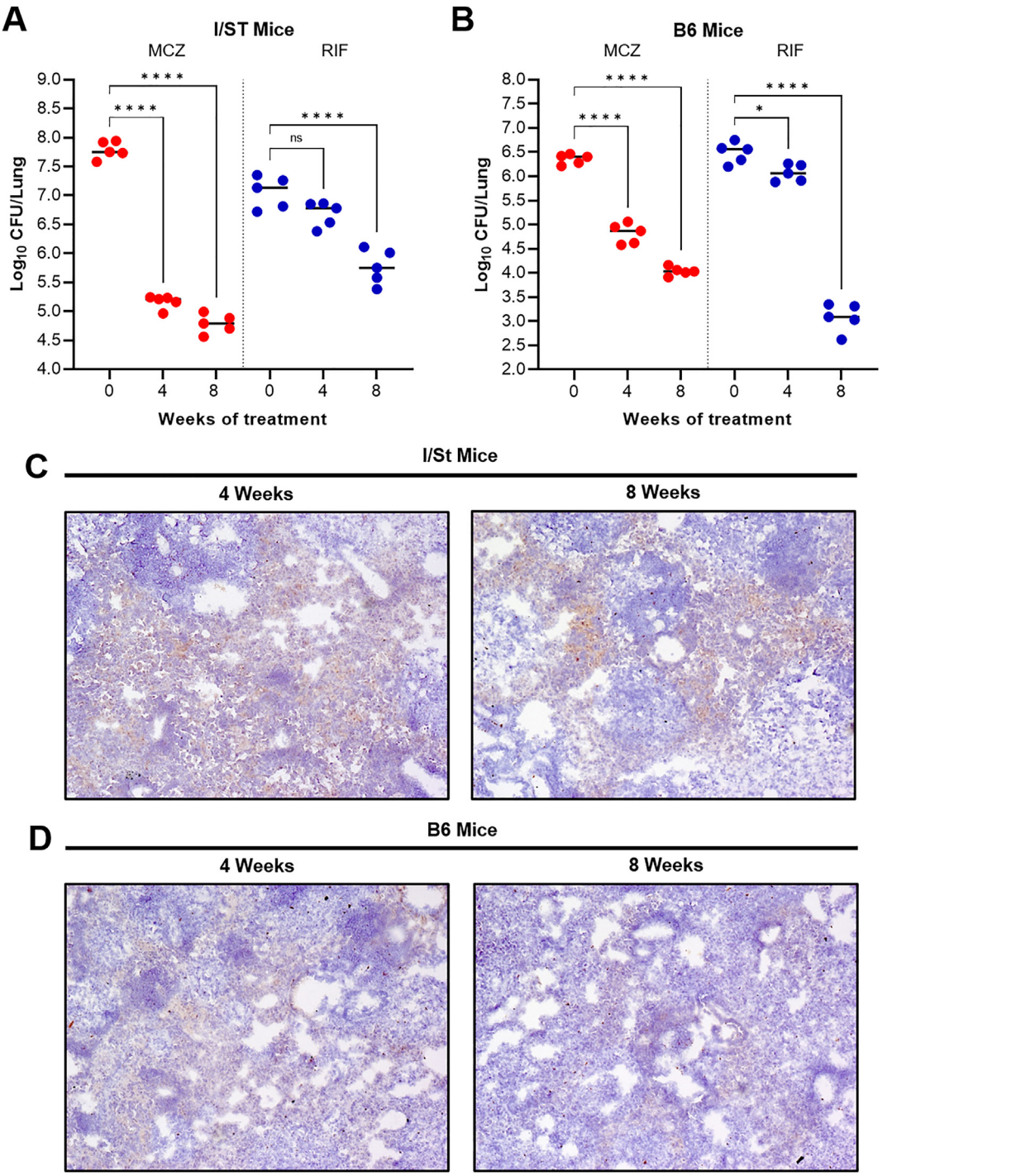

Fig. 5. Efficacy of macozinone in a mouse model of TB hypoxic granuloma. Eight weeks following a low-dose (1×10^2 CFU) aerosol *M. tuberculosis* H37Rv infection, mice were administered macozinone at a daily oral dose of 50 mg/kg or rifampicin at a daily oral dose of 10 mg/kg. Determination of Log_{10} CFU in the lungs of I/St (**A**) and B6 (**B**) mice after four and eight weeks of treatment with macozinone or rifampicin. The animal group labeled "0" represent mice infected within eight weeks. The RIF-receiving animal group served as a positive treatment group. Each dot represents a CFU value for each animal in the groups, and the line is the median The lower limit of CFU detection in the lungs and spleens was 1.0 log. Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$; ns, non-significant. Lung pathology of untreated I/St (**C**) and B6 (**D**) mice after four and eight weeks after treatment with macozinone. Lung cryosections were stained with pimonidazole to detect hypoxic areas. The slides were photographed using an Axioskop 40 microscope and an AxioCamMRc 5 camera, magnifications are × 10. Hypoxic lesions formed in the lungs of I/St mice are circled.

F1 mice highly resistant to *Mtb* infection (Fig. 4, Table S2). This finding may be related not only to the efficacy of macozinone but also to the host's natural suppressor activity. (AKR × I/St)F1-vaccinated mice recently demonstrated the highest survival rate compared to other mouse strains [20]. In any case, macozinone was able to reduce the lung and spleen bacterial burdens in all mouse models studied (Figs. 1 and 4, Tables S1, S2).

Comparison of the immune response of B6.I-139 and B6.I-100 mice to *Mtb* infection revealed that B6.I-100 mice, relatively susceptible to infection, developed more severe lung injury and

exhibited a higher recruitment of immune cells compared with B6.I-139 mice relatively resistant to infection. A 4-week treatment with macozinone or a control drug significantly reduced inflammation in the lungs of both strains. However, residual inflammation remained in the lungs of B6.I-100 mice after treatment with macozinone, indicating that distinct treatment regimens might be required for susceptible and resistant mice.

The hypoxic TB granuloma, a pathological hallmark of tuberculosis, can only be reproduced in a few animal models: I/St and C3HeB/FeJ mice have demonstrated extremely high susceptibility to *M. tuberculosis* strains H37Rv and Erdman, respectively [29,30]. These mice possess distinct genetic backgrounds: while the *Ipr* gene, located on chromosome 1 in C3HeB/FeJ mice, mediates innate immunity to tuberculosis infection [8], the H2Aβ gene, located on chromosome 17 in I/St mice, is involved in the adaptive immune response to infection [17]. Our study revealed that macozinone significantly reduced the lung bacterial load in the I/St hypoxic granuloma model four weeks after treatment, while rifampicin had a delayed effect (Fig. 5, Table S3). These findings contradict a recent paper by Robertson et al. [31], in which they reported that the lung CFU decrease in a C3HeB/FeJ mouse model of TB necrotic granuloma (Kramnik model) was only observed after eight-week treatment with macozinone. In addition, we demonstrated that macozinone notably reduced spleen CFUs after 4 weeks of treatment (Fig. 1), while Robertson and colleagues stated that PBTZ169 did not yield statistically significant CFU reduction in the spleen at any dose after 4 weeks. Although these mice have a distinctive genetic background [32] that provides a different response to drug treatment, this interesting difference in the macozinone treatment outcomes in different models of TB hypoxic granuloma warrants a detailed comparative investigation. Despite the sharp lung/spleen CFU decrease following macozinone treatment, histopathology analysis of the I/ST mouse lungs revealed that macozinone did not eliminate hypoxic lesions, at least within the 8-week treatment course.

The main objective of this study was to understand how host heterogeneity influences the efficacy of macozinone in monotherapy. As such, a potential limitation of our study is that we did not evaluate the combination of our drug candidate with standard TB drugs in these models, and this should be the subject of further studies.

Overall, our data demonstrate that macozinone decreased the bacterial burden across mouse models, manifesting as early as four weeks into the treatment regimen. Conventional TB treatment guidelines recommend a standard 6-month regimen of at least four-drugs [33,34], and shorter-duration drug regimens hold promise in mitigating development of rapid drug resistance. Initiatives like the Project to Accelerate New Treatments for Tuberculosis (PAN-TB) and the European Regimen Accelerator for Tuberculosis (ERA4TB) aim to overcome the challenges posed by current treatments by identifying promising new TB drug regimens. Along with the significant early bactericidal activity observed in the Phase IIa study (clinicaltrials.gov, NCT03334734), our findings strongly suggest that the inclusion of macozinone in investigational drug regimens may shorten TB treatment duration.

CRediT authorship contribution statement

Boris Nikonenko: Writing – original draft, Methodology, Investigation. **Nadezhda Logunova:** Writing – original draft, Resources, Methodology, Investigation, Data curation. **Anna Egorova:** Writing – original draft, Data curation. **Marina Kapina:** Methodology, Investigation, Formal analysis, Investigation. **Natalia Sterzhanova:** Investigation. **Irina Bocharova:** Project administration, Methodology, Investigation. **Elena Kondratieva:** Writing – original draft, Project administration, Methodology, Investigation, Data curation. **Olga Riabova:** Methodology, Investigation, Data curation. **Lyudmila Semyonova:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Vadim Makarov:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

V.M. is an inventor on macozinone patents.

Acknowledgements

We are deeply grateful to Dr Barry III Clifton (National Institute of Allergy and Infectious Disease), Dr Daniela Maria Cirillo, and Dr Nicola Ivan Lorè (San Raffaele Scientific Institute) for very helpful comments on the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2024.105376.

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