Establishment of an Inhalation Administration Noninvasive Murine Model for Rapidly Testing Drug Activity against *Mycobacterium tuberculosis*

Xirong Tian [1,2,3,4,*], Yamin Gao [1,2,3,4,*], Wanli Ma [1,2,3,4], Jingran Zhang [1,2,3,5], Yanan Ju [1,2,3,5], Jie Ding [1,2,3, 6], Sanshan Zeng [1,2,3,4], H.M. Adnan Hameed [1,2,3,4], Nanshan Zhong [7], Gregory M. Cook [4,8,9,10], Jinxing Hu [7, 11, #] and Tianyu Zhang [1,2,3,4,7,#]

[1] State Key Laboratory of Respiratory Disease, Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences (CAS), Guangzhou 510530, China, [2] Guangdong-Hong Kong-Macao Joint Laboratory of Respiratory Infectious Diseases, Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences (CAS), Guangzhou 510530, China, [3] University of Chinese Academy of Sciences (UCAS), Beijing 100049, China, [4] China-New Zealand Joint Laboratory on Biomedicine and Health, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China, [5] School of Life Sciences, University of Science and Technology of China, Hefei 230026, China, [6] Institute of Physical Science and Information Technology, Anhui University, Hefei, 230601, China, [7] Guangzhou National Laboratory, Guangzhou 510005, China, [8] Department of Microbiology and Immunology, School of Biomedical Sciences, University of Otago, Dunedin, 9054, New Zealand, [9] Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Private Bag 92019, Auckland, 1042, New Zealand, [10] Translational Research Institute, Queensland University of Technology, Brisbane, Queensland, 4102, Australia, [11] State Key Laboratory of Respiratory Disease, Guangzhou Chest Hospital, Guangzhou, China

[*] These authors contributed equally to this work.

[#] Corresponding author: Jinxing Hu (hujinxing2000@163.com) and Tianyu Zhang (zhang_tianyu@gibh.ac.cn)

ABSTRACT

Mycobacterium tuberculosis, as one of the most serious infectious pathogens, results in millions of deaths and a tremendous economic burden for both individuals and governments. Many potential drugs for the treatment of tuberculosis show excellent activity against *M. tuberculosis in vitro*, but lack significant activity *in vivo* when administered traditionally by either oral or injection delivery routes. Even though previous researches revealed that inhaled administration method can enhance the efficacy of drugs against mycobacteria, while this evaluating process was complicated and time-consuming by using wild type *M. tuberculosis*. Conversely, our research uncovered that the inhalation administration model using strains of autoluminescent *M. tuberculosis* may provide a more rapid, non-invasive, lung-targeted mid-throughput method, may avoid significant toxic effects on other organs, for evaluating the anti-tuberculosis activity of compounds in mouse models of infection. The live relative light units, quantifying the light emitted by autoluminescent *M. tuberculosis* $H_{37}Rv$, can be used to preliminarily evaluate the therapeutic effect of different drug doses, which can significantly shorten the evaluation time. This model offers the ability to assess the efficacy of new lead compounds and TB drugs that can be administered by inhalation.

KEYWORDS

Inhalation administration, Autoioluminescence, Tuberculosis, Murine model, Chemotherapy

INTRODUCTION

Mycobacterium tuberculosis (Mtb) primarily transmitted via respiratory droplets, stands as the causative agent of human tuberculosis (TB) and accounted for the highest mortality attributed to a single infectious agent prior to the 2019 coronavirus pandemic (McQuaid et al., 2021, Shah et al., 2022). Approximately one quarter of the global population is latently infected with Mtb, with an estimated 5-10% progressing to active TB over their lifetimes (Ahmad et al., 2022). The rise in prevalence of drug-resistant Mtb strains is largely attributable to antibiotic misuse and patient non-compliance, posing substantial public health risks and imposing considerable economic burdens (Dousa et al., 2020, Hameed et al., 2018, McGrath et al., 2014, Shoen et al., 2018). Consequently, there is a pressing demand for novel anti-TB medications characterized by enhanced efficacy, minimal toxicity, and a lack of cross-resistance with existing therapies to address these challenges (Grüber, 2020, Liu Pingxian et al., 2019).

In conventional *in vivo* efficacy evaluation experiments for anti-tuberculosis drugs, oral or various injection routes of administration are frequently employed (Namvarpour et al., 2019, Yu et al., 2020, Yu et al., 2021). However, these traditional administration methods fail to specifically target the lungs during absorption, transport, and metabolism, while also inducing significant toxicity in other organs (Xu et al., 2019, Yoon et al., 2017, Zhao et al., 2023). Over recent decades, inhalation therapy has become a preferred treatment method for respiratory diseases (Barnes et al., 2015, Sanchis et al., 2013). Despite previous attempts to

develop models for assessing inhaled drug administration, these models encounter various challenges necessitating further improvement.

The lung-targeted inhalation drug delivery method devised in this study effectively overcomes the aforementioned limitations. Drug activities administered via inhalation can be assessed in live mice infected with autoluminescent Mtb H37Rv (AlRv) in a time-efficient, non-invasive, and cost-effective mid-throughput manner. This model represents a valuable tool for evaluating the efficacy of inhalable lead compounds, frontline anti-TB medications, and therapeutic regimens, thereby providing clinicians with invaluable insights.

MATERIALS AND METHODS

Mycobacterial strains and culture conditions

The AlRv strain, genetically engineered to contain the *luxCDABE* gene cluster within its genome, was preserved at -80 □ (Zhang et al., 2012). AlRv displays a consistent emission of blue-green light independently, attributed to the enzymatic activity encoded by the *luxCDE* genes, which continuously produces the necessary substrate aldehyde (Zhang et al., 2012). Remarkably, AlRv demonstrates a susceptibility to drugs comparable to that of the wild type Mtb H37Rv strain (Zhang et al., 2012). The quantification of colony forming units (CFUs) was replaced with the assessment of relative light units (RLUs), maintaining a ratio of approximately 10:1 (Zhang et al., 2012).

The AlRv was subcultured in Middlebrook 7H9 broth (Difco, Detroit, MI, USA), supplemented with 10% oleic-acid-albumin-dextrose-catalase enrichment medium

(BBL, Sparks, MD, USA), 0.2% glycerol, and 0.05% Tween 80 at 37 □. The AlRv was cultured until the optical density, measured at 600 nm, reached 0.6-0.8, with a corresponding RLUs level of 5×10^6/mL. The optical density was measured using a V-1000 spectrophotometer (AOE, Shanghai, China), and the RLUs were quantified using the GLOMAX 20/20 luminometer (Promega, Madison, USA).

Antimicrobials

All pharmaceutical compounds, including rifampicin (RIF), isoniazid (INH) and ethambutol (EMB) were purchased from Meilun (Dalian, China) with a minimum purity threshold of ≥ 95%. RIF and INH were solubilized in sterilized water, serving as the solvent, to attain stock concentrations of 2 mg/mL, which were subsequently diluted to 0.5 and 0.25 mg/mL, respectively. EMB were similarly dissolved into sterilized water at its original concentration of 10 mg/mL, then further diluted to 2.5 and 0.625 mg/mL. The prepared solutions of the drugs were refrigerated at 4°C until administration.

Aerosol infection determination of infection bacterial burden

The female BALB/c mice (Gempharmatech, Foshan, China), aged six to eight weeks, were subjected to an acclimatization period lasting five to seven days before the initiation of experimental procedures. Subsequently, all mice were exposed to homogenous AlRv culture of 10 mL using an inhalation exposure system (Glas-Col, Terre Haute, IN) (Yu et al., 2020, Yu et al., 2021). The total exposure duration was two hours, comprising preheating (15 minutes), atomization (60 minutes), defogging (15 minutes), and disinfection (25 minutes) procedures.

Determination of the initial bacterial number

After infection, the mice were randomly divided into treatment and solvent groups, each consisting of six individuals. On the initial day of treatment administration, anesthesia was administered individually to six mice via exposure to a container containing 0.5 mL of isoflurane. Loss of consciousness typically occurred within approximately 10 seconds. Subsequently, the live RLUs of mice were measured by aligning their chests with the detection position of the GLOMAX 20/20 luminometer. Upon detection of the live RLUs, six mice were euthanized via cervical dislocation and positioned on a foam board with large head pins to secure them, ensuring their abdomens were oriented upwards. The mice were sterilized by spraying 75% ethanol and dissected to obtain the lungs. The organs were then placed into 2 mL sterilized phosphate-buffered saline, washed once with phosphate-buffered saline, and homogenized using glass tissue grinders. The lung suspensions were diluted using a 10-fold serial dilution method. Subsequently, the RLUs of 0.5 mL of undiluted and 10-fold diluted lung suspensions were measured using the GLOMAX 20/20 luminometer. Based on the detected RLUs and the ratio of CFUs to RLUs, the CFUs of the lung suspension were briefly speculated upon. The o.5 mL properly diluted lung suspension was plated on 7H10 plates containing trimethoprim, actidione, carbenicillin and polymyxin B to prevent contamination. All plates were then incubated in a 37□ thermostatic incubator for four weeks, following which the CFUs were determined.

Chemotherapy

Each group of six mice received daily administration of either 4 mL of solvent or drug solutions using the Tow Systems Nose-Only Exposure Units (Tow-Int, Shanghai, China), as previously described (Fan et al., 2022, Yang et al., 2021, Zhao et al., 2023). The device of administration is illustrated in Figure 1. Subsequently, each mouse was secured using the holder and connected to the exposure cabinet. The drug solution or solvent were injected into the atomizer, which utilizes high frequency oscillation technology through microporous screens to generate nebulized aerosols with diameters ranging from 1 to 5 micrometers for inhalation. The drug exposure duration for each group spanned approximately 20-25 minutes, until 4 mL of the solutions had been administered.

The study consists of two main experiments, labeled as Experiment 1 and Experiment 2. The experimental protocols remained largely consistent, with the primary variations involving adjustments in drug dosages and the timing of drug administration post-infection. Detailed protocols for Experiment 1 and 2 were provided in Tables 1 and 2, respectively. The live RLUs of the solvent group were continuously monitored from the day 11 for Experiment 1 (or day 13 for Experiment 2) post-infection. Once the live RLUs of the solvent -treated mice exceeded 400, the RLUs of all groups were measured. Based on our experiences, live RLUs exceeding 400 indicated a higher level of lung RLUs and CFUs, suitable for evaluating drug efficacy. Treatment of mice was discontinued upon reaching live RLUs exceeding 400. The total duration of administration for Experiment 1 and 2 were 14 and 15, respectively. Subsequently, all mice were euthanized the following day to determine

lung RLUs and CFUs following the method mentioned above. All plates were then incubated in a 37℃ thermostatic incubator for four weeks, after which CFUs were determined.

Statistical analysis

Log-transformed data using GraphPad Prism version 8.3.0 was used to determine drug efficacy through a two-way analysis of variance with $P < 0.05$ as statistical significance.

RESULTS

The results of experiment 1.

In experiment 1, the initial CFUs of the infected bacteria approached approximately 4000. The live RLUs of all treatment groups were significantly lower than that of the solvent group, with all differences exceeding 200 (Figure 1, $P < 0.0001$). Additionally, these live RLUs were comparable to those of the before-treatment (BT) group (Figure 1, $P > 0.05$). Furthermore, the lung RLUs of all drug-treated groups were substantially lower than those of the solvent group, with all $\log_{10}$RLUs/lung differences exceeding 1.5 (Figure 1, $P < 0.0001$). Specifically, the lung RLUs of the RIF or EMB-treated groups were nearly identical to those of the BT group (Figure 1, $P > 0.05$), while the INH-treated group exhibited slightly lower lung RLUs than the BT group (Figure 1, $P < 0.05$). Moreover, the lung CFUs of all treatment groups were markedly lower than that of the solvent group, as all differences were over 1.5 (Figure 1, $P < 0.0001$). The RIF group had higher lung CFUs, whereas the INH group had lower lung CFUs compared to the BT group (Figure 1, $P < 0.01$ and $P <$

0.0001, respectively). The lung CFUs of EMB-treated group were almost similar to those of the BT group (Figure 1, $P > 0.05$). The data from experiment 1 indicated that the autoluminescence-based inhalation delivery method can be utilized to establish a noninvasive model for testing drugs activities *in vivo*, using the same batch of live mice within 18 days after infection. Furthermore, the effectiveness of drugs can be further evaluated as either bacteriostatic or bactericidal by comparing lung CFUs of the different treatment groups rather than RLUs, as lung RLUs of the BT group were close to the background values.

The results of experiment 2.

Based on the findings of Experiment 1, two additional dosages of each drug were added in Experiment 2. The initial CFUs of the infected bacteria, in the Experiment 2, were found to be approximately equivalent to those recorded in Experiment 1. The live RLUs of the groups treated with RIF 2, INH 2, INH 0.5, EMB 10, EMB 2.5 and EMB 0.625 mg/mL were significantly lower compared to the solvent group (Figure 2a, $P < 0.0001$, $P < 0.0001$, $P < 0.01$, $P < 0.001$, $P < 0.001$ and $P < 0.05$, respectively). Conversely, the live RLUs of RIF 0.5, RIF 0.125 and INH 0.125 mg/mL groups did not exhibit significant differences from those of the solvent group (Figure 2a, $P > 0.05$). These findings were also largely supported by both the lung RLUs and CFUs (Figure 2b and 2c), except for the lung CFUs of the RIF at 0.5 mg/mL group, which were slightly significantly lower than that of the solvent group (Figure 2c, $P < 0.05$). Collectively, these findings indicate a negative correlation between live RLUs and administered dosages, suggesting that live RLUs could serve

as an initial criterion for assessing the therapeutic efficacy.

The lung RLUs of the RIF 2 mg/mL group exhibited a significant reduction compared to those in the solvent group, with a $\log_{10}$RLUs/lung difference exceeding 1.5 and even similar to that of the BT group (Figure 2b, $P < 0.0001$ and $P > 0.05$, respectively). Conversely, the lung RLUs in the RIF 0.5 and 0.125 mg/mL groups were similar to that in the solvent group, indicating a lack of evident anti-Mtb activity at these dosages (Figure 2b, $P > 0.05$). The lung RLUs of the INH 2 and 0.5 mg/mL groups were markedly lower than that of the solvent group (Figure 2b, $P < 0.0001$), as all differences in $\log_{10}$RLUs/lung exceeded 1.5 and comparable to that of the BT group (Figure 2b, $P > 0.05$). The lung RLUs of INH 0.125 mg/mL group were similar to that of the solvent group (Figure 2b, $P > 0.05$). In comparison, the lung RLUs of the EMB 10 and 2.5 mg/mL groups exhibited a pronounced decrease compared to those in the solvent group, with $\log_{10}$RLUs/lung differences exceeding 1.5 and 1, respectively (Figure 2b, $P < 0.0001$). Meanwhile, the lung RLUs of the EMB 10 mg/mL group were basically equivalent to that of the BT group (Figure 2b, $P > 0.05$), whereas the lung RLUs in the EMB 0.625 mg/mL group showed less significant differences from the solvent group (Figure 2b, $P < 0.05$). In conclusion, the lung RLUs results are consistent with those obtained from the live RLUs, providing further support for the utility of detecting live RLUs in determining drug activity.

The lung CFUs in the RIF 2 mg/mL group exhibited a significant reduction compared to those in the solvent group, given that the difference of $\log_{10}$CFUs/lung

exceeded 1.5 (Figure 2c, $P < 0.0001$). The lung CFUs in the RIF 0.5 and 0.25 mg/mL groups were approximately comparable to that of the solvent group (Figure 2c, $P <$ and close to 0.05 and $P > 0.05$, respectively). Notably, no colonies were observed on the INH 2 mg/mL group's plates, indicating potential complete clearance of AlRv from the mice lungs, given that only a fraction of the lung suspension from each mouse was plated. The CFUs of the INH 0.5 mg/mL group were also considerably lower than that of the solvent group, with a difference of $\log_{10}$CFUs/lung exceeding 1.5 and even lower than BT group (Figure 2c, $P < 0.0001$). Furthermore, all EMB dosages resulted in significantly reduced lung CFUs compared to the solvent control (Figure 2c, $P < 0.0001$, $P < 0.0001$ and $P < 0.001$, respectively). While only the $\log_{10}$CFUs/lung difference between the EMB 10 mg/mL and solvent group exceeded 1.5, with the lung CFUs in the EMB 10 group nearly identical to those in the BT group (Figure 2c, $P > 0.05$). These CFU results were consistent with the findings derived from live or lung RLUs data. Overall, based on the comprehensive experimental data presented, we have successfully established an autoluminescence-based inhalation administration murine model for evaluating drugs activity against Mtb *in vivo*.

DISCUSSION

Mtb poses a substantial burden of morbidity and mortality, primarily in developing countries (Chong and Lim, 2009, Grüber, 2020). Despite concerted efforts, the development of effective anti-Mtb drugs remains confronted with numerous challenges and uncertainties, including the complexity of the drug activity evaluation

process and the continued emergence of drug-resistant strains, particularly multidrug-resistant and extensively drug-resistant Mtb (McGrath et al., 2014, Olaru et al., 2015). Over several decades of continuous research, only bedaquiline, pretomanid and delamanid have received approved for clinical use (Pontali et al., 2013). Recently, inhaled administration has emerged as a promising approach for the treatment of plumonary infections, offering improved cure rates and bacterial eradication (Riveiro et al., 2023). However, the evaluation of inhalable drugs still presents challenges such as complex manipulation and time-consuming processes. Addressing these challenges is crucial to optimizing the efficacy and accessibility of inhaled drug delivery in clinical settings. Therefore, the development of a rapid, accurate and efficient activity evaluation model with impressive clinical applicability performance has the potential to significantly accelerate drug development progress.

In this study, we implemented an autoluminesence-based inhalation administration murine model to assess the *in vivo* anti-Mtb activity of three first-line TB drugs. The assessment process can be significantly shortened by detecting either the live RLUs of mice or the RLUs of lung suspensions, thereby yielding prompt results. Our findings demonstrate the successful establishment of an autoluminescence-based murine model for assessing respiratory administration efficacy against Mtb, offering substantial reductions in time, cost, and labor. The autoluminesence-based inhalation administration murine model established holds promise for improving the assessment of anti-Mtb drug efficacy *in vivo*, thus preventing the omission of potentially effective anti-Mtb drugs and minimizing the risk of serious side effects.

The drug's activity can be determined by comparing the lung suspension RLUs of different groups within a shorter duration than the current 17-day period (from infection to get the results), since the lung RLUs become significantly higher than the background value a few days earlier than the live RLUs do. Additionally, drug efficacy could potentially be observed within a considerably shorter timeframe in live mice if higher infection dosages were administered, such as via injection, although this may result in a higher standard deviation (Liu Yang et al., 2019, Olaru et al., 2015). Further investigation is warranted to determine whether other anti-tuberculosis drugs or potent compounds demonstrate efficacy using this model. Exploring alternative formulations or solvents could also be of interest to enhance the drug's effectiveness beyond sterilized water. In conclusion, the autoluminesence-based inhalation administration murine model for evaluating anti-Mtb drug efficacy *in vivo* has the potential to expedite the development of innovative anti-Mtb drugs and treatment regimens.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal care and experimental protocols were approved by the Laboratory Animal Ethics Committee of the Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

Conceived the project: Tianyu Zhang, Jinxing Hu, Xirong Tian, Yamin Gao; Designed the research: Tianyu Zhang, Jinxing Hu, Xirong Tian, Yamin Gao; Performed the studies: Xirong Tian, Yamin Gao, Jingran Zhang, Yanan Ju, Jie Ding, Sanshan Zeng, Wanli Ma; Interpreted the results: Tianyu Zhang, Xirong Tian, Yamin Gao; Drafted the manuscript: Tianyu Zhang, Jinxing Hu, Xirong Tian, Yamin Gao, H.M. Adnan Hameed, Nanshan Zhong, Gregory M. Cook; Final approval of the manuscript: all authors.

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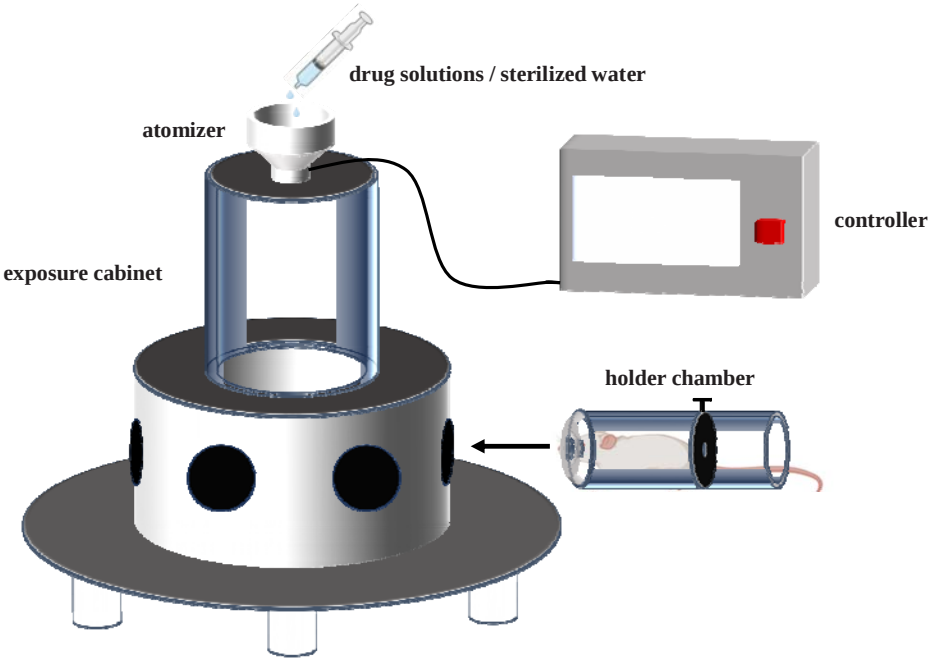


Figure 1. The operating mechanism of the Tow Systems Nose-Only Exposure Units.

Table 1. Scheme of experiment 1.

Drug/Dosage[1] (mg/mL)	Number of mice sacrificed at the given time points				Total
	D0[2]	D3[3]	D11-17	D18[4]	
Solvent	Infection	6	Detecting chest RLUs of live mice in solvent group daily from day 11 until live RLUs exceed 400	6	12
RIF 2		Treatments initiation		6	6
INH 2				6	6
EMB 10				6	6
Total	30	6		24	30

[1] RIF, rifampicin; INH, isoniazid; EMB, ethambutol; Solvent, sterilized water; the number after the drug represents the dosage.

[2] D0, a total of 30 mice were infected with AlRv by aerosol.

[3] D3, the mice were randomly divided into groups and treated daily from the day 3 to day 16. The live RLUs, the lung suspension RLUs and CFUs of six mice were detected before treatment initiated.

[4] D18, the mice were sacrificed and the lungs were removed for detecting RLUs and CFUs.

All CFUs were read 4 weeks after incubation at 37 □.

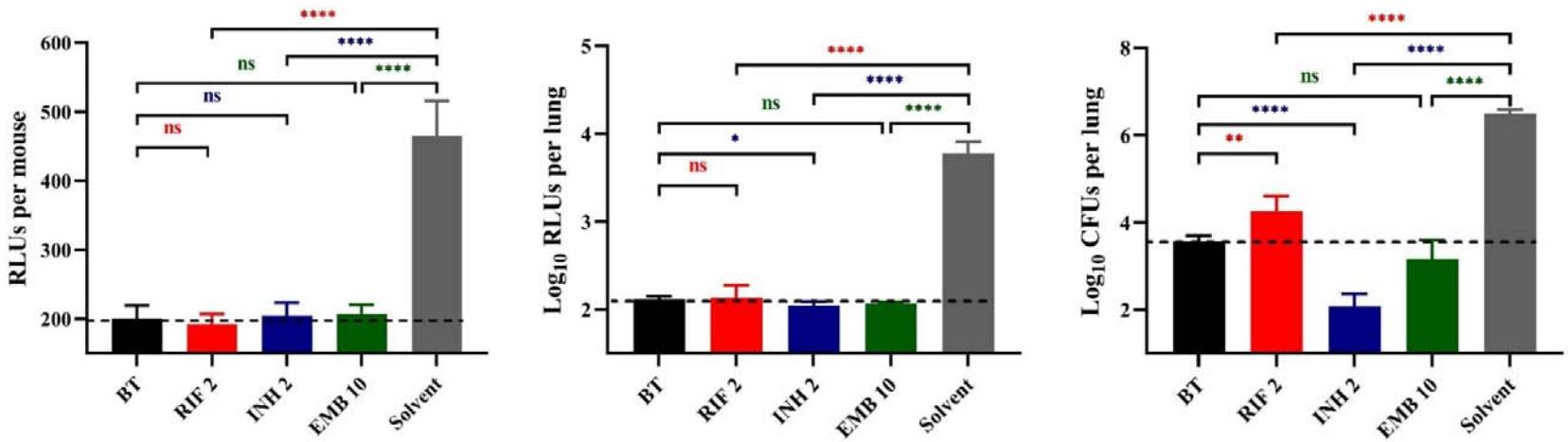


Figure 2. Anti-TB drug activities by inhalation administration in Experiment 1.

BT, before treatment; RIF, rifampicin; INH, isoniazid; EMB, ethambutol; Solvent, sterilized water; the number after the drug represents the dosage; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, $P > 0.05$, no significance. The dotted lines represent the background values.

Table 2. Scheme of experiment 2.

Drug/Dosage[1] (mg/mL)	Number of mice sacrificed at the given time points				Total
	D0[2]	D1[3]	D13-16	D17[4]	
Solvent		6		6	12
RIF 2				6	6
RIF 0.5			Detecting chest	6	6
RIF 0.125			RLUs of live mice	6	6
INH 2			in solvent group	6	6
INH 0.5	Infection	Treatments initiation	daily from day 13	6	6
INH 0.125			until live RLUs	6	6
EMB 10			exceed 400	6	6
EMB 2.5				6	6
EMB 0.625				6	6
Total	66	6		60	66

[1] RIF, rifampicin; INH, isoniazid; EMB, ethambutol; Solvent, sterilized water; the number after the drug represents the dosage.

[2] D0, a total of 66 mice were infected with AlRv by aerosol.

[3] D1, the mice were randomly divided into groups and treated daily from day1 to day 15. The live RLUs, the lung suspension RLUs and CFUs of six mice were detected before treatment initiated.

[4] D17, the mice were sacrificed and the lungs were removed for detecting RLUs and CFUs.

All CFUs were read 4 weeks after incubation at 37 □.

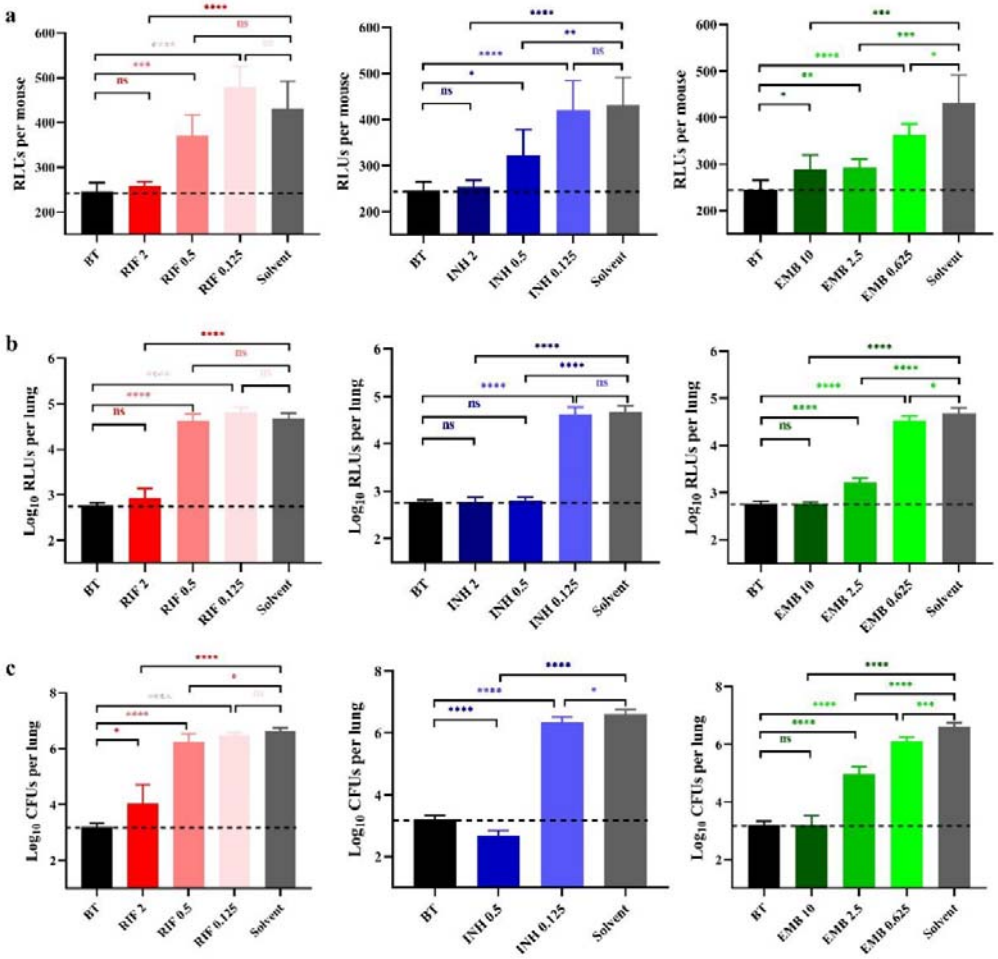
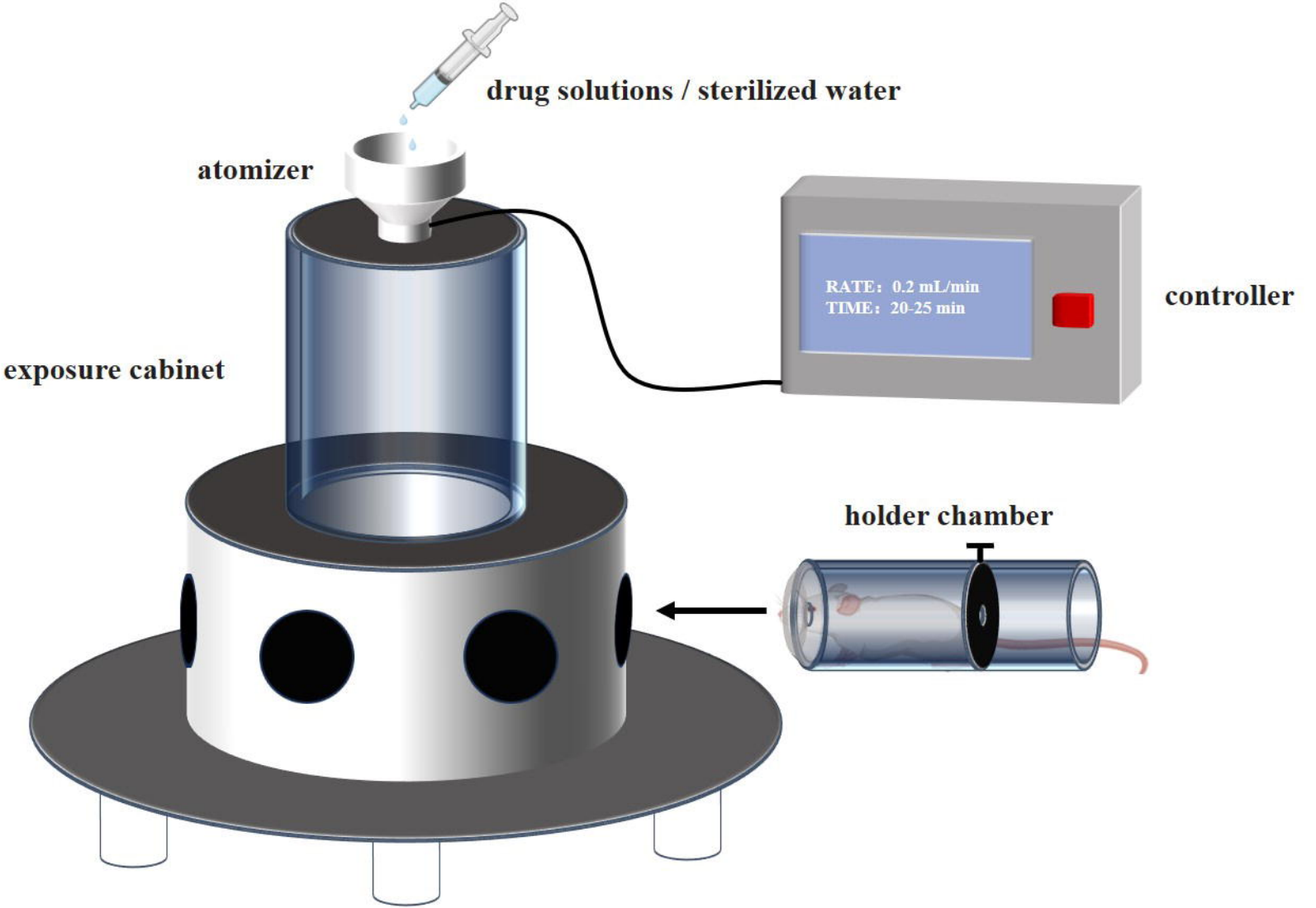
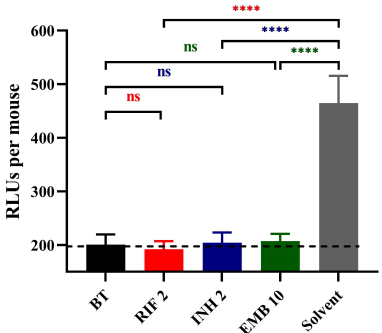
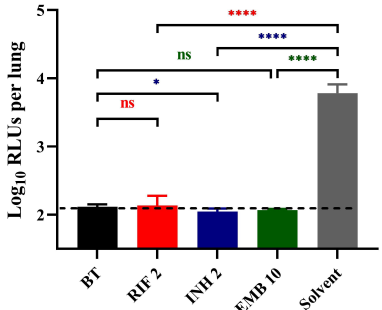


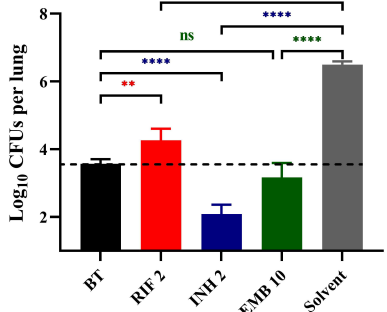
Figure 3. Anti-TB drug activities by inhalation administration in Experiment 2. (a) Light intensities of live mice. (b) The suspension light intensities. (c) The lung Mtb burden.

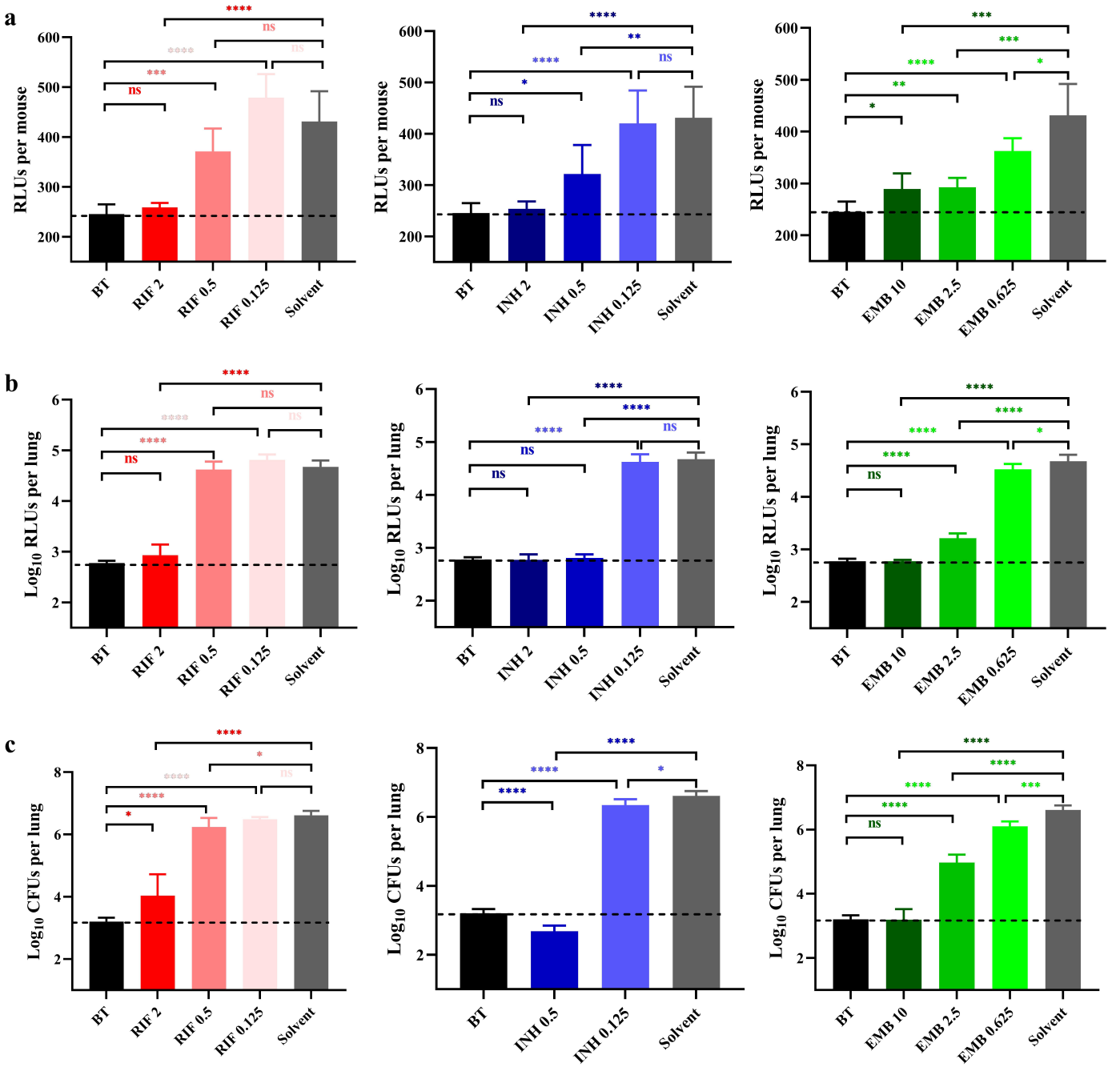
BT, before treatment; RIF, rifampicin; INH, isoniazid; EMB, ethambutol; Solvent, sterilized water; the number after the drug represents the dosage; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, $P > 0.05$, no significance. The dotted lines represent the background values.


drug solutions / sterilized water
atomizer
RATE：0.2 mL/min
TIME：20-25 min
controller
exposure cabinet
holder chamber


RLUs per mouse
600
500
400
300
200
****
****
ns
****
ns
ns
BT
RIF 2
INH 2
EMB 10
Solvent


Log10 RLUs per lung
5
4
3
2
****
****
ns
****
*
ns
BT
RIF 2
INH 2
EMB 10
Solvent


Log10 CFUs per lung
8
6
4
2
****
****
ns
****
****
**
BT
RIF 2
INH 2
EMB 10
Solvent


a
RLUs per mouse
BT
RIF 2
RIF 0.5
RIF 0.125
Solvent
INH 2
INH 0.5
INH 0.125
EMB 10
EMB 2.5
EMB 0.625
b
Log10 RLUs per lung
c
Log10 CFUs per lung