Infection and Drug Resistance

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ORIGINAL RESEARCH

Nintedanib Enhances the Antibacterial Activity of Bedaquiline Against Non-Tuberculous Mycobacteria: In vitro and in Mice Models

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Aim: Non-tuberculous mycobacteria (NTM) are ubiquitous in the natural environment. Globally, incidence of non-tuberculous mycobacterial disease is on the rise. To date, over 190 species of NTM have been identified, many of which possess inherent natural resistance to antibiotics, developing new drugs is a challenging process with a high risk of failure. The repurposing of existing drugs may be a quick and effective approach. This study investigates the combined effects of bedaquiline (BDQ) and nintedanib (a multi-target tyrosine kinase inhibitor) on NTM, with the aim of providing a novel therapeutic approach against NTM.

Methods and Results: The number of colony-forming units (CFUs) in macrophages was determined to assess the antibacterial activity of nintedanib against *Mycobacterium avium* and *Mycobacterium abscessus*. To evaluate the efficacy of nintedanib in combination with BDQ in a mouse model of pulmonary infection. The mechanism of the synergistic antibacterial effect of nintedanib and BDQ was investigated using assays of immune cells and immune factors, ATP measurement, transcriptomic sequencing and RT-PCR. Nintedanib significantly enhanced the intracellular antibacterial activity of BDQ against *M. avium* and *M. abscessus*. In mice infection model, nintedanib enhanced the efficacy of BDQ, reducing the extent of inflammatory damage and fibrosis in the mice's lung tissue. The mechanism by which nintedanib enhances the antibacterial activity of BDQ is as follows: on the one hand, it boosts adaptive immunity in mice, promoting anti-inflammatory and tissue-repairing immune responses; on the other hand, it enhances BDQ's ability to inhibit ATP production and disrupts bacterial metabolic pathways.

Conclusion: In vitro and in vivo studies have shown that nintedanib enhances the antibacterial activity of BDQ, accelerates pathogen clearance and reduces lung damage. Nintedanib adjuvant therapy modulates the body's adaptive immune response, promoting resolution of inflammation and tissue repair. Nintedanib may enhance the efficacy of BDQ by interfering with bacterial ATP synthesis and metabolic pathways. These findings highlight the potential of BDQ combined with nintedanib for the treatment of NTM infections and provide a crucial theoretical basis for the development of more effective therapeutic strategies for NTM infections.

Keywords: antibacterial activity, non-tuberculous mycobacteria, nintedanib, bedaquiline, drug combination

Introduction

Based on the growth rate of NTM, it is divided into two categories: fast-growing and slow-growing types, those comprising the *Mycobacterium avium* (MAC), *Mycobacterium intracellulare* (*M. intracellulare*) and the subspecies of *Mycobacterium abscessus* (MAB) are among the most common encountered clinically.[1–3] Non-tuberculous mycobacterial pulmonary disease (NTM-PD) is a difficult-to-treat condition caused by infection with NTM.[4] NTM species show significant heterogeneity in their sensitivity to standard antituberculosis drugs. Treatment of NTM-PD usually involves the use of macrolides and injectable aminoglycosides.[5] Guidelines mandate at least 12–24 months of triple-drug combination therapy.[3] Despite the publication of well-established international guidelines, treatment of NTM-PD is mostly empirical and not entirely successful. Clinical experience indicates that nearly 40% of patients experience treatment-related adverse reactions, with high rates of treatment failure (varying significantly between species, ranging from approximately 25% to 80%) and recurrence (50%).[6–9] Consequently, the development of novel therapeutics for NTM-PD is urgently required.

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NTM has been demonstrated to interfere with host immune responses, including preventing phagosome acidification and maturation or escaping from phagosomes into the cytoplasm. Counteracting pathogen-induced immunomodulation through host-directed therapy (HDT) represents a promising adjunctive approach to antibiotic treatment for mycobacterial infections. HDT effectively targets antibiotic-resistant mycobacteria with minimal risk of resistance development, shortening treatment duration and reducing adverse reactions.[10] Although HDT is an active area of research for tuberculosis, its applicability to NTM infections remains unknown.

As the first new anti-tuberculosis drug to be developed and marketed globally in nearly 50 years, Bedaquiline (BDQ) has been classified by the World Health Organisation as the drug of choice for the long-term treatment of rifampicin- or multidrug-resistant tuberculosis (MDR-TB) in 2021.[11] The core target of BDQ's antimycobacterial action is to inhibit the bacterial ATP synthase, depleting the energy to kill the bacteria.[12] In recent years, BDQ has been repurposed for NTM. Clinically, BDQ has emerged as one of the most promising drugs for treating macrolide-resistant MAC infections, and it has become a core component of the *Mycobacterium abscessus* pulmonary disease (MAB-PD) multidrug combination regimen.[13] Whilst adding BDQ to failed regimens for MAC and MAB infections may alleviate symptoms, it neither prevents microbiological treatment failure nor suppresses the emergence of BDQ resistance.[14,15] Consideration could be given to enhancing the efficacy of BDQ through combination therapy with other drugs.

Drug repurposing is an effective strategy for accelerating drug development. As is well known, Tyrosine kinases regulate multiple immunomodulatory pathways. Recent studies have revealed that tyrosine kinase inhibitors (TKIs) represent a promising strategy to supplement standard antibiotic therapy for MDR-TB. For example, dasatinib reduces intracellular MTB growth, while imatinib in mice and the novel Abl/Src inhibitor AZD0530 in guinea pigs both demonstrate beneficial effects against in vivo mycobacterial infection.[16,17] These findings have encouraged the use of TKIs for host-directed therapy against mycobacteria.

Nintedanib is an oral small-molecule tyrosine kinase inhibitor that was first approved by the FDA in 2014 for the treatment of idiopathic pulmonary fibrosis.[18] The drug acts by competitively binding to the ATP-binding sites of the platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR) and vascular endothelial growth factor receptor (Vascular Endothelial Growth Factor Receptor, VEGFR), thereby blocking downstream signalling pathways, inhibiting the proliferation, migration and transformation of fibroblasts, and slowing the decline in lung function.[19,20] Nintedanib exhibits broad-spectrum pharmacological properties including anti-inflammatory, anti-fibrotic, and anti-tumor activities. Dinnon et al explored that early intervention with nintedanib altered the severity of pulmonary infections[21] Previous studies have confirmed the efficacy of nintedanib as a candidate host-directed therapeutic agent in the treatment of tuberculosis.[22] Although MTB and NTM differ in virulence and infectivity, they belong to the same genus and share numerous similarities at fundamental biological levels, such as cellular structure and metabolic pathways. Therefore, the "targets" of drugs designed for MTB may also exist in NTM.[23]

Data on the efficacy of nintedanib against NTM remain limited. This study evaluated the efficacy of nintedanib combined with BDQ and other antibiotics in treating NTM through in vitro and in vivo experiments, and investigated its mechanism of action. These findings provide new therapeutic strategies for NTM-PD.

Nintedanib Enhances the Antibacterial Activity of Anti-NTM Drugs Within Macrophages

We investigated whether nintedanib indirectly inhibits the growth of NTM by enhancing macrophage activity in vitro. In the preliminary phase, we evaluated the cytotoxicity of nintedanib. The IC_{50} value of nintedanib in J774A.1 cells was 11.29 μg/mL, leading us to establish a safety concentration limit of 4 μg/mL for nintedanib. Expose J774A.1 macrophages to different concentrations of nintedanib for 72 hours, then detect MAC intracellular survival using the CFU assay. As shown in Figure 1a, nintedanib inhibits the growth of MAC in J774A.1 macrophages, and exhibit dose-dependence. Nintedanib 2μg/mL and 4μg/mL caused a reduction in CFU/mL by up to 0.69 Log_{10}CFU/mL and 0.99 Log_{10}CFU/mL, respectively. We further investigated whether nintedanib could enhance the intracellular bactericidal capacity of four commonly used antibiotics when combined. In J774A.1 macrophages, nintedanib reduced CFU/mL by 0.55 ± 0.13 Log_{10}CFU/mL, 0.47 ± 0.29 Log_{10}CFU/mL, 0.33 ± 0.18 Log_{10}CFU/mL and 0.22 ± 0.1 Log_{10}CFU/mL in groups of clarithromycin plus nintedanib, moxifloxacin plus nintedanib, clofazimine plus nintedanib and bedaquiline plus nintedanib, respectively, compared with these drugs alone (Figure 1b).

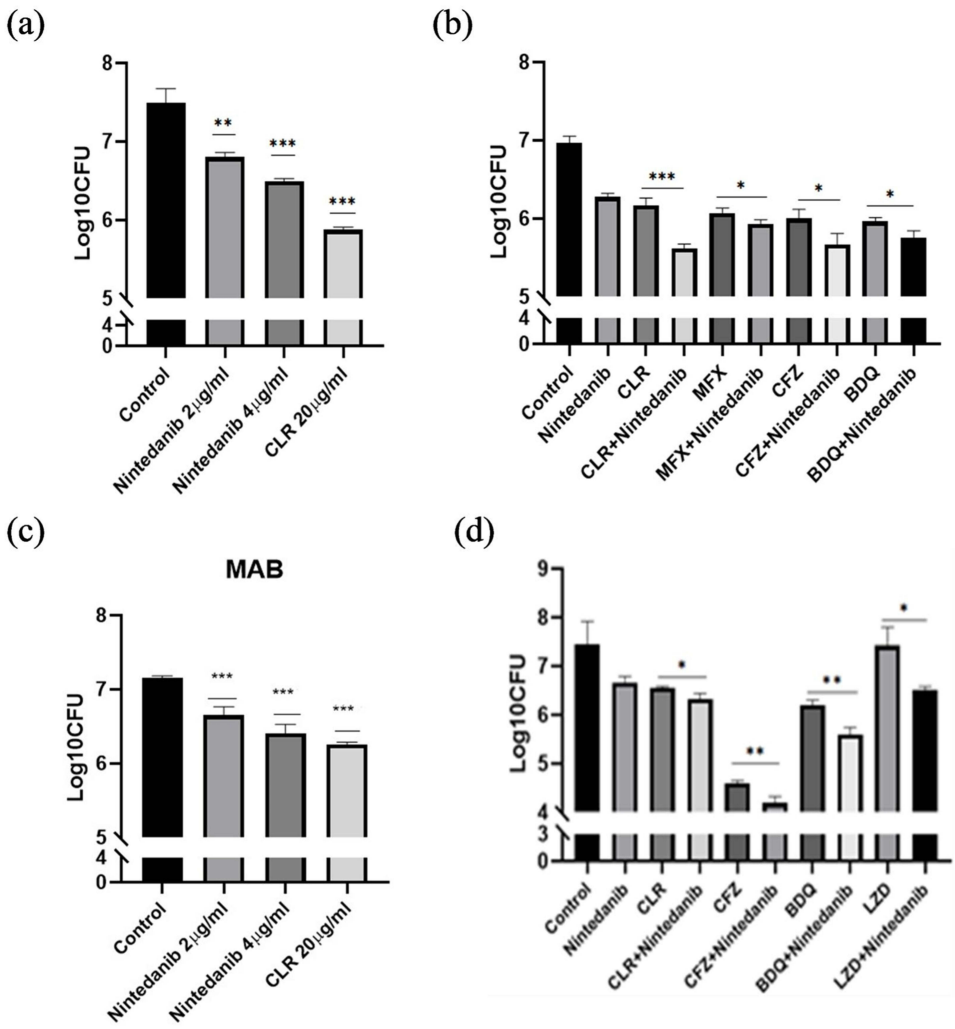

Figure 1 Antibacterial activity of nintedanib in combination with anti-NTM drugs in macrophages. J774A.1 macrophages infected with different bacterial strains were treated with varying concentrations of nintedanib for 72 hours, lysed with 0.1% SDS, and finally spread on 7H10 medium. Intracellular survival rates in MAC (**a**) and MAB (**c**)-infected J774A.1 macrophages treated with single agents nintedanib (2 μg/mL and 4 μg/ mL) or clarithromycin (CLR, 20 μg/ mL). (**b**) Intracellular survival rate of MAC in J774A.1 macrophages treated with combination therapy of nintedanib (2 μg/ mL) plus CLR (20 μg/ mL), moxifloxacin (MFX, 4 mg/L), clofazimine (CFZ, 1 μg/mL), and bedaquiline (BDQ, 1 μg/mL). (**d**) Intracellular survival rate of MAC in J774A.1 macrophages treated with nintedanib (2 μg/mL) combined with CLR (20 μg/mL), clofazimine (CFZ, 4 μg/mL), Bedaquiline (BDQ, 1 μg/mL), and Linezolid (LZD, 64 mg/L). Intracellular survival rates were determined by colony-forming unit (cfu) assays 72 hours after combined treatment (n=3; mean ± standard deviation, **P*<0.05, ***P*<0.01, ****P*<0.001).

Similar results were observed in J774A.1 macrophages infected with MAB. After 72 hours of drug treatment, clarithromycin, the "cornerstone drug" of MAB therapy, decreased the bacterial load within macrophages by 2.01 Log_{10}CFU/mL. Following treatment with 2μg/mL and 4μg/mL nintedanib, the bacterial load within J774A.1 decreased by 0.50 ± 0.11 and 0.74 ± 0.12 Log_{10}CFU/mL, respectively (Figure 1c). Similarly, we found that nintedanib enhances the intracellular antimicrobial activity of inhibitory MAB antibiotics. Compared with the monotherapy groups of clarithromycin, clofazimine, bedaquiline and linezolid, the bacterial load within macrophages decreased by 0.22 ± 0.12, 0.41 ± 0.25, 0.61 ± 0.18 and 0.91 ± 0.39 Log_{10}CFU/mL, after combination therapy with nintedanib, respectively (Figure 1d). Research findings indicate that nintedanib enhances the antibacterial activity of Commonly used anti-NTM drugs within macrophages.

Nintedanib Enhances the Antibacterial Efficacy of BDQ in Mice

Although the addition of nintedanib to combination therapy shows promise for its antibacterial effects in macrophages, It's protective effect in the host organism needs to be confirmed in animal models. We separately investigated the efficacy of nintedanib in animal models of slow-growth and fast-growth NTM infections. Figures 2A and 3A are flowcharts illustrating

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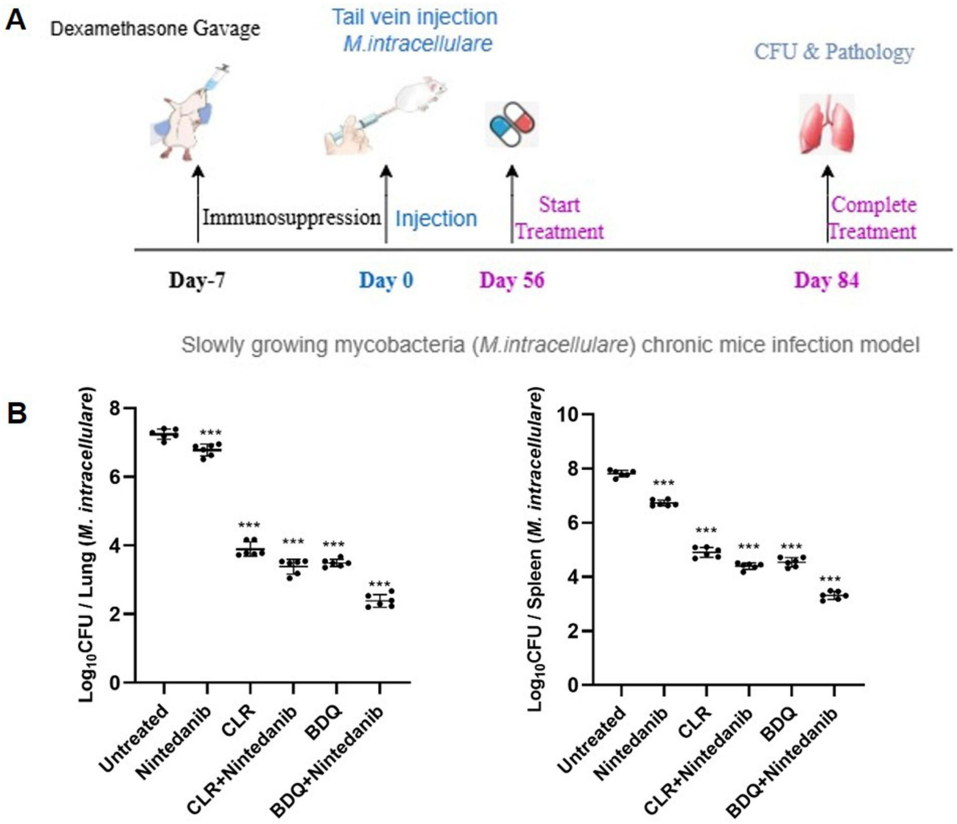

Figure 2 Nintedanib enhances the antimicrobial activity of BDQ in mice infected with *M. intracellulare*. (**A**) Thirty-six mice infected with *M. intracellulare* via tail vein injection were randomly divided into six groups. Administering dexamethasone via gastric lavage for 7 consecutive days prior to infection suppresses the immune response in mice. Each group received nintedanib (30 mg/kg/day) combined with recommended BDQ (15 mg/kg) during days 56–84 post-infection. (**B**) Bacterial load comparison in lung tissue and spleen tissue at indicated time points; all colony counts were log-transformed to $\log_{10}$ (cfu), with detection limits determined based on culture medium inoculation ratios. Statistical analysis performed using one-way ANOVA. ***$P < 0.001$.

the establishment and treatment of the mouse infection model. As shown in Figures 2B and 3B results, nintedanib further reduces the bacterial load in the lungs of BALB/c mice by day 28 of treatment. Four weeks of nintedanib combination treatment significantly reduced the *M. intracellulare* mycobacterial burden in the lung and spleen (Figure 2B) compared to monotherapy group. We observed the same results in the MAB infected mice model. Compared to the monotherapy group, the nintedanib combination therapy group showed a significant reduction in bacterial load in both the lungs and spleen of mice (Figure 3B). HE and MASSON staining revealed that compared to untreated mice, nintedanib mice significantly reduced in inflammatory pathology and fibrosis in the lungs, the difference was particularly evident in the comparison between the BDQ and BDQ plus nintedanib mice (Figure 4A). Lung injury scores and the proportion of areas showing pulmonary fibrosis further support this view (Figure 4B). Similarly, in the mice model of MAB infection, significant improvement in the degree of inflammation and fibrosis in lung tissue (Figure 5A), quantitative measures of the proportion of lung area affected by injury and fibrosis lend greater credibility to the findings (Figure 5B).

Nintedanib Enhances the Adaptive Immune Response in Mice

We then investigated the mechanism by which nintedanib enhances the antibacterial activity of BDQ. We examined this from two perspectives: the host's immune function and the direct effects on the bacteria themselves. We investigated the status of immune cells in treated mice. $CD4^+$ T cells play an extremely important role in the immune system. The $CD4^+$ T cell levels in the nintedanib with BDQ group were significantly higher than those in the BDQ group (Figure 6A). Flow cytometry results from mouse spleen cells show that nintedanib enhanced the immune capacity of the adaptive immune capacity of mice (Figure 6B).

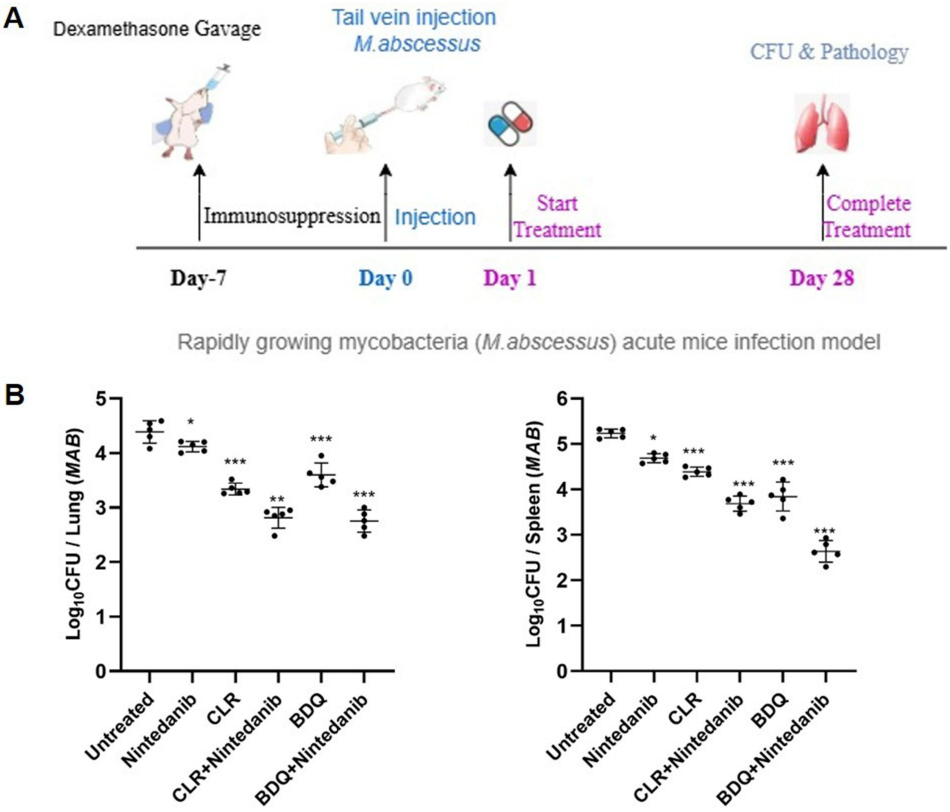

Figure 3 Nintedanib enhances the antimicrobial activity of BDQ in mice infected with MAB. (**A**) Thirty-six mice infected with MAB via tail vein injection were randomly divided into six groups. Administering dexamethasone via gastric lavage for 7 consecutive days prior to infection suppresses the immune response in mice. Each group received nintedanib (30 mg/kg/day) combined with recommended anti-NTM drugs (CLR 50 mg/kg, BDQ 15 mg/kg) during weeks 1–4 post-infection. (**B**) Bacterial load comparison in lung tissue and spleen tissue at indicated time points; all colony counts were log-transformed to log10(CFU), with detection limits determined based on culture medium inoculation ratios. Statistical analysis performed using one-way ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

BDQ in Combination with Nintedanib Regulates the Balance of the Cytokine Network

To analyse the dynamic changes in relevant immune factors, we established a mouse model of mycobacteria avium infection, changes in bacterial load in the lungs and spleen during treatment are shown in Supplementary Table 1. The immune response following NTM infection typically follows the following dynamic course: in the early stages of infection (the first two weeks), the body's immune system is highly active, with elevated levels of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-6) to control the infection; in the later stages of infection, bacterium-induced immunosuppressive mechanisms (such as IL-10 and TGF-β) gradually become dominant, and the body may enter a state of "immune paralysis", making it difficult to clear the infection and leading to tissue fibrosis.[24–26] This study assessed changes in serum cytokine levels during the course of treatment. IFN-γ: Levels were elevated to varying degrees in all groups during the first two weeks of treatment, with the BDQ plus nintedanib group reaching peak levels at week 2 (Figure 7A). TNF-α: The BDQ monotherapy group maintained relatively high levels throughout the treatment period; by the end of treatment (week 4), TNF-α levels in the BDQ plus nintedanib group were significantly lower than in the other groups (Figure 7B). IL-6: IL-6 levels in the Nintedanib, BDQ and BDQ plus Nintedanib groups all showed a gradual downward trend as treatment progressed. By week 4, IL-6 levels in the BDQ plus Nintedanib group were 50% lower than those in the BDQ monotherapy group (Figure 7C). IL-10: IL-10 levels in all four groups of mice gradually increased with the duration of treatment. By week 4, IL-10 levels in the BDQ plus Nintedanib group were significantly higher than in the other groups (Figure 7D). The results revealed that the combination of BDQ and nintedanib achieves a dynamic balance between controlling infection and mitigating tissue damage by regulating the cytokine network—specifically, by enhancing the Th1-type immune response (IFN-γ) whilst subsequently synergistically suppressing pro-inflammatory factors (TNF-α, IL-6) and promoting the secretion of the anti-inflammatory repair factor IL-10.

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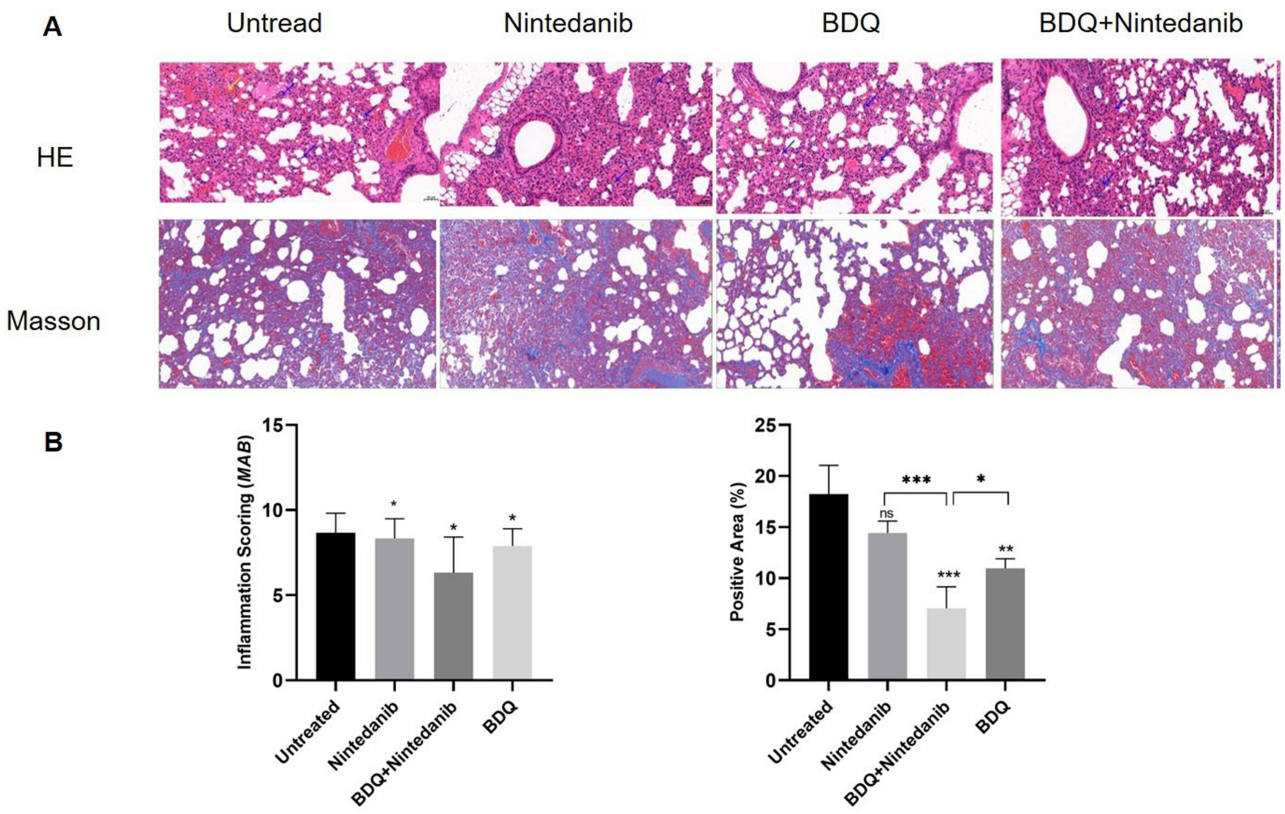

Figure 4 Nintedanib reduces lung tissue damage and fibrosis in mice infected with *M. intracellulare*. (**A**) Pathological analysis (HE and Masson staining) of lung tissue in mice from each group following treatment (n=3 per group). (**B**) Indicators for assessing the extent of pulmonary tissue infiltration and damage: (1) inflammatory cell infiltration; (2) alveolar wall thickening; (3) haemorrhage; (4) congestion. The scoring criteria for each parameter were as follows: 0 points for findings within the normal range; changes just exceeding the normal range: 1 point (very mild); lesions are observable but not yet severe: 2 points (mild); lesions are marked and likely to be more severe: 3 points (moderate); lesions are very severe (lesions have taken over the entire organ): 4 points (severe); the sum of the scores for the above items constitutes the lung tissue injury score (Inflammation scoring). Assessment of pulmonary fibrosis: Quantification was performed using Image J software to calculate the percentage of positive area for pulmonary fibrosis, i.e. the positive area (%) (positive area (%) = positive area/total tissue area). Statistical analysis performed using one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$.

Nintedanib Enhances the Inhibition of ATP Synthesis by BDQ on NTM

Our findings indicate that nintedanib effectively suppresses the growth-inhibitory effects of BDQ on NTM in vivo, regardless of whether the strain is fast-growing or slow-growing. Time-kill curves were determined using MAB strain ATCC 19977. As shown in Figure 8A, the combination of nintedanib and BDQ produced superior bacteriostatic effects. We have preliminarily explored the mechanism by which nintedanib enhances the efficacy of BDQ. Since BDQ exerts its antibacterial effects by targeting the c subunit of ATP synthase and inhibiting ATP synthesis, we investigated the impact of BDQ alone and in combination with nintedanib on MAB ATP flux (Figure 8B), results indicate that BDQ significantly reduced ATP flux in MAB, with the most pronounced depletion observed in the BDQ-nintedanib combination. A marked dose-dependent effect was demonstrated, characterized by a significant increase in the inhibition rate of ATP synthesis. According to Mackenzie et al,[27] BDQ reveals the vulnerability of *Mycobacterium tuberculosis* during glycolysis by reprogramming central metabolism By analysing the expression of key genes in metabolic pathways, we have conducted a preliminary investigation into the mechanism by which the combination of nintedanib and BDQ affects NTM metabolism. The genes gmpA, gpsA, leuD, purB, and pyk participate in regulating bacterial glycolysis, purine nucleotide metabolism, and glutamine biosynthesis. 24 hours after drug treatment, we validated the expression levels of these genes within MAB cells. We find that compared with the BDQ monotherapy group, the expression levels of these target genes were significantly downregulated following combination therapy with nintedanib (Figure 9). These findings suggest that nintedanib may enhance the antibacterial activity of BDQ by interfering with bacterial metabolic processes such as glycolysis (gpmA, pyk), amino acid synthesis (leuD) and membrane lipid synthesis (gpsA).

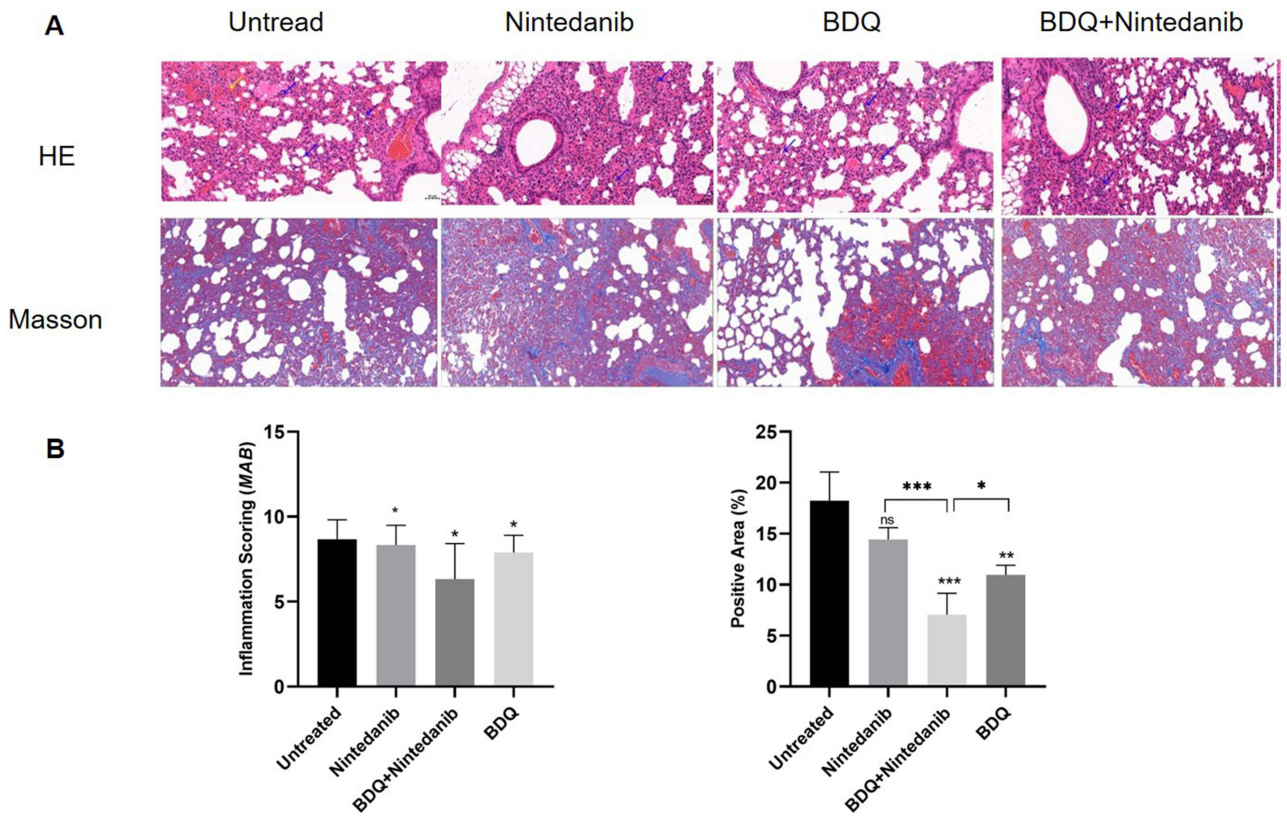

Figure 5 Nintedanib reduces lung tissue damage and fibrosis in mice infected with MAB. (**A**) Pathological analysis (HE and Masson staining) of lung tissue in mice from each group following treatment (n=3 per group). (**B**) Indicators for assessing the extent of pulmonary tissue infiltration and damage. For quantitative standards, refer to Figure 4. Statistical analysis performed using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001.

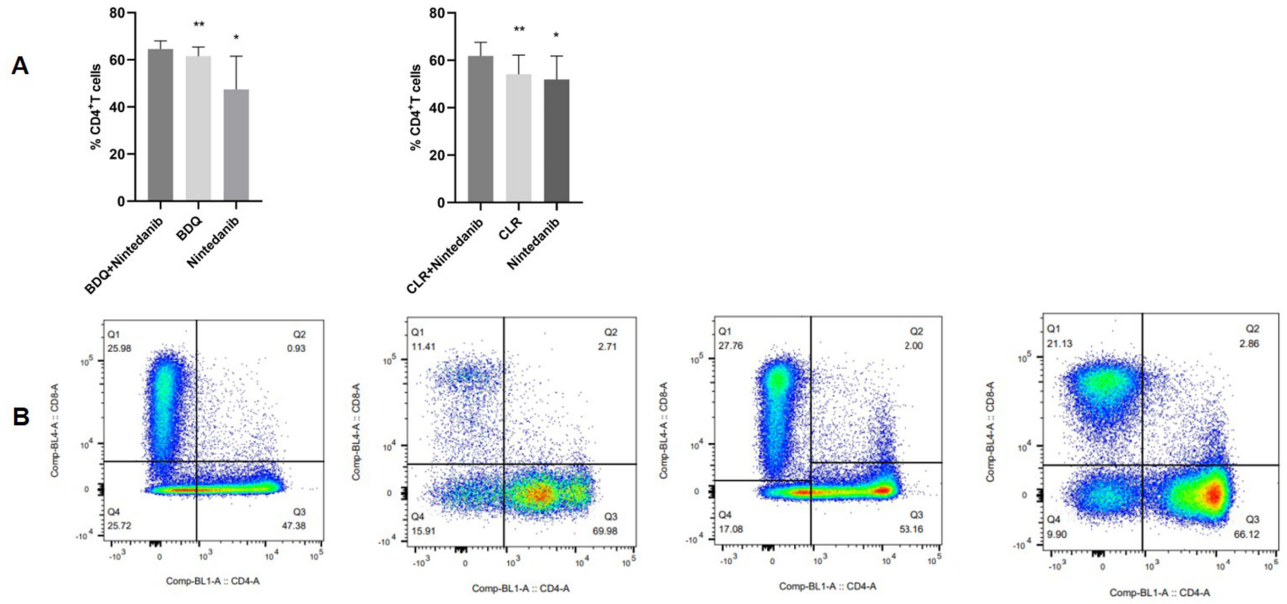

Figure 6 Nintedanib enhances the adaptive immune response in mice. (**A**) From left to right: CD3+ T cells, CD4+ T cells, and CD8+ T counts. (**B**) From left to right: untreated group, nintedanib, BDQ, and nintedanib with BDQ combination therapy group. ns: P>0.05, *P < 0.05, **P < 0.01, ***P < 0.001.

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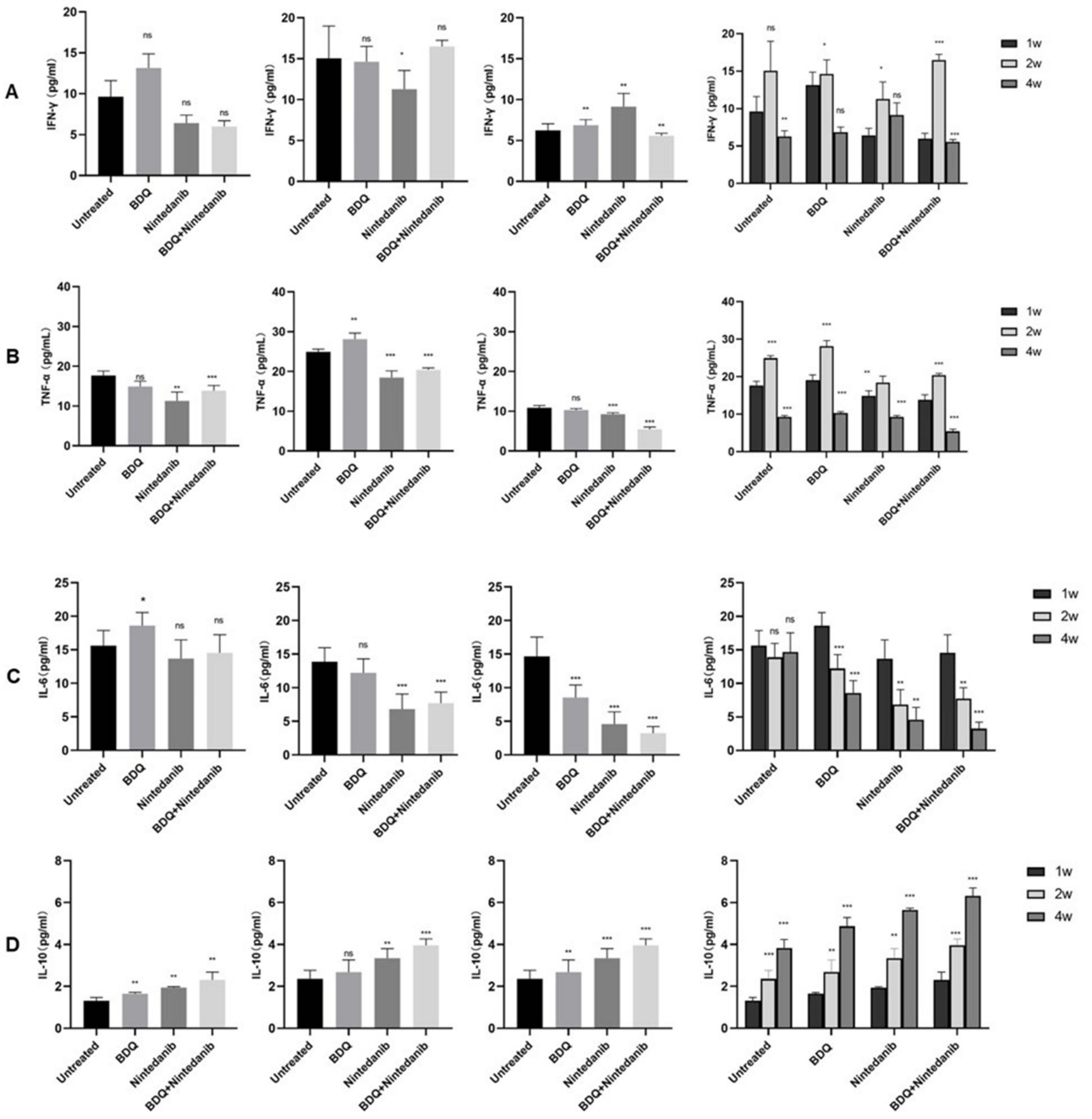

Figure 7 Assay of cytokines (IFN-γ, TNF-α, IL-6 and IL-10) in mice serum. (**A**–**D**) represent the cytokines IFN-γ, TNF-α, IL-6 and IL-10, respectively. (**A**–**D**) show, from left to right, cross-sectional comparisons between the various treatment groups at 1 week, 2 weeks and 4 weeks; as well as longitudinal comparisons within each group over the course of treatment. ns: $P>0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Materials and Methods

Animals and Mycobacterial Strains

Animal experiments were approved by the Animal Ethics Committee of Beijing Chest Hospital, Capital Medical University. All animal protocols were conducted in accordance with the animal care guidelines of the Institutional Animal Care and Use Committee of Capital Medical University (Beijing, China).

Standard strains of non-tuberculous mycobacteria include slow-growing types such as *Mycobacterium avium* (ATCC 25291) and *Mycobacterium intracellulare* (ATCC 13950), and fast-growing types such as *Mycobacterium abscessus*

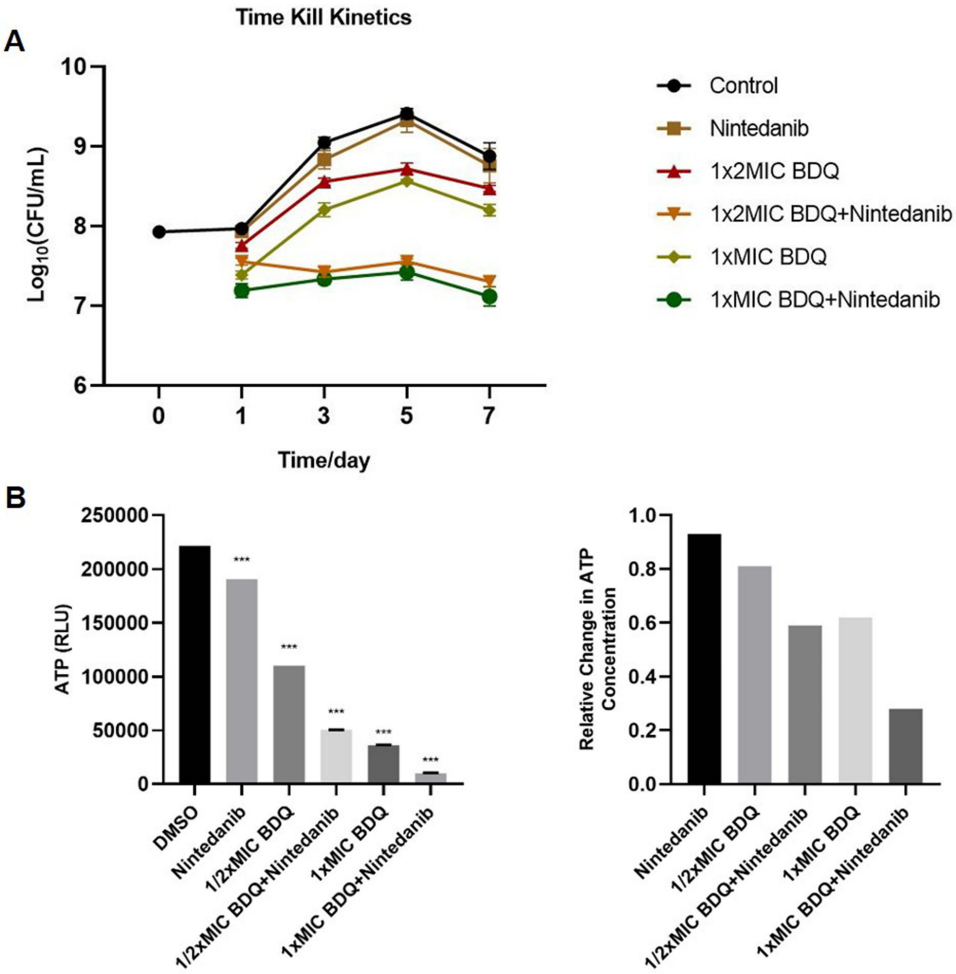

Figure 8 Nintedanib enhances the inhibition of ATP synthesis by BDQ on NTM. (**A**) Nintedanib potentiates the efficacy of BDQ against MAB in vitro. Time-Kill Kinetics: Growth curves of MAB treated with BDQ alone or in combination with nintedanib for 7 days, compared to DMSO-treated controls. Data represent the mean ± standard deviation of triplicate. (**B**) After 24 hours of treatment with BDQ at concentrations of 1/8, 1/4, and 1/2 x MIC, with or without nintedanib, intracellular ATP levels were measured and the ATP values have not been standardised against the bacterial count. *$P < 0.05$, **$P < 0.01$.

(ATCC 19977). These strains originate from the Drug Research Laboratory of the Beijing Institute of Tuberculosis and Thoracic Oncology. The experimental strains mentioned above are stored at the Department of Pharmacology, Beijing Chest Hospital, Capital Medical University.

Cells and Chemicals

J774A.1 cells (ATCC) were cultured in DMEM medium supplemented with 10% fetal bovine serum. Cells were cultured statically in a 37°C incubator containing 5% CO_2.

Clarithromycin, bedaquiline, linezolid, moxifloxacin clofazimine and dexamethasone were purchased from Biochempartner. Nintedanib was purchased from Sigma-Aldrich.

The following antibodies were used CD8a Monoclonal Antibody (53–6.7), PE-Cyanine7, eBioscience™, CD3 Monoclonal Antibody (17A2), APC, eBioscience™, CD4 Monoclonal Antibody (GK1.5), FITC, eBioscience™.

Quantitative RT–PCR (qRT–PCR) Analysis

Total RNA was isolated using TRIzol (Invitrogen, USA). cDNA was then generated using cDNA Synthesis SuperMix (YEASEN, Shanghai, China). qRT–PCR was performed using SYBR Green Real-time PCR Master Mix (Yeasen) with specific primers. The following primers were used:

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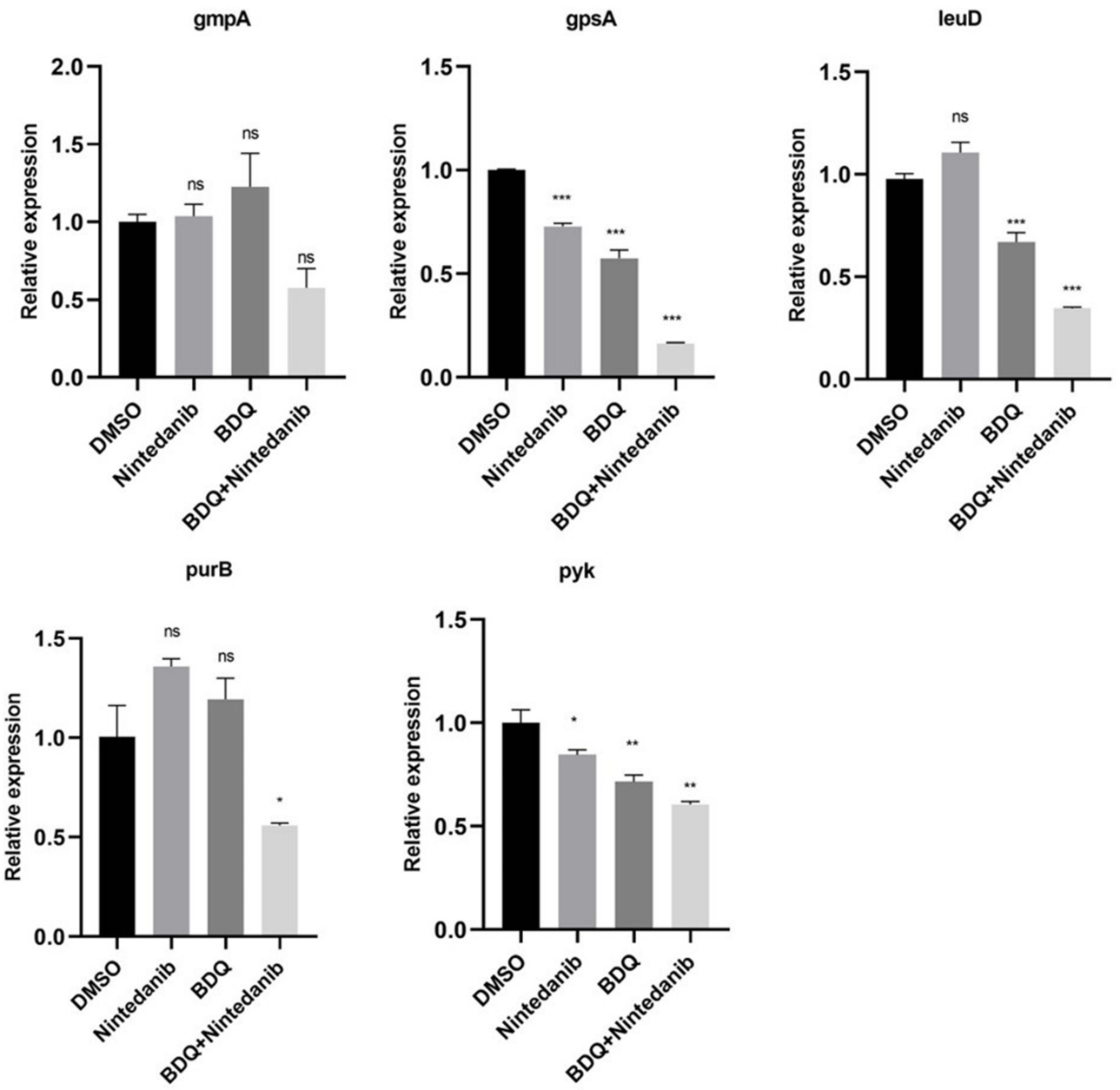

Figure 9 qPCR validation of changes in the expression levels of metabolism-related target genes induced by the combination of nintedanib and BDQ. Using the DMSO treated group as the control (with relative expression set to 1), the relative expression levels of the target genes in each drug-treated group are presented as bar charts. Nintedanib, BDQ and BDQ + Nintedanib denote the respective drug-treated groups. *P*>0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

gpmA-forward: TGTCGACCTGACGGAAAAGG,
reverse: TCGCCATATTTGGCCTTGGT;
gpsA-forward: TCATCGAGCCGAATTCCTCG,
reverse: GTAAGGACGGAAGTAGCGGG.
leuD-forward: ACCGACCCCTCCTTCATTCT,
reverse: TCCAAACCCGGATTCTGCTC.
purB-forward: TCAACGGAGACGTGGTCAAG,
reverse: GTTCATCTTGTGCGGCATCG
pyk-forward: ACCAAACGTGGGGTCATCTC
reverse: GTTGAATCATGCCGTCGGTG ®

internal control gene- forward: ACGCCGAAGAGGAAGTTGAG
internal control gene- reverse: TGAATCAGGTCCAGGAACGC

Intracellular Antimicrobial Activity Assay

Digest J774A.1 cells with trypsin to a final concentration of 4×10^6 cells/mL. Infect cells with MAB at a MOI of 1 and with MAC at a MOI of 0.5 for 6 hours. Remove extracellular bacteria by washing three times with sterile 1×PBS, then add the corresponding drugs to the medium and incubate cells for an additional 72 hours. Wash infected macrophages with 1xPBS and lyse with SDS lysis buffer. Perform 10-fold serial dilutions of the original dilution of the culture suspension and plate on 7H10 solid medium. Fast-growing strains were cultured for 1 week, while slow-growing strains were cultured for 3 weeks.

Mouse Model of Infection

This study first subjected all BALB/c mice (male, 8-weeks old) to one week of immunosuppression using dexamethasone. All mice in this study were infected via tail vein injection of NTM. (MAC: 1×10^6 CFU/mL, MAB: 2.5×10^7 CFU/mL, 0.2 mL per unit). The specific groups and experimental protocols are shown in the legend Mice were euthanised at the conclusion of treatment to determine bacterial counts in the lungs and spleen.

Euthanasia of Mice

At the conclusion of the experiment, mice were placed in a dedicated sealed chamber and euthanised by carbon dioxide inhalation at a rate of 30–70% of the chamber volume per minute. The euthanasia procedure was conducted in accordance with the American Veterinary Medical Association's Guidelines for the Humane Euthanasia of Animals (2020 edition). The liver and spleen were subsequently collected and weighed.

Histological Examinations

Lung tissue was harvested from mice treated in each group and from untreated control animals, then fixed in formalin. Paraffin sections from each specimen were stained using hematoxylin-eosin staining and Masson's trichrome stain.

Flow Cytometric Analysis

Preparation of Mouse Splenic Single-cell Suspension

After euthanasia, the mouse spleen was dissected and a portion was immediately placed into a pre-chilled 70 μM cell strainer. The tissue was ground with pre-chilled 1 × PBS. The resulting cell suspension was collected and centrifuged at 400×g for 5 min at 4 °C, and the supernatant was discarded. The cell pellet was gently resuspended in 2 mL of red blood cell lysis buffer and incubated for 2–3 min at 4 °C in the dark. The lysis was stopped by adding 10 mL of pre-chilled 1× PBS, followed by gentle mixing. The cells were centrifuged again at 400×g for 5 min at 4 °C, and the supernatant was removed. The pellet was resuspended in 1× PBS, and the cells were counted using trypan blue staining. The concentration was adjusted to 1×10^7 cells/mL with 1× PBS, and the cell suspension was kept on ice until use.

Cell Surface Staining

100 μL of the splenic cell suspension (containing 1×10^6 cells) was transferred to a 1.5 mL sterile EP tube. The cells were stained with Fixable Viability Dye diluted 1:1000 in 1× PBS (1 mL per tube) and incubated for 30 min at 4 °C in the dark. After incubation, the cells were centrifuged at 400×g for 5 min at 4 °C, and the supernatant was discarded. The cells were washed once with 1 mL of Staining Buffer, centrifuged again, and the supernatant was removed. Subsequently, 100 μL of an antibody cocktail (anti-mouse CD3, CD4, CD8 antibodies diluted 1:200 in Staining Buffer) was added, mixed thoroughly, and incubated for 30 min at 4 °C in the dark. After staining, 200 μL of Staining Buffer was added to each tube, and the cells were centrifuged and washed. The wash was repeated once. Finally, the cell pellet was resuspended in 300 μL of Staining Buffer and transferred to a flow cytometry tube.

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Flow Cytometry Acquisition and Analysis

Samples were acquired using a BD FACSCanto II flow cytometer. Before acquisition, the cell suspension was thoroughly mixed, and at least 50,000 events were collected per tube. Single-stained controls were used to adjust photomultiplier tube (PMT) voltages and to perform fluorescence compensation for spectral overlap. Data were analyzed with FlowJo V10 software. The gating strategy was as follows: lymphocytes were first gated on an FSC-A vs. SSC-A scatter plot; then, doublets were excluded using FSC-A vs. FSC-H to obtain single cells. $CD3^+$ T cells were gated on a CD3 vs. SSC plot. Within the $CD3^+$ T cell gate, $CD4^+CD8^-$ cells (ie., $CD4^+$ T cells) were identified on a CD4 vs. CD8 plot. Results were expressed as the percentage of $CD4^+$ T cells among $CD3^+$ T cells.

Statistical Analysis

Statistical analysis and graphical representation of data were performed using GraphPad Prism 8.0 and SPSS 26 software. CFU counts underwent logarithmic transformation prior to analysis. The data are presented as "mean ± standard deviation". Comparisons between two groups employed *t*-tests, while multiple comparisons used one-way ANOVA (Analysis of Variance). A p-value < 0.05 was considered statistically significant.

Impact Statement

NTM cause pulmonary infections, particularly in immunocompromised patients. Their inherent resistance to multiple anti-tuberculosis drugs poses significant challenges for both patients and clinicians, thereby driving the need for novel drug discovery. This paper describes the combined action of BDQ and nintedanib against NTM. In vitro and in vivo studies demonstrate that nintedanib enhances BDQ's inhibitory effects. Furthermore, we investigated synergistic effects at the metabolic level. Consequently, these findings highlight the potential of BDQ-nintedanib combination therapy against NTM infections, and provide a theoretical basis for clinical application.

Discussion

The classic classification scheme proposed in 1959 categorises mycobacteria into fast-growing and slow-growing types based on their growth rate; the key distinction is that RGMs typically form colonies within 3 to 7 days, whereas SGMs require more than 7 days.[28] By the end of 2019, the number of globally recognised RGM species had reached 109; among clinical isolates, *M. abscessus* is the most common RGM pathogen.[29] SGM also comprises an extremely diverse range of species, predominantly comprising avian and intracellular mycobacteria.[30] Unlike *Mycobacterium tuberculosis*, NTMs exhibit a remarkably wide range of ecological adaptability, being able to survive in everything from extreme natural environments to the human microbiome. There are significant differences among NTM species in terms of infectivity, modes of transmission and pathogenic potential.[31,32] The increasing prevalence of NTM infections, coupled with limited treatment options and poor therapeutic outcomes, supports the search for novel therapeutic approaches, including therapies aimed at enhancing host defenses as adjuncts to conventional antimicrobial treatment.[10,33–35] The drug susceptibility test (DST) of NTM is detached from the actual physiological environment and ignores host factors and bacterial status, so the correlation between the DST results of NTM and clinical efficacy is much less clear than that of *Mycobacterium tuberculosis*, and the clinical reference has great limitations. Intracellular assay can largely compensate for this deficiency of traditional DST, and its results have a positive predictive value of 74% and a negative predictive value of up to 82% for the therapeutic response of NTM-infected patients,[36] so it is necessary to go beyond pure in vitro experiments and explore the antimicrobial activity assay of NTM deeper into the physiologically relevant environment of macrophages. The relationship between NTM and macrophages is complex, with macrophages acting as both a defender for the removal of NTM and a site for its survival and dissemination. NTM escapes the immune response of host cells through various immune escape mechanisms and is able to survive within macrophages, leading to chronic infections.[37]

NTM has been shown to modulate host immune responses, including preventing phagosome acidification and maturation or escaping from phagosomes into nutrient-rich cytoplasm. Counteracting pathogen-induced immune modulation through HDT represents a promising adjunctive approach to antibiotic treatment against intracellular mycobacterial infections. HDT may also contribute to the elimination of non-replicating and drug-resistant bacteria that are tolerant or resistant to antibiotic

treatment. Furthermore, adjunctive HDT holds the potential advantage of shortening the duration of current treatment regimens, which could reduce adverse drug reactions and lower the likelihood of inducing mycobacterial drug resistance, as it targets the host rather than bacterial pathways.

BDQ, as a novel anti-tuberculosis drug, has demonstrated significant potential in the treatment of drug-resistant NTM infections due to its unique mechanism of action targeting ATP synthase.[38]

Macrophages play a decisive role in the host's response to intracellular bacteria.[39,40] In this study, we propose that nintedanib is a novel candidate drug for the treatment of NTM-associated host immunity. Nintedanib enhances the host's control over intracellular NTM, thereby reducing the intracellular survival rate of NTM. No direct antibacterial activity was observed at concentrations that promote the clearance of intracellular NTM, suggesting that nintedanib must act via host signalling pathways. Previous studies suggest nintedanib's potential host-mediated antibacterial mechanism may accelerate mycobacterial clearance by upregulating macrophage autophagy pathways.[22] In a mouse model of NTM infection, treatment with nintedanib in combination with BDQ significantly reduced bacterial load in the lungs and alleviated pulmonary pathological damage, thereby further enhancing the therapeutic efficacy of BDQ.

Both in vitro and in vivo studies indicate that nintedanib enhances the antibacterial activity of BDQ, which exerts its antibacterial effect by targeting ATP synthase to deplete ATP. We observed that co-administration with nintedanib enhances the depletion of bacterial ATP flux. The gpmA and purB genes are involved in purine nucleotide synthesis, gpsA in glycerophospholipid and triglyceride synthesis, leuD in leucine synthesis, and pyk is associated with glycolysis. RT-qPCR validation confirmed that both nintedanib monotherapy and combination therapy with BDQ downregulate the expression of gpmA, gpsA, pyk, leuD and purB. This suggests that nintedanib may target glycolysis, purine nucleotide and lipid metabolic pathways, providing new insights into the synergistic effects of this drug combination.

This study has several limitations. The broad applicability of the conclusions remains to be confirmed, given the heterogeneity of NTM strains. Due to constraints of the current research model, immunocompromised mice were used. Given the critical role of T cell-mediated adaptive immunity in host defense, subsequent studies should employ immunologically intact animal models and construct NTM infection models more closely aligned with clinical features by introducing phenotypes of underlying diseases (eg., chronic lung disease). Therefore, additional mouse models should be utilized before clinical application. Furthermore, the long-term safety and efficacy of nintedanib in combination with anti-NTM drugs require confirmation.

In summary, our research demonstrates that nintedanib enhances the antibacterial activity of BDQ, accelerating pathogen clearance and mitigating pulmonary injury. Nintedanib exerts a synergistic effect in potentiating BDQ's therapeutic efficacy by interfering with bacterial ATP synthesis and metabolic pathways. Although the results are encouraging, further clinical studies are needed to validate the safety and efficacy of combination therapy in patients. This research provides a crucial theoretical foundation for developing more effective treatment strategies for NTM infections.

Data Availability Statement

The datasets generated during and analyzed during the current study are not publicly available due to their large size and specialized format, but are available from the corresponding author on reasonable request.

Ethics Approval

This study has been approved by the Laboratory Animal Welfare Ethics Committee of the Beijing Institute of Tuberculosis and Thoracic Oncology (Beijing, China; Approval No.: XK2025-123; Licence No.: SCXK (Jing) 2021-0006). All animal experiments were conducted in accordance with the AVMA Guidelines for the Euthanasia of Animals (2020) and institutional animal welfare policies.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. Shaoyu Dong: Writing – original draft, Validation, Methodology, Formal analysis, Conceptualization. Xinda Li: Methodology, Investigation. Weiyan Zhang: Methodology, Investigation. Xiaoyou Chen: Supervision, Project administration, Data curation, Conceptualization. Yu Lu: Supervision, Project administration, Data curation, Conceptualization.

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Disclosure

The authors declare that no conflict of interest exists.

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