RESEARCH ARTICLE

In vitro and in vivo local tolerability of a synergistic anti-tuberculosis drug combination intended for pulmonary delivery

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Abstract

A drug combination, vancomycin (VAN) plus tetrahydrolipstatin (THL), has demonstrated an effective synergistic action in vitro against *Mycobacterium tuberculosis* (Mtb). The poor oral bioavailability of VAN and THL and the predominant tropism of Mtb infection to the lungs make their pulmonary administration very attractive. To evaluate their local tolerability, bronchial cells, alveolar cells and monocytes were exposed to concentrations around and above their minimal inhibitory concentration (MIC). The VAN had no inhibitory activity on the tested human cell lines, even at a concentration 125 times higher than its MIC, whereas the THL, alone or in combination with VAN, presented a cytostatic action. Monolayer epithelium showed no significant irreversible damage at concentrations up to 100 times the combination MIC. BALB/cAnNRj mice exposed to concentration of 50 times the combination MIC delivered endotracheally 3 times a week for 3 weeks showed no clinical signs or significant weight loss. The increase of proinflammatory biomarkers (i.e., IL-1, IL-6, TNF-α and proportion of inflammatory cells) and cytotoxicity in bronchoalveolar lavage fluid (BALF) were non-significant. Lung histopathology did not show significant tissue damage. The VAN/THL combination at doses up to 50 times the combination MIC is found to be thus well tolerated by pulmonary route. This study is a promising result and encouraging further investigations of pulmonary administration of VAN/THL combination as dry powder for anti-tuberculosis treatment.

KEYWORDS
cytotoxicity, *Mycobacterium tuberculosis*, pulmonary delivery, pulmonary tolerance, tetrahydrolipstatin, vancomycin

1 | INTRODUCTION

Tuberculosis (TB) is a mycobacterial infection affecting mainly the lungs, caused by the pathogen *Mycobacterium tuberculosis* (Mtb). It represents the second cause of death by an infectious agent (after the severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]) in the world. Based on the data reported by the World Health Organization (WHO), in 2021, Mtb infected over 10 million people and caused 1.3 million deaths worldwide (World Health Organization, 2021).

Current treatments require at least four antimicrobial drugs for a long-term treatment (6-month regimen), leading to poor therapy adherence, antibiotic resistance with the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) (Tan et al., 2020). Moreover, the Bacillus Calmette-Guerin (BCG) vaccine does not provide a complete protection, especially for adolescents and adults (Mangtani et al., 2014). Hence, a new treatment strategy is needed to comply with WHO's goal to end the global TB epidemic by 2035 (Uplekar et al., 2015).

The new anti-TB treatment approach is a combination of different drugs to enhance the treatment efficacy, reducing dose and frequency of the treatment and improving patient's compliance (Tan et al., 2020). The activity of vancomycin (VAN) and tetrahydrolipstatin (THL) alone and the VAN/THL combination have already been tested in vitro on Mtb (Rens et al., 2016). It was demonstrated that VAN alone has an inhibitory activity against 99% of Mtb at a concentration of 250 μg/ml. THL alone is active at 25 μg/ml but combined, the minimal inhibitory concentration (MIC) of the VAN and THL drops to 10 and 1 μg/ml, respectively. These MIC values are referred as 'combination MIC' in this paper. This synergistic action of the drug combination was observed at a 10:1 ratio. VAN, a large and rigid molecule, is a tricyclic glycopeptide antibiotic widely used to treat infection caused by gram-positive bacteria (e.g., staphylococci and enterococci). This antibiotic (chemical formula: $C_{66}H_{75}Cl_2N_9O_{24}$), with a molecular weight of 1449.2 Da, is a hygroscopic white solid soluble in water (over 100 g/L), moderately soluble in methanol and insoluble in organic solvents (i.e., acetone and ether) (Matzke et al., 1986). In the presence of Mtb cell wall lipid biosynthesis inhibitor, such as cerulenin targeting acyl-ACP or THL targeting phthiocerol dimycocerosate (PDIM) biosynthesis, VAN can diffuse through the mycobacterial wall and exert its inhibitory action against Mtb (Soetaert et al., 2015). The absorption of this drug per os to the systemic compartment is very low in most patients due to its poor oral permeability (Coutsouvelis et al., 2011; Matzke et al., 1986, 1987; Patel et al., 2021; Rao et al., 2011). The intravenous infusion of VAN has shown a good distribution throughout the body, except in the lungs (Cruciani et al., 1996; Matzke et al., 1986). Cruciani et al. (1996) hypothesized that this poor lung distribution could be explained by the physicochemical characteristics of the VAN (i.e., polar compound, high molecular weight and partially ionized at physiological pH) or by the fact that antibiotics tend to bind to plasma proteins, preventing their distribution to the lungs. THL, a derivative of lipstatin, acts as an inhibitor of gastric and pancreatic lipases. THL (chemical formula: $C_{29}H_{53}NO_5$), also called orlistat, is a small molecule with a molecular weight of 495.8 Da. It is practically insoluble in water (i.e., 9.19×10^{-5} mg/ml) but soluble in organic solvents such as methanol and ethanol (DrugBank Online, 2005). This substance alters mycobacterial lipid metabolism (Soetaert et al., 2015). It has been demonstrated that its absorption from the intestine to the systemic compartment is extremely low, as this drug acts locally in the gastrointestinal tract (Zhi et al., 1995, 1996). This poor bioavailability of VAN and THL from oral route to systemic compartment and/or distribution from systemic compartment to the lungs does not allow the use of this combination via the oral route.

The administration of this combination using the pulmonary route could be a good alternative as this route is non-invasive and as Mtb is mainly located in the lungs. Pulmonary administration could enhance therapeutic effects, as drugs directly delivered to the lungs will act at higher drug concentration at the site of infection, with lower systemic side effects (Hickey et al., 2016; Tan et al., 2020). To date, VAN delivered by pulmonary route has proven its safety in humans (de Jesús Valle et al., 2007; Morais et al., 2020; Sullivan et al., 2015; Waterer et al., 2020), but no safety data have been reported on THL delivered to the lungs until now.

The aim of this work was to demonstrate the lung tolerance of the synergistic anti-TB combination VAN/THL at high concentration in vitro and in vivo. The first step was to study in vitro cytotoxic potential of the drugs, alone and in combination (Mosmann, 1983; Scherließ, 2011). Cells selected were models of airway epithelium: Calu-3 from an adenocarcinoma as a model of bronchial cells and epithelium (Kreft et al., 2015) and A549 from a lung carcinoma that showed characteristics of alveolar cells, which could be infected by Mtb (Mariotti et al., 2020). Moreover, THP-1 monocyte cells were also used as a model for macrophages, which are frequently infected by Mtb (Guirado et al., 2013; Weiss & Schaible, 2015). As this combination is intended to be chronically delivered by pulmonary route, a subacute exposure (i.e., three times a week during three consecutive weeks) was subsequently applied at high concentration on healthy BALB/c mice. The dispensing of the combination in solution was made using an endotracheal device to investigate in vivo pulmonary tolerance of the VAN plus THL combination.

2 | MATERIALS AND METHODS

2.1 | Materials

A549 (CRM-CCL-185), Calu-3 (HTB-55) and monocytes THP-1 (TIB-202) cells were purchased from ATCC (Manassas, USA). VAN, THL and Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The dimethyl sulfoxide (DMSO), Roswell Park Memorial Institute 1640 (RPMI) medium and the Dulbecco's modified Eagle's medium (DMEM) were purchased from Carl Roth (Karlseruhe, Germany). Ultrapure Milli-Q water was generated from a Pure-Lab Ultra purification system (Elgan, Lane End, UK). Minimal essential medium (MEM), phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS) and trypsin–ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Thermo Fisher, Dilbeek, Belgium). Penicillin/streptomycin, L-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium pyruvate (Na-PY) and May-Grunwald Giesma coloration were purchased from Merck (Sigma-Aldrich, St. Louis, USA). Millicell-HA 12 mm inserts were purchased from Merck Millipore (Darmstadt, Germany). Sterile syringe filter with 0.2 μm cellulose was purchased from VWR (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) powder, lipopolysaccharide (LPS) and propylene glycol were purchased from Sigma-Aldrich. Sodium pentobarbital (Nembutal®) was purchased from Ceva Santé Animale (Libourne, France). Isoflurane (Isoflo®) was purchased from Zoetis (Malakoff, France). Mouse IL-1β DuoSet ELISA, Mouse IL-6 DuoSet ELISA and Mouse TNF-α DuoSet ELISA kits were purchased from RnD Systems (Abingdon, UK). Bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Fisher.

2.2 | In vitro evaluation

2.2.1 | Cell culture

The human Calu-3 adenocarcinoma cell line was cultured in adherence in MEM supplemented with 10% FBS, L-glutamine (100 μg/ml), sodium pyruvate (100 μg/ml) and penicillin/streptomycin at 10 μg/ml. The human A459 lung carcinoma cell line was cultured in adherence in DMEM supplemented with 10% FBS, L-glutamine (100 μg/ml) and penicillin/streptomycin at 100 μg/ml. The human THP-1 monocyte cell line was cultured in suspension in RPMI medium supplemented with 10% FBS, L-glutamine (100 μg/ml), sodium pyruvate (100 μg/ml) and HEPES buffer at 100 μg/ml. THP-1 cell line was used until passage 20 to reduce the risk of phenotypic change (i.e., differentiation potency and sensitivity to bacterial agents).

All cell cultures were incubated in an incubator (Binder, Germany) at 37°C with a humid atmosphere controlled at 5% CO_2 and were handled under a laminar vertical hood (Esco, Class II, Type A2, Labculture). The culture medium was renewed every 2 or 3 days in flasks with a surface area of 25 or 75 cm^2. The number of cells in the flasks was determined on 10 μl of cell suspension, transferred to a Neubauer counting chamber to be counted using an optical microscope (Euromex, Holland).

2.2.2 | Viability assessment: MTT colorimetric assay

The cell viability of Calu-3, A459 and THP-1, after exposure to THL, VAN or VAN/THL, was evaluated using the MTT colorimetric assay as previously described (Mosmann, 1983). The colorimetric assay that converts the tetrazolium MTT (yellow) into insoluble formazan via the enzyme NAD(P)H-dependent cellular oxidoreductase allows to assess the amount of viable cell in the presence or absence of various molecule concentrations. All experiments were repeated in triplicate in three independent assays ($n = 9$).

For suspension cells (THP-1), 4×10^4 cells per well were seeded inside each well of a 96-well plate using a volume of 100 μl and exposed or not for 72 h to drug(s) at various concentrations (i.e., VAN alone 2–1250 μg/ml, THL alone 0.2–125 μg/ml or VAN/THL 2–1250 μg/ml for VAN and 0.2–125 μg/ml for THL) in a final volume of 200 μl.

For adherent cells (A549 and Calu-3), cells were seeded at a density of 4×10^4 cells per well for A549 and of 3×10^4 cells per well for Calu-3. Medium was refreshed after 24 h culture with 200 μl supplemented culture medium including or not drug(s) at various concentrations (i.e., VAN alone 2–1250 μg/ml, THL alone 0.2–125 μg/ml or VAN/THL 2–1250 μg/ml for VAN and 0.2–125 μg/ml for THL).

After an additional 72 h incubation at 37°C, 20 μl of MTT (0.5 mg/ml) were added to each well. Plates were incubated for 4 h at 37°C and then centrifuged at 1200 rpm for 5 min for adherent cell strains or at 1900 rpm for 10 min for cells in suspension. The medium was then removed, and formazan crystals were washed once with 100 μl of PBS. Finally, formazan crystals were solubilized within 50 μl of DMSO prior to spectrophotometer analysis (Synergy HT, Bio-Tek and KC4 software Version 3.4, Bio-Tek) at 570 nm (reference wavelength 610 nm). The optical density (OD) analysed was $OD_{570\,nm}-OD_{630\,nm}-OD_{blank}$. The results expressed the cell viability in percentage, calculated by dividing the absorbance at a given concentration of the product by the absorbance of the control (without product), depending on the concentration of drug. The inhibitory concentration (IC_{50}), representing the concentration that inhibited 50% of the cell viability, was also calculated.

2.2.3 | Cytocidal assessment: LDH assay

Calu-3 cells were selected for assessing cytocidal effect of THL, VAN or VAN/THL. Calu-3 were seeded on a 96-well plate at a density of 1×10^5 cells per well in a final volume of 200 μl of medium and incubated for 24 h for cell adhesion. After incubation, the plate was centrifuged at 1300 rpm for 5 min and the medium was replaced by 200 μl of HBSS. Twenty microlitres of Triton X-100 were added for maximum release positive control wells, and 20 μl of assay buffer were added for spontaneous release negative control wells. The background was determined using wells containing only medium. The remaining wells with cells were treated at different concentrations of VAN (100–2000 μg/ml), THL (10–200 μg/ml) or VAN/THL (100–1000 μg/ml for VAN and 10–100 μg/ml for THL).

The plate was incubated at 37°C for 24 h before LDH cytotoxicity assay assessment, following the kit standard procedure. Briefly, the plate was centrifuged at 1300 rpm for 5 min before transferring 100 μl of all cell supernatants into a new 96-well plate. A volume of 100 μl of reaction solution was added to each well before incubation at 37°C, under gentle shaking on an orbital shaker for 30 min in the dark (KS 4000 I control, Ika). The absorbance was read at 490 nm (A490). The percentage of cytotoxicity was then calculated using the following formula:

$$\% \text{ cytotoxicity} = \left[\frac{(experimental\ value\ A490) - (spontaneous\ release\ A490)}{(maximum\ release\ A490) - (spontaneous\ release\ A490)} \right] * 100.$$

2.2.4 | Epithelial integrity assay

To assess the effects of the combination of VAN and THL on cells organized as an epithelium, the integrity of monolayer epithelium at a liquid–liquid interface using the Calu-3 cells was investigated (Srinivasan et al., 2015). Calu-3 were seeded on the Millicell-HA 12 mm insert (pore size 0.45 μm, surface area 0.6 cm^2) in a 24-well plate at an initial density of 7.5×10^5 cells/cm^2 on the apical surface of the insert (liquid–liquid method). Cells were grown with 400 μl of culture medium in the apical surface and submerged inside 600 μl of culture medium in the basolateral chamber (well). Every 2–3 days, the MEM was changed. The evaluation of the resistivity was measured using an epithelial voltohmmeter EVOM^2

using chopstick electrodes STX2 (World Precision Instrument Inc., USA) at the apical–basal interfaces. The measurement was done three times for each insert at different sides of the well using the electrodes. After approximatively 7 days, when the ohmic signal was above 300 Ω, monolayer epithelium was ready on the insert to perform the assay.

To prepare the monolayer epithelia before treatment, medium was discarded, and the monolayer epithelia were washed twice with HBSS solution. Then MEM without FBS and phenol red was added on each insert and well of the plates that were incubated 30 min to equilibrate. The transepithelial electrical resistance (TEER) was measured before the treatment. MEM from the apical side of each insert was thrown away and was replaced by the adapted concentrations of drugs diluted in MEM (i.e., THL alone 50–100 μg/ml, VAN alone 500–1000 μg/ml or VAN/THL 500–1000 μg/ml for VAN and 50–100 μg/ml for THL). The TEER was measured 1, 2, 3 and 24 h after treatment. After the last sampling, monolayer epithelia were washed twice with HBSS solution. Then MEM without FBS and phenol red was added to each insert and well of the plates that were incubated at 37°C for 48 h. Two last measurements were made 24 and 48 h later (i.e., 48 and 72 h post-treatment) after removing the drugs.

To correctly interpret the results, it was necessary to subtract the measured value from the blank value on a single insert without cells (equivalent to approximately 140 Ω per insert). The resistivity of the membrane expressed in ohm (Ω) was obtained as raw data. Furthermore, the surface of the insert must also be taken in account. The resistivity of the membrane was then multiplied by this surface (equivalent to 0.6 cm^2). The result was therefore expressed in Ω·cm^2.

2.3 | In vivo assessment

2.3.1 | Animals

Eight to 10-week-old male ($n = 36$, approximately 24.5 g) and female ($n = 36$, approximately 19.5 g) BALB/cAnNRj mice, purchased from Janvier Labs (Le Genest-Saint-Isle, France), were used in this study after a quarantine period of 5 days. The animal had free access to food (Carfil Quality, Oud-Turnhout, Belgium) and water and were kept in controlled standard conditions (day/night cycle of 12 h, temperature of 22 ± 2°C, humidity rate of 55 ± 10%). Four to eight animals of the same gender were placed in type III cages (area: 830 cm^3, dimensions: 42 × 27 × 15 cm from Ehret, Germany). The animal protocol, registered under ethical protocol 727N (registration number LA 1230568), was approved by animal ethical committee of the ULB institute (CEBEA, Comité d'Ethique et du Bien-Etre Animal) and was in accordance with EU Directive 2010/63/EU. Body weights were recorded three times per week. The animals were examined once per day for appearance, posture and behaviour. During the study, limit points were defined by a scoring (respiratory function, weight loss and behaviour).

2.3.2 | Pulmonary administration

Due to the low solubility of THL in aqueous solutions, a liquid vehicle composed of 25% propylene glycol and 75% phosphate buffer (0.02 M, pH 5.0) was used. Limpid drug solutions were formulated daily and were filtered through 0.2 μm filter. The volume of administered liquid to the mice was 50 μl.

Three groups of 16 mice (8 females and 8 males) were administered either THL alone, VAN alone or VAN/THL, 3 times a week for 3 consecutive weeks (Administration Days 1, 3, 5, 7, 9, 11, 13, 15 and 17) in the following quantities: 50 μg/g of lung or 50 mg/kg body weight for the THL-treated group, 500 μg/g of lung or 500 mg/kg body weight for the VAN-treated group or 500 μg/g of lung (or 500 mg/kg body weight) for VAN and 50 μg/g of lung (or 50 mg/kg body weight) for THL for the VAN/THL-treated group corresponding to 50 times the combination MIC (1 μg/ml for THL and 10 μg/ml for VAN considering that 1 g of lung tissue represented approximatively 1 ml). These groups were compared with the vehicle control (Ctrl, $n = 16$, 8 males and 8 females), which only received the liquid vehicle used to solubilize drugs. The LPS group ($n = 8$, 4 females and 4 males) was exposed to a solution of 1 μg of LPS from *Escherichia coli* 18 h before sampling.

Isoflurane anaesthesia (2%–4%) in oxygen (2%) was applied to anaesthetize the mice for 5 to 7 min during endotracheal administration of 50 μl of solution using the IA-1C® Microsprayer™ tip connected to a FMJ-250-high-pressure syringe (Penn-Century, USA) according to the endotracheal procedure described by Bivas-Benita et al. (2005) and adapted by Chraibi et al. (2021).

2.3.3 | Sampling, histopathology and evaluation of the pulmonary tolerance

The evaluation of the pulmonary tolerance was performed as described by Chraibi et al. (2021). Indeed, a lethal dose (10 mg/kg) of sodium pentobarbital was intraperitoneally administered to the treated groups and the vehicle control 24 h after the last administration. The LPS group was sampled 18 h after LPS administration, following the inflammatory biomarker's kinetics (Bondue et al., 2012).

After blood collection by retro-orbital puncture, the trachea was cannulated, and the lungs were flushed three times with 0.7 ml of fresh PBS solution to collect the bronchoalveolar lavage fluid (BALF). A volume of 20 μl of the BALF was used to evaluate the number of viable cells per millilitre by a Countess II FL (Life Technologies, Thermo Fisher Scientific, Dilbeek, Belgium). The rest of the BALF was centrifuged for 5 min at 200 g at 4°C. The supernatant was aliquoted and stored at −80°C until further analysis for the proinflammatory biomarkers and cytotoxicity. The cell pellet was resuspended in 200 μl of fresh PBS and used for cytospin preparation to fix cells on slides. The cytospin was set for 5 min at 800 rpm at room temperature. Cells were then stained with the May-Grunwald Giesma coloration to determine the cell proportion between macrophages, eosinophils and neutrophils as a percentage of 200 BALF cells.

Lungs were then collected, washed in fresh PBS solution and fixed in 4% formaldehyde solution for 24 h. The lungs (10 were selected for each group, i.e., THL-treated, VAN-treated, VAN/THL-treated and vehicle control, and 8 lungs were sent for the LPS group) were embedded in paraffin wax and stained with haematoxylin and eosinophil coloration. The investigation of lung damage of these 48 slides (one section per lung) was conducted by an independent pathologist, as a randomized and blinded study (Organization for Economic Co-operation and Development, 2018). The frequency of each observation (congestion, intra-alveolar haemorrhage [IAH], pneumocyte hyperplasia [HYP] and bronchiolar epithelial vacuolation [BEV]) was calculated as the number of animals for whom the observation occurred (Chraibi et al., 2021).

Investigations of inflammation and cytotoxicity on BALF were conducted to evaluate the local tolerance. Quantification of selected proinflammatory biomarkers (IL-1β, IL-6 and TNF-α) was done by the ELISA method, as described by the manufacturer. Pierce BCA protein assay kit was used to quantify the total protein content. Measurement of cytotoxicity via the LDH activity was also done using the LDH Cytotoxicity Assay from Cayman Chemicals.

2.4 | Statistical analyses

GraphPad Prism® (Version 8.4.3) software was used to perform in vitro and in vivo statistical analyses. To study the in vitro local tolerance, the mean of each treated group (i.e., THL, VAN and VAN/THL) ± standard deviation (SD) and/or standard error of mean (SEM) was compared with the mean of the negative control group for MTT, TEER and LDH ratio measurements for in vitro experiment. A one-way analysis of variance (ANOVA) and Bonferroni's multiple comparisons tests were applied when stipulated. For in vivo experiment, the mean ± SD of each treated group (i.e., THL, VAN and VAN/THL) and the LPS group were compared with the mean of the vehicle control for IL-1β, IL-6, TNF-α, LDH ratio and protein content. A one-way ANOVA and Bonferroni's multiple comparisons tests were applied. The results were expressed as follows: non-significant for $p > 0.05$, statistically significant (*) for $p < 0.05$, very significant (**) for $p < 0.01$ and extremely significant for $p < 0.001$ (***) and for $p < 0.0001$ (****).

3 | RESULTS

3.1 | In vitro tolerance

3.1.1 | Effects on cell viability

In the presence of VAN, the amount of cells was not affected, identical for the three cell lines tested (i.e., Calu-3, A549 and THP-1), even at the highest dose. Cell viability was above 80% in comparison with the negative control after exposure to the different concentrations of VAN (Figure 1A). VAN was well tolerated with low toxicity for bronchial cells, alveolar cells and monocytes.

However, THL (Figure 1B) and VAN/THL (Figure 1C) demonstrated a dose-dependent effect on viable cell amount. Table 1 presents the THL IC_{50} mean values ± SD for each strain based on the results obtained for THL- and VAN/THL-treated cells.

Calu-3, A549 and THP-1 cells demonstrated similar IC_{50} after exposure to THL alone or the VAN/THL combination. The presence of VAN did not change significantly the IC_{50}. Therefore, THL seems to be the only limiting factor in the VAN/THL combination but at concentrations 34–48-fold higher than the combination MIC. Cell viability fluctuations (SD comprised between 0.2% and 29.5%, or SEM comprised between 0.1% and 17.0%) were observed for all cell lines,

TABLE 1 IC_{50} values (means ± SD) for THL alone or in combination with VAN in the MTT assay ($n = 3$)

Cell lines	IC_{50} for THL alone (μg/ml)	IC_{50} for THL in combination with VAN (μg/ml)
Calu-3	51 ± 10	34 ± 2
A459	51 ± 9	45 ± 8
THP-1	57 ± 14	48 ± 6

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; THL, tetrahydrolipstatin; VAN, vancomycin.
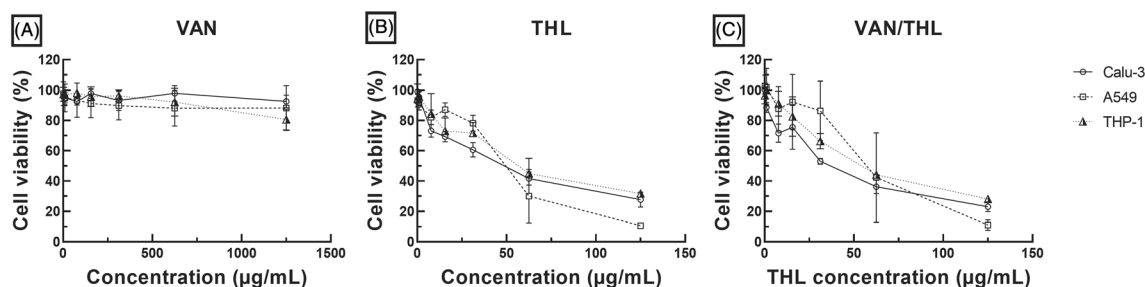

FIGURE 1 Dose–response curve of viable cell amount in percentage versus the negative control on three cell lines (Calu-3, A549 and THP-1). Vancomycin (VAN) (A) did not affect cell proliferation, but a dose-dependent effect was observed with tetrahydrolipstatin (THL) (B) and VAN/THL (C). Concentrations are expressed in μg/ml. All results are expressed as means of three wells per condition ± SD ($n = 3$).

probably due to a fluctuation in terms of number of cells seeded per well and the basal cell death rate.

3.1.2 | Cytocidal or cytostatic effects

It is important to investigate whether reduced viable cell amount resulted from cytocidal activity (cell lysis) or cytostatic activity (antiproliferative activity) in the MTT test. As the effects of THL on cell viability could be induced by cytotoxicity and/or cytostatic effects, an LDH assay was performed on Calu-3 bronchial cell line. If THL induces cytotoxicity on Calu-3 cells, the cytoplasmic LDH will be released outside the cells and will be quantified. After 24 h of cell exposure to different treatments (i.e., THL, VAN or VAN/THL) at various concentrations (i.e., 10–200 times the combination MIC for THL and VAN alone and 10–100 times the combination MIC for VAN/THL), cell cytotoxicity was determined by quantifying the LDH release. The range of concentrations tested in this assay was large enough to detect a potential cytotoxic effect of THL even at higher dose than 50 times the combination MIC.

Cells treated with the assay buffer represented the spontaneous release of LDH and were considered as a negative control (0% cytotoxicity). On the other hand, cells treated with Triton X-100 were considered as the positive control with the maximum LDH release (100% cytotoxicity).

As presented in Figure 2, the cytotoxicity rate of the treated cells was around the baseline of the negative control (spontaneous LDH release) after 24 h. No cytotoxicity was observed in the VAN-treated cells (from −5 ± 6% at 10 times the combination MIC to −5 ± 2% at 200 times the combination MIC), in the THL-treated cells (from −9 ± 0% at 10 times the combination MIC to −1 ± 6% at 200 times the combination MIC) and in the combination-treated cells (from −8 ± 1% at 10 times the combination MIC to −7 ± 2% at 100 times the combination MIC), as LDH release was not significatively different between treated groups and negative control. The extracellular LDH activity was therefore non-detectable, showing a very low level of cell lysis by toxic impact of the various drugs on the Calu-3 cells. This was
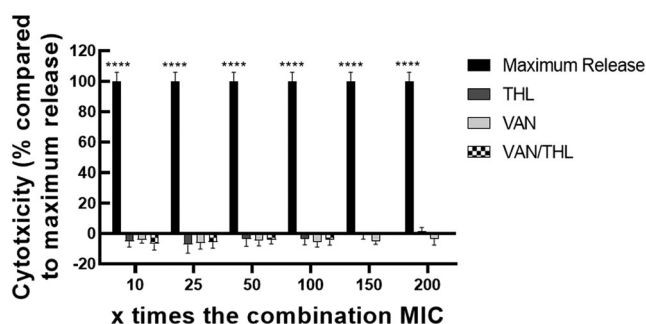
observed at the concentrations tested and even at the highest dose (200 times the combination MIC).

3.1.3 | Bronchial epithelium integrity

A transepithelial electrical assay was used to evaluate the integrity of the monolayer epithelium cellular barrier formed by Calu-3 cells after exposure to THL, VAN or VAN/THL at various concentrations (i.e., THL: 50–100 μg/ml, VAN: 500–1000 μg/ml and VAN/THL: 50–100 μg/ml for THL and 500–1000 μg/ml for VAN) representing 50–100 times the MIC combination. Before treatment (at 0 h), the resistivity values of all monolayer epithelia on the inserts were between 526 ± 168 and 597 ± 188 $\Omega \cdot cm^2$ (Figure 3). Treatments were applied and only the highest drug concentrations (i.e., 1000 μg/ml for VAN, 100 μg/ml for THL and 1000/100 μg/ml for VAN/THL) are represented in Figure 3.

Throughout the post-treatment time (after 1, 2 and 3 h), a nonsignificant decrease in monolayer resistivity was observed for all the different conditions in comparison with the negative control. This trend was also observed in the negative control. After 3 h post-treatment, the monolayer resistivity was between 376 ± 86 and 423 ± 51 $\Omega \cdot cm^2$, demonstrating still resistant junctions of the monolayer epithelium during the first hours of treatment. At 24 h post-treatment, values dropped in a non-significant way. At this time, the monolayer epithelium treated with the VAN/THL combination at 500/50 and 1000/100 μg/ml reached resistivities of 194 ± 62 and 239 ± 147 $\Omega \cdot cm^2$, respectively, and with THL at 100 μg/ml, it reached

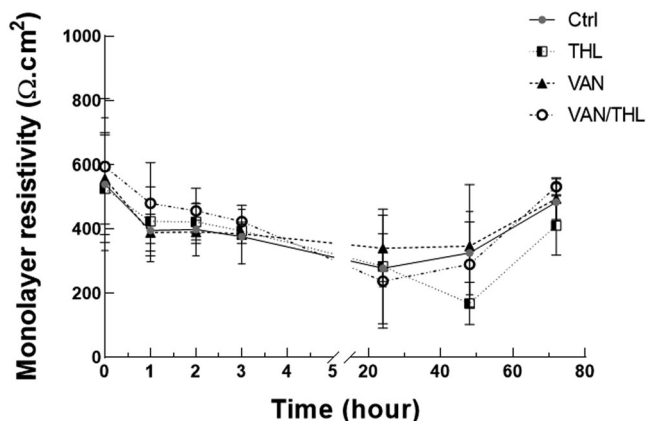**FIGURE 3** Transepithelial electrical resistance (TEER) monolayer epithelium integrity (in $\Omega \cdot cm^2$) as a function of time (in hours) of Calu-3 monolayer epithelium before treatment (0 h), after treatment (1, 2, 3 and 24 h post-treatment measurements) and after removing the treatments and replacing them by fresh medium (48 and 72 h post-treatment) with tetrahydrolipstatin (THL) at 100 μg/ml, with vancomycin (VAN) at 1000 μg/ml and with the VAN/THL combination at 1000:100 μg/ml. Monolayer epithelia without treatment were used as control (Ctrl). Monolayer epithelium integrity in all conditions decreased with time but fully recovered after the removal of treatments. All results are expressed as means of three wells per condition ± SD (n = 3).

FIGURE 2 Vancomycin (VAN), tetrahydrolipstatin (THL) and the combination VAN/THL did not present any cytotoxicity on Calu-3 cells (**** for p < 0.0001), compared with maximum release by Triton X-100. All results are expressed as means of three wells per condition ± SD (n = 3).

284 ± 179 Ω·cm². The negative control demonstrated a similar resistivity after 24 h (278 ± 57 Ω·cm²), and the differences observed with the combination were not significant in comparison with the negative control.

The TEER values measured during recovery are presented in Figure 3. The treatment was removed after 24 h and replaced by fresh culture medium. A first measurement 24 h after removing treatment (48 h post-treatment) demonstrated an increase of the monolayer epithelium resistivity beyond 290 ± 133 Ω·cm² for all conditions, except for cells treated with 100 μg/ml THL (i.e., 169 ± 66 Ω·cm²). This decrease is however not statistically significant versus the negative control at the same sampling time. At 48 h after removing treatments (72 h post-treatment), all epithelia demonstrated a full TEER recovery (from 411 ± 92 to 532 ± 25 Ω·cm²), even for cells treated with 100 μg/ml THL (significant increase from 24 and 48 h after treatments removed, $p = 0.02$).

3.2 | In vivo tolerance in mice

Because similar results were obtained for each assay between each gender, all the results presented and discussed in this paper are from the female and the male groups without any gender distinction, unless clearly stated.

3.2.1 | Pulmonary administrations and clinical signs

During the study, no mice reached the end points defined by the scoring in the ethical protocol. The respiratory functions were normal, and no abnormal behaviour was noticed.

Mean body weights of the mice were presented in Figure 4. At the beginning of the treatment, a slight weight loss was observed for the THL-treated group (at Days 3 and 5), with a reduction below 5%,
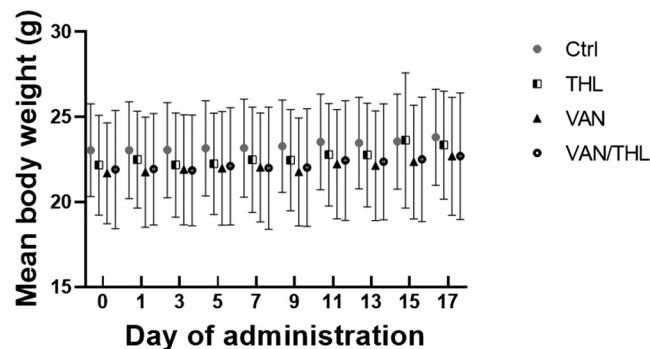

FIGURE 4 Mean body weight of the mice (in grams) did not reach limit points during the treatment period for vehicle control (Ctrl), tetrahydrolipstatin (THL), vancomycin (VAN) and VAN/THL groups. All results are expressed as means ± SD ($n = 16$). A statistical analysis was performed between the treated groups and the vehicle control using one-way analysis of variance (ANOVA) and Bonferroni's multiple comparisons tests for both genders ($p > 0.05$).

which was not significant versus the vehicle control. Indeed, a fast weight recovery was observed after the first week of treatment. No significant weight loss was observed in the other treated groups versus the vehicle control. No external clinical signs were noticed during and after the pulmonary administration of THL, VAN or VAN/THL solutions.

3.2.2 | Inflammation evaluation

The subacute pulmonary inflammation of THL, VAN or VAN/THL was evaluated in the BALF 24 h after the last administration. The total number of cells of each treated group was not significantly different versus the vehicle control (1.3 ± 0.9, 1.6 ± 0.7, 1.1 ± 0.7 and 1.4 ± 0.8 × 10⁵ cells/ml for THL, VAN, THL/VAN-treated groups and vehicle control, respectively). The proportion of cells (i.e., alveolar macrophages, neutrophils and lymphocytes) was also assessed on a total of 200 BALF cells. As expected, mice presented a significant decrease (32.5 ± 20.1%) of the number of alveolar macrophages in the LPS group versus the vehicle control (99 ± 0%) and, therefore, a higher proportion of neutrophils (LPS group 67 ± 20% vs. vehicle control 0.0 ± 0.0%). Most cells present in the treated groups (i.e., THL, VAN and VAN/THL) and in the vehicle control were usually alveolar macrophage cells (approximatively 99% of cells proportion). However, some neutrophils (from 0.3 ± 0.5% to 1.0 ± 2.8% of cells proportion) and lymphocytes (from 0.1 ± 0.2% to 0.2 ± 0.4% of cells proportion) were also counted in these treated groups. No significant differences were observed versus the vehicle control.

The quantification of the proinflammatory biomarkers (i.e., IL-1β, IL-6 and TNF-α) was also performed in the BALF (Figure 5). As expected, the LPS group presented a significant increase in inflammation biomarkers such as IL-1β (i.e., 279 ± 62 vs. 146 ± 53 pg/ml for vehicle control), IL-6 (48 ± 15 vs. 28 ± 11 pg/ml for vehicle control) and TNF-α (237 ± 96 vs. 94 ± 49 pg/ml for vehicle control). Within the treated groups (i.e., THL, VAN and VAN/THL), no significant increase was observed versus the vehicle control for IL-1β, IL-6 and TNF-α.

3.2.3 | Cytotoxicity evaluation

The cytotoxicity induced by the treatment was evaluated by measuring the protein concentration levels and the LDH activity in the BALF versus the vehicle control. No significant increase in terms of protein content in the BALF, which could indicate damage to the alveolar capillary barrier junctions of the lungs, was observed in treated groups (i.e., THL, VAN and VAN/THL) versus the vehicle control.

However, a significant increase in LDH activity was only observed in the THL-treated group versus the vehicle control (for both genders: $p = 0.04$, THL-treated group: 2.0 ± 1.3, vehicle control: 1.0 ± 0.5) (see Figure 6). This significant increase of LDH release in the THL-treated group was observed in two male cases, which presented very high levels of LDH release. By taking gender into account, LDH release
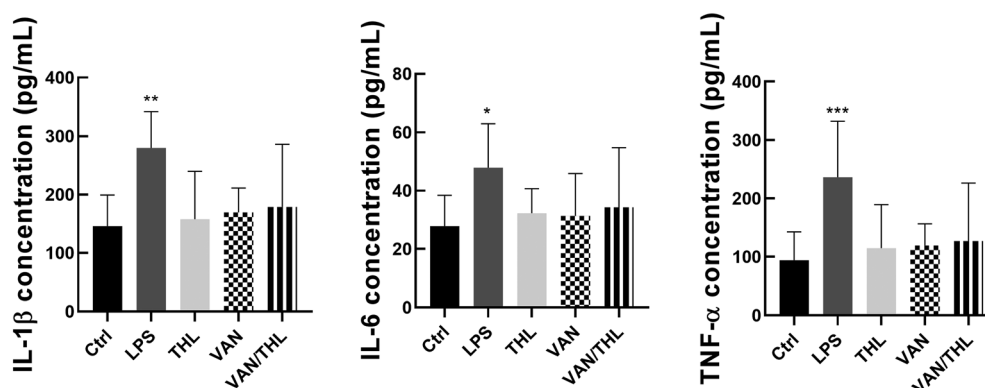
FIGURE 5 Mouse proinflammatory biomarkers IL-1β (A), IL-6 (B) and TNF-α (C) concentrations (pg/ml) in the bronchoalveolar lavage fluid (BALF) 24 h post-treatment (scheme of three times a week during three consecutive weeks) not increased for tetrahydrolipstatin (THL)-treated, vancomycin (VAN)-treated or VAN/THL-treated groups versus the vehicle control (Ctrl) but increased for lipopolysaccharide (LPS) group in the BALF 18 h post-administration. All results are expressed as means ± SD (n = 8–14). A statistical analysis was performed between the treated groups and the negative control group using one-way analysis of variance (ANOVA) and Bonferroni's multiple comparisons tests for both genders (*** for $p < 0.001$, ** for $p < 0.01$ and * for $p < 0.05$).
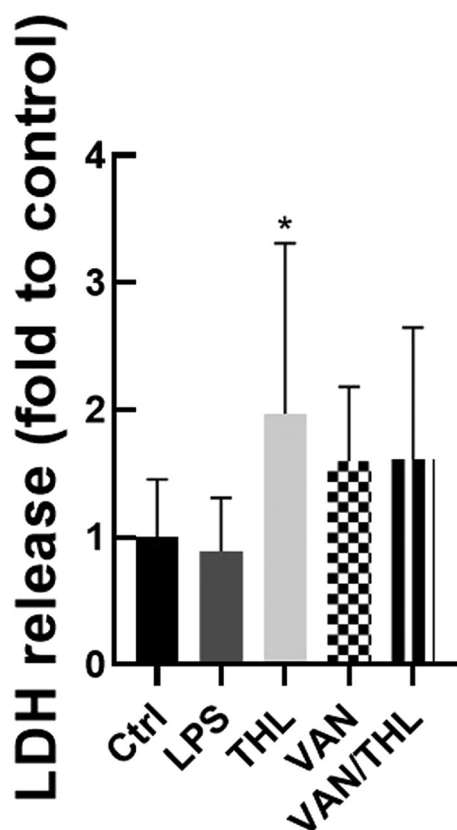

FIGURE 6 Lactate dehydrogenase (LDH) release increased for tetrahydrolipstatin (THL)-treated group versus the vehicle control in bronchoalveolar lavage fluid (BALF) 24 h post-administration. Vancomycin (VAN)-treated and VAN/THL-treated groups (in BALF 24 h post-administration) and lipopolysaccharide (LPS) group (in BALF 18 h post-administration) did not increase LDH activity. All results are expressed as means ± SD (n = 8–14). The statistical analyses were performed versus the negative control group using one-way analysis of variance (ANOVA) and Bonferroni's multiple comparisons tests for both genders (* for $p < 0.05$).

was not significantly different for females versus the vehicle control. However, males demonstrated a significant increase of the LDH release versus the vehicle control ($p = 0.03$). Although THL alone increased LDH release, VAN/THL-treated group did not engender any significant LDH increase.

3.2.4 | Lung histopathology

To reduce the number of analyses, only 8 to 10 animals per group were selected for histology. All the groups displayed damages (see Figure 7).

The most consistent finding was IAH, reported for all groups, including the vehicle control. Pulmonary congestion (Cong) was also observed for all the groups. The LPS group was the only group demonstrating HYP (50% of mice) and BEV (37.5% of mice).

4 | DISCUSSION

Lipids in the outer membrane of mycobacteria have revealed their role in resistance to large antibiotics, including glycopeptides such as VAN (Soetaert et al., 2015). It was observed that the absence of lipid, in particular PDIM, increases the sensitivity to glycopeptides, potentially by favouring its diffusion through the mycobacterial wall to exert their inhibitory action, such as inhibition of peptidoglycan synthesis and thus inhibition of mycobacterial growth. The screening of lipid inhibitors able to synergize with VAN to inhibit mycobacteria growth led us to identify THL (Soetaert et al., 2015). It was then demonstrated that the inhibition of the PDIM biosynthesis by THL could act synergistically with VAN to inhibit Mtb, at a 10:1 ratio (Rens et al., 2016). Due to the poor membrane permeability of VAN and THL, they must be delivered at their site of action using a non-invasive route of
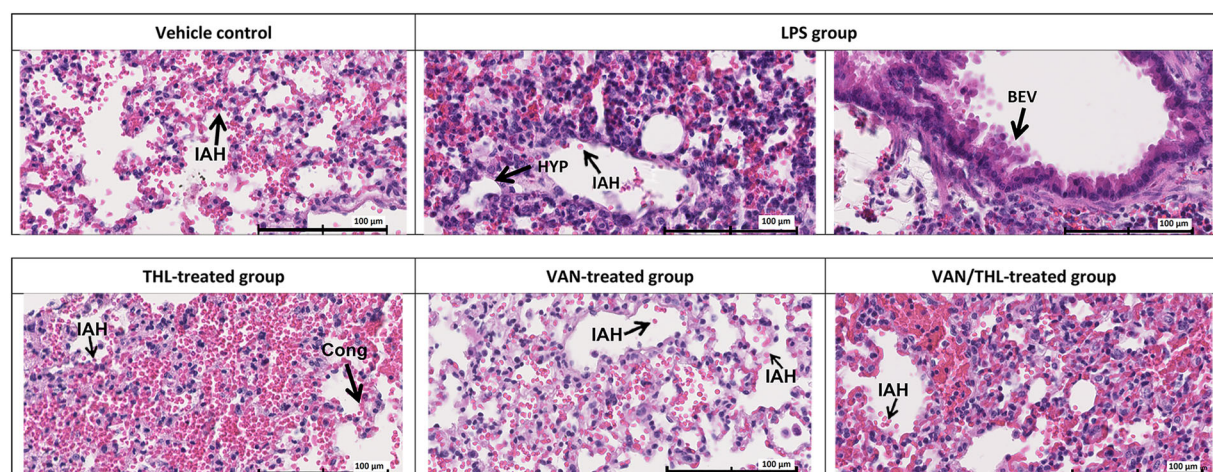
FIGURE 7 Representative images of lung tissue exposed to vehicle control, lipopolysaccharide (LPS), tetrahydrolipstatin (THL), vancomycin (VAN) or VAN/THL groups ($n = 8$–10) (optical microscopy, ×40). Intra-alveolar haemorrhage (IAH; black arrow points intra-alveolar erythrocytes) and pulmonary congestion (Cong) were observed in all groups. Only LPS group presented pneumocyte hyperplasia (HYP) and bronchiolar epithelial vacuolation (BEV).

administration, such as by pulmonary delivery. In the absence of previously reported tolerance studies on THL alone or in combination with VAN, in vitro and in vivo pulmonary tolerability studies were conducted to assess whether the VAN/THL combination could be administered via the pulmonary route and be involved in future pharmaceutical developments.

In order to evaluate the tolerance of VAN and THL alone or in combination on cells, on monolayer epithelia in vitro and in the mouse lungs in vivo, solutions were prepared in a liquid vehicle: FDA-approved co-solvent (25% propylene glycol) (Wauthoz & Amighi, 2015) within a phosphate buffer at pH 5.0. The liquid solution for pulmonary delivery was developed as a limpid solution that did not precipitate despite high dilution (i.e., 1:100 dilution). VAN and THL were completely solubilized in this solution, allowing the maximum pharmacological action of both drugs. As the formulation delivered into the lungs has certainly reached higher drug concentration at the beginning of the bronchial tree than at the end (i.e., in the alveolar ducts and air sacs) where alveolar macrophages resided, the strategy was to test high drug(s) concentration. According to the architecture of the lung, it is expected that during pulmonary administration, bronchial cells and monolayer epithelia (the conducting zone) will encounter higher concentration than alveolar cells (the respiratory zone) (Chaurasiya & Zhao, 2021). In addition, the targeted alveolar drug concentration should be around 10 times the drug concentration (Momin et al., 2018) at which it inhibited Mtb proliferation to 99% (MIC). The MIC were previously established (Rens et al., 2016). In combination, the synergic activity of the antibiotic VAN and the lipase inhibitor THL allowed to use in vitro 10 μg/ml VAN and 1 μg/ml THL (ratio of 10:1).

The colorimetric MTT assay allowing to detect the number of living cells with preserved mitochondrial function (van Meerloo et al., 2011) showed that VAN was well tolerated by the cells, even at the highest tested dose (1250 μg/ml corresponding to 125 times the

concentration MIC). The cell viability profile was identical for the three tested cell lines (i.e., Calu-3, A549 and THP-1). VAN never reached the IC_{50} at the tested concentrations. Cells treated by THL and VAN/THL combination presented a different profile, with a dose-dependent effect after 72 h of treatment (the highest THL dose tested during this assay was 125 μg/ml, i.e., 125 times the combination MIC). The IC_{50} was reached at 30 to 50 times the THL combination MIC. THL genotoxicity was previously documented on lymphocytes from human peripheral blood at 250 μg/ml (Chakrabarti et al., 2017). To our knowledge, our work is the first study on the THL effect on airway epithelium Calu-3 and A549 cell lines and THP-1 monocyte cell line. The nature of THL effect (cytostatic or cytocidal) observed during the MTT assay was unknown and required additional tests to identify the reason of the decrease of cell viability (such as LDH and TEER assays).

Calu-3 cells were more sensitive to THL in the viability assay than A549 and THP-1 (see Table 1). Moreover, the bronchial tree is exposed to a much higher drug concentration than alveolar cells or monocytes during pulmonary administration. To be in the most limiting conditions, it was decided to do further investigations on VAN, THL or VAN/THL on bronchial cells and bronchial epithelium (Calu-3 cells): Cell viability assay was completed with the measurement of the cell lysis (LDH assay) and the study of the monolayer epithelium integrity (TEER assay). In the TEER assay, it is also necessary to work on cells that can form tight monolayers such as Calu-3, which is not the case for A549 and THP-1 cells.

To assess whether the reduced cell viability observed in the presence of THL resulted from a cytocidal or a cytostatic activity, we investigated Calu-3 cell membrane integrity by measuring the released LDH activity in the culture medium. LDH is a cytoplasmic enzyme capable of converting lactic acid into pyruvic acid. During membrane damage or cell lysis, this enzyme is released into extracellular medium and can be quantified by measuring its activity (Holder et al., 2012).

LDH plays the role of an indicator of membrane integrity and constitutes a measure of cytotoxicity (cell death, whether necrotic or apoptotic without distinctions) (Holder et al., 2012). No cytotoxicity effect was observed, even in presence of the highest dose of THL (200 µg/ml, i.e., 200 times the combination MIC). This suggested that the reduced amount of cells detected in the MTT assay would result from an antiproliferative/cytostatic effect rather than a cytocidal effect.

The TEER assay measured the tightness and the integrity of monolayer cell epithelium. The liquid–liquid interface method presents higher TEER values than the air–liquid interface method and is more suitable for integrity studies (Kreft et al., 2015). A tight epithelium is confirmed with a measure of TEER > 500 $\Omega\cdot cm^2$ for a 1-cm^2 insert (i.e. >300 $\Omega\cdot cm^2$ for a 0.6-cm^2 insert) (Kreft et al., 2015; Srinivasan et al., 2015; Wan et al., 2000). After a long contact period (24 h), VAN had very little effect on the monolayer cell epithelium resistivity, although a slight damage could be observed (even in the negative controls). With high concentration of THL (i.e., 100 µg/ml) or THL combined with VAN, the monolayer epithelium resistance decreased under 300 $\Omega\cdot cm^2$, showing also slight damage to the monolayer epithelium integrity. Nevertheless, these slight damages seem due to the conditions of analysis rather than due to the drugs as the negative control showed a similar resistivity after 24 h. It is probably due to the stress induced by the change of temperature and environment (ambient temperature and low CO_2 level under a laminar vertical hood vs. 37°C with a humid atmosphere controlled at 5% CO_2 in the incubator) and the absence of FBS during the analysis, inducing a stress on the monolayer epithelium. These conditions affected the membrane tight junction integrity of the monolayer epithelium and are therefore not related to a direct cytotoxicity action of drug(s) (Ndayishimiye et al., 2021). After drug removal, cell regeneration was fast and complete after 24 h (48 h post-treatment) for all conditions, except with THL. Indeed, a decrease of the resistivity of the monolayer of cells treated with THL 100 µg/ml was observed. The effect of THL at this concentration could be explained by the cytostatic effect of THL, slowing monolayer epithelium regeneration. Forty-eight hours after removing treatment (72 h post-treatment), the monolayer epithelium treated with THL 100 µg/ml demonstrated almost full regeneration. This regeneration is attributed to the lower stress applied to the monolayer epithelium with analysis spaced by 24 h in comparison with analysis made every hour for the treated groups (optimal growth condition: 10% FBS, 37°C, 5% CO_2, without interruption) and in the absence of cytostatic compounds. Moreover, THL seems to slow down the regeneration, without any irreversible impact on the monolayer epithelium integrity, even at 100 µg/ml THL. This effect of THL (cytostatic effect and reducing tight junction) is thus completely reversible.

Various biases should be considered in this kind of in vitro assays. Besides the activity of the active ingredient, possible explanations for differences may be the nature of the cell line, the variation of the exact number of cells per well, the stage of growth reached by the cells, the basal cell death rate, enzymatic interference and so forth. However, the in vitro results remained in the range of those observed in other cell assays (Ahmad et al., 2006; van de Loosdrecht et al., 1994) and provided significant basis in this work to continue investigating pulmonary tolerance of these drugs in an in vivo model. As the anti-TB treatments must be administered during a long period of time (6 months), a subacute tolerance study was assessed in vivo. Healthy mice were exposed to THL, VAN and their combination (i.e., three times a week during three consecutive weeks) following an endotracheal administration. The drug concentration administered in vivo was determined according to the in vitro results being 50 times the combination MIC, the maximum dose tolerated in vitro. As the drug concentration in delivered solutions should reach about 50 times the combination MIC, 50 µg/g$_{lung}$ of THL and/or 500 µg/g$_{lung}$ of VAN were administered in a 50-µl liquid volume. Indeed, this was done considering that a 20-g female mouse has a lung mass of about 200 mg and a 25-g male mouse has a lung mass of about 250 mg. Despite the fact that male mice are more susceptible to Mtb infection (higher bacilli burdens and higher mortality compared with female and castrated male mice) (Zhang et al., 2020), this tolerance study was carried out on both genders. No clinical sign as well as no significant body weight loss was noticed in the treated mice during or after the pulmonary administrations. A general good tolerance was thus observed in mice after 3-week administration of three doses (VAN, THL or VAN/THL) per week. The results obtained with VAN agree with previously published results. The good tolerance and safety of pulmonary delivered VAN was previously reported in rabbits treated with VAN administered using an endotracheal insufflator-catheter at 1 or 5 mg/kg (with a fine particle fraction of 32 ± 10%) (Sullivan et al., 2015). This was also observed in female piglets when treated with a single VAN nebulization dose at 37.5 mg/kg (62% of the dose reached the respiratory tract) (Morais et al., 2020). Several clinical trials have been conducted on VAN pulmonary delivered for the treatment of cystic fibrosis or bacterial pneumonia in the last past years (Boyle & Chmiel, 2019; Elliott, Dasenbrook, and MHS, 2020; Flume, 2021; Giulia and Paiola, 2020; Jouhikainen, 2014; Palmer, 2013; Yim, 2019). For example, AeroVanc™, a dry VAN powder suitable for pulmonary route, was developed by an Australian lab group in 2020 to treat cystic fibrosis for methicillin-resistant *Staphylococcus aureus* (MRSA) patients (Waterer et al., 2020). The dose-escalating phase I study has shown that an 80-mg dose of pulmonary delivered VAN was well tolerated and enabled VAN levels above MIC values for MRSA to be achieved for up to 24 h in sputum. However, the present work represents the first in vivo tolerance study on pulmonary delivered THL (with or without VAN), and the results obtained demonstrated also a good pulmonary tolerance at the THL tested concentration.

In the inflammatory response, leukocytes (such as neutrophils, macrophages and lymphocytes) are activated by the damaged tissue, releasing substances specialized in the mediation of inflammatory process (such as eicosanoids and proinflammatory cytokines) (Abdulkhaleq et al., 2018). In this study, we evaluated directly in BALF, whether THL, VAN or VAN/THL could induce inflammation. Sampling was taken 24 h after the last treatment for treated groups and the vehicle control and 18 h after the LPS administration for the LPS

group, based on the inflammatory biomarker's kinetics (Bondue et al., 2012). The total cell number was not significantly different in treated groups versus the vehicle control, demonstrating no particular inflammation. This result is in line with the fact that the cell recruitment was identical for all groups, except for the LPS group. Indeed, a higher proportion of neutrophils were found in the LPS group, demonstrating a more intense inflammation process for this LPS group. Indeed, among the leukocytes, neutrophils are the first inflammatory cells recruited at the site of inflammation (Curcic et al., 2015). Proinflammatory cytokines (IL-1β, IL-6 and TNF-α) concentrations were also quantified. According to the results previously obtained in vitro with THL, and its higher but not significant impact on monolayer epithelium, an increase in cytokines in THL- and VAN/THL-treated groups would be expected. However, in our study, none of the treated groups presented a higher lung inflammation in comparison with the vehicle control, except the LPS group.

Lung cytotoxicity was assessed by the measurement of protein content and released LDH activity in BALF. These analyses permit to quantify the release of cell contents in the extracellular compartment, which occurs during cytotoxicity and reflects cell lysis. During tissue damage, the plasma outflows from the capillaries to the tissue matrix. This is due to the increase of the vascular permeability. The tight junctions between endothelial cells are opened, and proteins are able to pass through membrane and enter in the airspaces (Matute-Bello et al., 2008). When IAH occurs, protein content in BALF increases. The quantification of protein content in BALF reflects vascular permeability in the lungs and is a good indicator of the integrity of the alveolar capillary barrier. The measurement of protein content demonstrated no significant increase in treated groups versus the vehicle control, reflecting no pulmonary cell lysis and no haemorrhage. The measurement of LDH activity in BALF reflects cytotoxicity of pulmonary cells (Drent et al., 1996). Although the VAN/THL combination presented no lung cytotoxicity, a significant increase of LDH in the THL-treated mice group was observed, as 2 out of 14 treated mice showed high released LDH activity. This result is difficult to interpret as no other symptoms showing cytotoxicity or abnormal assay results were found in these two mice. The intra-assay variability of this result is high. But this range of variability was already demonstrated on zebrafish liver cells with an LDH detection kit (Bopp & Lettieri, 2008). Moreover, no cytotoxic effect was detected in the VAN/THL-treated mice group, receiving the same doses of THL. The susceptibility to THL effect cannot be ruled out in this study, despite the number of animals. Therefore, the reason for an elevated LDH assay result in the THL-treated mice group may demonstrate an idiosyncratic reaction. As no other toxicities have been observed (in vitro or in vivo, in this group and other groups), this point was not further investigated. It was concluded that the VAN/THL combination, at the dose and the regimen tested, could be used for in vivo efficacy studies, keeping in mind this result. The LPS group did not show any significant difference of released LDH from the vehicle control. As LPS is a positive control model of inflammation (does not lyse the epithelium), and not a control model of cytotoxicity, the LDH result obtained for the LPS group is therefore expected.

Histological analysis reported IAH for all groups. As this phenomenon also occurred in the vehicle control, it could be traces of blood remaining in the lungs after washing, and not completely removed before paraffin-embedding (Chraibi et al., 2021). This hypothesis is also reinforced by the fact that there was no significant increase in protein content in BALF. Congestion was also observed for all groups. These marks of stagnant blood into the tissue could be due to the treatment (endotracheal administration) or to the sodium pentobarbital euthanasia (Laferriere & Pang, 2020). In the treated groups and the vehicle control, IAH and congestion were not associated with the presence of luminal alveolar macrophages, alveolar oedema, HYP or BEV. Assessment of the severity of each observation is limited because only one section per lung was analysed, not enabling the calculation of the severity score. Because no major or significant lung damage was observed by the histopathology analysis in the lung slides of the treated mice, it was concluded that the VAN, THL and the VAN/THL combination did not induce any major lung inflammation and were generally well tolerated in the mouse model.

The results getting from the vehicle control proved the absence of toxicity of the vehicle used in the tested solution. This liquid vehicle was formulated at a pH of 5.0 to find the best compromise between the VAN stability and lung tolerance but also to avoid possible bronchospasms that may occur at lower pH (Mathew & Das Gupta, 1995). Propylene glycol at 25% and phosphate buffer at the concentrations tested have been already used in human by nebulization (Bisgaard et al., 1987; Corcoran, 2006). The results obtained are in accordance with these previous studies, showing a good lung tolerance of these excipients. Therefore, although these in vitro studies allowed identifying a reversible cytostatic effect of THL at 30–50 times the combination MIC, this in vivo study demonstrated that those drugs, alone or in combination, are generally well tolerated in terms of inflammation, cytotoxicity and histopathology analysis. Comparison between in vitro and in vivo could be problematic as cells used in vitro are not from the same species than mice used in vivo. Also, cell culture model is not able to transcribe the complex regulations and interactions of the animal model. However, for evaluation of drugs' cytotoxicity, cell culture models are very useful. For example, Calu-3 cells have already demonstrated to be a very good model of in vivo lung epithelium (Mathias et al., 2002). In this work, in vitro and in vivo tests are two complementary methods that help us to better understand the local tolerance of VAN and THL in the lungs.

In conclusion, this study demonstrated for the first time that the combination VAN/THL administrated via pulmonary delivery had no cytotoxic effect on pulmonary cells in vitro, had no irreversible significant effect on a monolayer epithelium integrity in vitro and did not induce inflammation, cytotoxicity or other histological changes in vivo, in mouse lung, during subacute exposition. A potential cytostatic action of THL was only observed in vitro when concentration reached 50 μg/ml.

As this synergistic and innovative combination was well tolerated and should be considered in Mtb therapy development, further pharmaceutical investigation will be performed to achieve optimal pulmonary drug delivery. Future studies will assess the efficacy, the

mechanistic and the pharmacodynamic of the formulated VAN/THL combination on a murine TB model to support results obtained in this work.

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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