

## ***In vitro* and *in vivo* evaluations of the tolerance of a new and innovative anti-tuberculosis drug combination by inhalation**

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### **Summary**

A drug combination, vancomycin (VAN) plus tetrahydrolipstatin (THL), has demonstrated an effective synergistic action *in vitro* against *Mycobacterium tuberculosis* (Mtb), inhibiting the growth of the bacilli as well as its resistant forms. Given the poor oral bioavailability of VAN and THL, and the tropism of tuberculosis for lungs, this combination is more suitable for administration by inhalation. To evaluate the local tolerability of this combination, bronchial cells, alveolar cells, and monocytes were exposed to different concentrations around the combination's minimal inhibitory concentration (MIC) of the drugs (i.e., 10 µg/mL for VAN and 1 µg/mL for THL). The VAN-treated group never reached the half MIC (IC<sub>50</sub>), even at a concentration 80 times higher than the combination MIC. However, for THL-treated and VAN/THL-treated groups, the IC<sub>50</sub> measured 30 to 50 times the combination MIC, due to the action of THL. It was demonstrated, using a lactate dehydrogenase assay, that this effect was related to a cytostatic and not a cytotoxic action. Subsequently, an *in vivo* experiment was performed at a concentration of 50 times the combination MIC, administered 3 times a week for 3 weeks on different groups of healthy mice, using an endotracheal device. Pro-inflammatory biomarkers (i.e., IL-1β, IL-6, and TNF-α) in bronchoalveolar lavage fluid remained insignificant vs. the negative control group, and lung histopathology did not show significant tissue damage. The VAN/THL combination at doses up to 50 times the combination MIC seems very well tolerated *in vitro* and *in vivo*; a promising result which encourages us to continue the development of an inhalation form of this combination to fight Mtb.

### **Key Message**

The present work shows local tolerance of a potential anti-tuberculosis drug combination *in vitro* and *in vivo*. To date, this represents the first tetrahydrolipstatin study with administration by inhalation that provides the absence of cytotoxic activity and leads to multiple possibilities of use.

### **Introduction**

Tuberculosis (TB) is a mycobacterial infection caused by the pathogen *Mycobacterium tuberculosis* (Mtb), and represents one of the top 10 causes of death in the world [1]. Current treatments use at least four antimicrobials for a long-term treatment (1-6 months), leading to potential poor therapy adherence, also to antibiotic resistance and the emergence of multidrug-resistant TB and extensively drug-resistant TB [2]. The need to develop a new treatment strategy to eradicate this bacterial disease could be addressed by combining different drugs to enhance treatment efficacy.

The activity of vancomycin (VAN) and tetrahydrolipstatin (THL) alone and the VAN/THL combination have already been tested *in vitro* on *Mycobacterium tuberculosis* (Mtb) [3]. It was shown that the 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. Combined, the minimal inhibitory concentration (MIC) of the VAN was 10 µg/mL and 1 µg/mL for THL. This synergistic action of the combination was proved at a 10:1 ratio. This ratio was used in the following assays to test the lung tolerance of the drugs *in vitro* and *in vivo*. VAN, a large and rigid molecule, is a tricyclic glycopeptide antibiotic widely used to treat infection caused by gram-positive bacteria, inhibiting cell wall peptidoglycan synthesis. The absorption of this drug *per os* from the systemic compartment to the lungs is quite low [4]. THL is a derivative of lipstatin and acts as inhibitor of gastric and pancreatic lipases. It has been shown that its absorption from the intestine to the systemic compartment is extremely low [5]. This poor permeability of VAN and THL to the lungs or to the systemic compartment does not allow this combination to be considered for the oral route.

Inhalation of this combination could be a good alternative as Mtb is mainly located in the lungs. Local administration decreases systemic side effects and increases the drug concentration at the site of action. To date, inhaled VAN has proven its safety [6] but no safety data has been published on inhaled THL. The lungs tolerance evaluation must be processed for THL and for its combination with VAN.

The aim of this work was to demonstrate the good lung tolerance of the potential anti-tuberculosis combination VAN/THL at high concentrations under *in vitro* and *in vivo* conditions.

## **Experimental methods**

### *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay*

The cell proliferation of lung carcinoma (alveolar) A549 cells, adenocarcinoma (bronchial) Calu-3 cells and monocyte THP-1 cells, after exposure to THL, VAN, or VAN/THL, was determined using an MTT colorimetric assay. Cells were seeded in a 96-well plate and treated with drug(s) at various concentrations (i.e., THL alone 0.125-78 µg/mL, VAN alone 0.4-625 µg/mL, or VAN/THL 2-1250 µg/mL for VAN and 0.2-125 µg/mL for THL) in 200 µL per well. The plate was then incubated at 37 °C for 72 h. After the incubation time, 20 µL of MTT 0.5 mg/mL were added to each well. Plates were incubated for 4 h at 37 °C. Formazan crystals were washed and solubilized with dimethyl sulfoxide prior to analysis on a spectrophotometer (Synergy HT, Bio-Tek and KC4 software version 3.4, Bio-Tek) at 570 nm (reference wavelength 610 nm). The inhibitory concentration (IC<sub>50</sub>) was then calculated, representing the concentration which inhibited 50% of the cell proliferation vs. the negative control group.

### *Lactate dehydrogenase (LDH) assay*

Calu-3 cells were seeded on a 96-well plate and incubated for 24 h. After incubation, the medium was replaced by 200 µL of Hank's balanced salt solution. Following this, 20 µL of Triton X-100 were added to maximum release wells, and 20 µL of assay buffer were added to spontaneous release wells. The background was determined using wells containing only the 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. The LDH cytotoxicity assay was then used following the kit standard procedure (LDH Cytotoxicity Assay kit, Cayman Chemicals, USA). The plate was centrifuged and 100 µL of cell supernatant were transferred to a new 96-well plate. Each well received 100 µL of reaction solution and the plate was then incubated at 37 °C, with gentle shaking on an orbital shaker for 30 min in the dark (KS 4000 I control, Ika). The absorbance was measured at 490 nm (A490) and the percentage of cytotoxicity was then calculated using the formula:

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

### *In vivo lung tolerance evaluation*

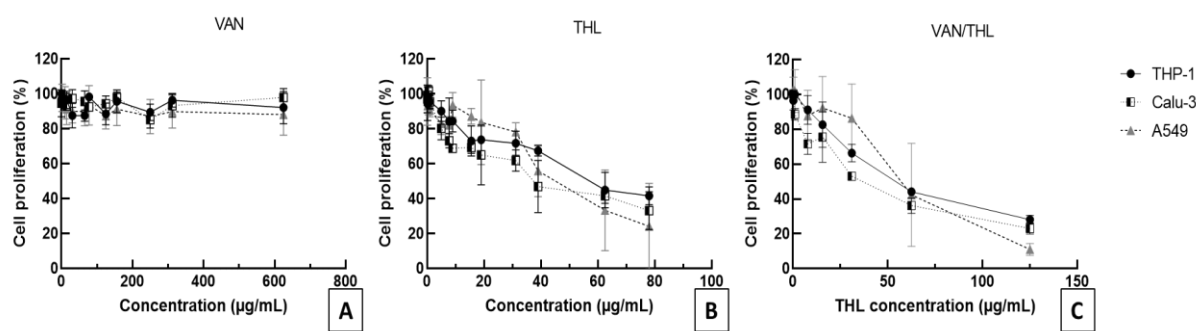
Eight to 10-week-old BALB/cAnNRj mice (approximately 19.5-24.5 g) purchased from Janvier Labs (Le Genest-Saint-Isle, France) were used in this study in accordance with EU Directive 2010/63/EU for animal experiments, under ethical protocol 727N. Three groups of 16 mice (8 males and 8 females) were administered either THL alone, VAN alone or VAN/THL 3 times a week for 3 consecutive weeks in the following quantities: 1 mg/kg for the THL-treated group; 10 mg/kg for the VAN-treated group; 1 mg/kg for THL and 10 mg/kg for VAN for the VAN/THL-treated group. These groups were compared to the negative control group (n = 16, 8 males and 8 females), which received only the liquid vehicle used to solubilize drugs (25% propylene glycol + 75% phosphate buffer 0.02 M, pH 5.0). The positive control group (n = 8, 4 males and 4 females) was exposed to a solution of 1 µg lipopolysaccharide (LPS) from *E. coli*. Isoflurane anaesthesia (2-4%) in oxygen (2%) was administered to allow a direct endotracheal administration of 50 µL of the solution using a model IA-1C® Microsprayer™ connected to an FMJ-250-high-pressure syringe (Formerly: Penn-Century, Wyndmoor, PA, USA). Mice from all groups were euthanized using pentobarbital (10 mg/kg in intra-peritoneal) 24 h, or 18 h for the LPS positive control group, after the last administration. The trachea was cannulated, and the lungs were flushed with phosphate buffered saline solution to collect the bronchial-alveolar liquid fluid (BALF). Investigations of inflammation in BALF were conducted to evaluate the local tolerance. Quantification of selected pro-inflammatory biomarkers (IL-1β, IL-6, and TNF-α) was done by the ELISA method as described by the manufacturer (DuoSet, RnD Systems, Abingon, UK). Lungs were also collected, embedded in paraffin wax, and stained with haematoxylin and eosinophil. The investigation of lung damage was conducted

by an independent pathologist analysing the frequency of each observation (intra-alveolar haemorrhage, pneumocyte hyperplasia, tissue inflammation, and bronchiolar epithelial vacuolation) and was calculated as the number of animals for whom the observation occurred.

## Results and discussion

The aim of the *in vitro* part was to assess the impact of THL, VAN and the VAN/THL combination on lung cell viability and cytotoxicity for the range of drug concentrations at which the molecules inhibited the proliferation of Mtb to 99%.

Cells were exposed to different concentrations of drugs (i.e., VAN from -25 to 62.5 times the combination MIC, THL from -8 to 78 times the MIC, or VAN/THL from -5 to 125 times the combination MIC). Cell proliferation was not affected by the presence of VAN, even at the highest dose tested (see Figure 1.A). VAN never reached the IC<sub>50</sub> at the tested concentrations, showing a good tolerance, with low toxicity for alveolar and bronchial cells and monocytes. However, THL (Figure 1.B) and VAN/THL (Figure 1.C) presented a dose-dependent effect. For THL-treated cells, the IC<sub>50</sub> mean values  $\pm$  SD for each strain was calculated: A549 cells: IC<sub>50</sub> = 48  $\pm$  16  $\mu$ g/mL; Calu-3 cells: IC<sub>50</sub> = 30  $\pm$  13  $\mu$ g/mL; THP-1 cells: IC<sub>50</sub> = 54  $\pm$  8  $\mu$ g/mL. For VAN/THL-treated cells, the values for each strain were: A549 cells: IC<sub>50</sub> = 45  $\pm$  8  $\mu$ g/mL; Calu-3 cells: IC<sub>50</sub> = 34  $\pm$  2  $\mu$ g/mL; THP-1 cells: IC<sub>50</sub> = 48  $\pm$  6  $\mu$ g/mL. The IC<sub>50</sub> was therefore reached for THL and VAN/THL at concentrations 30-55 times the combination THL MIC.

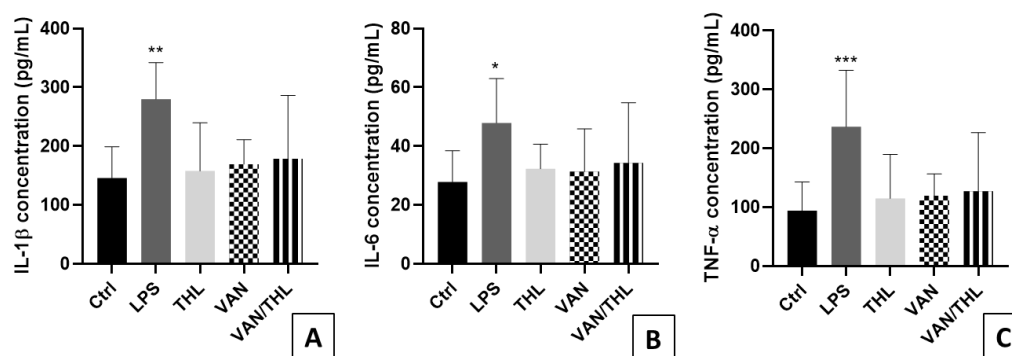


**Figure 1 - Dose-response curve of cell proliferation (in % in comparison to the negative control group) depending on the concentration of VAN (A), THL (B), or the combination of VAN and THL (C) in  $\mu$ g/mL. All results are expressed as means of three wells per condition  $\pm$  standard deviation ( $n = 3$ ).**

After 24 h of Calu-3 cell exposure to different treatments (i.e., THL, VAN or VAN/THL) at different 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 cytotoxicity rate of the treated cells was around the baseline of the negative control (spontaneous LDH release) after 24 hours. No cytotoxicity was found in the VAN-treated cells (from  $-5 \pm 6\%$  at 10 times the combination MIC to  $-5 \pm 2\%$  at 200 times the combination MIC). As THL did not present any cytotoxicity in the LDH assay (from  $-9 \pm 0\%$  at 10 times the combination MIC to  $-1 \pm 6\%$  at 200 times the combination MIC), it was concluded that the effect observed in the MTT assay was caused by a cytostatic effect rather than a cytotoxic effect. The combination also did not cause a cytotoxic effect at the tested concentrations (from  $-8 \pm 1\%$  at 10 times the combination MIC to  $-7 \pm 2\%$  at 100 times the combination MIC).

An *in vivo* experiment was performed to evaluate the local tolerance of the maximum dose tolerated *in vitro* (i.e., 50 times the combination MIC) in subacute exposure to each drug and their combination.

During the study, no significant weight loss was observed in the treated groups vs. the negative control group and no mouse reached the limit points defined by the scoring (i.e., normal respiratory function and behaviour). Quantification of the pro-inflammatory biomarkers (i.e., IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) was evaluated in BALF (see Figure 2). As expected, the LPS positive control group showed a significant increase in IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . Within the treated groups (i.e., THL, VAN and VAN/THL), no significant increase was observed vs. the negative control group for all biomarkers IL-1 $\beta$ , IL-6 and TNF- $\alpha$ .



**Figure 2 - Mouse pro-inflammatory biomarkers IL-1 $\beta$  (A), IL-6 (B), and TNF- $\alpha$  (C) concentrations (pg/mL) in the BALF 24 h post-administration for the negative control, THL-treated, VAN-treated, or VAN/THL-treated groups and 18 h post-administration for the LPS positive control group. All results are expressed as means  $\pm$  SD (n = 8-14). A statistical analysis was performed between the treated groups and the negative control group using one-way ANOVA and Bonferroni's post-hoc test for both sexes (\*\* for p < 0.01, and \* for p < 0.05).**

Moreover, no major lung damage was observed by pathologic examination for the treated groups (i.e., THL, VAN, and VAN/THL) vs. the negative control group. However, intra-alveolar haemorrhage was reported for all groups. As it also occurred in the negative control group, this damage could have been caused by the blood collection technique, and therefore not related to real tissue damage, as already reported [7]. Other phenomena were observed only in the LPS positive control group, such as pneumocyte hyperplasia displayed for 50% of mice, tissue inflammation for 12.5% of mice, and bronchiolar epithelial vacuolation for 37.5% of mice. VAN, THL and the VAN/THL combination did not induce any inflammation and were well-tolerated in the mouse model.

## Conclusion

The potential cytostatic action of THL on cells at high doses *in vitro* was established. This study demonstrated for the first time that THL, associated with VAN at a 10:1 ratio, was not toxic in pulmonary cells *in vitro* or *in vivo* in mice during a subacute exposition. As this combination was well-tolerated, further works will be considered for inhalation drug delivery. Future studies will assess the efficacy of formulated combinations on a murine TB model.

## References

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