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Short Communication

Tetrahydropyridine derivative as efflux inhibitor in *Mycobacterium abscessus*

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ABSTRACT

Objectives: This study aimed to evaluate a tetrahydropyridine derivative (THP) as a potential inhibitor of the efflux mechanism and modulator of the high level of antimicrobial resistance usually observed in members of the *Mycobacterium abscessus* (*M. abscessus*) group.
Methods: The strain *M. abscessus* subsp. *abscessus* (ATCC 19997) was used as reference, in addition to three clinical isolates: *M. abscessus* subsp. *abscessus* (AT 07), and two *M. abscessus* subsp. *bolletii* (AT 46 and AT 52). The minimum inhibitory concentration (MIC) of amikacin (AMI), ciprofloxacin (CIP), clarithromycin (CLA), verapamil (VP), and THP derivative (NUNL02) was determined.
Results: The NUNL02 showed activity against *M. abscessus*; the MIC of AMI against ATCC 19997 was reduced more than 16-fold, and the MIC of CIP against AT 52 was reduced four-fold. When combined with CLA, the MIC was reduced against all tested strains. In addition, to detect and quantify the activity of the efflux mechanism, the intracellular accumulation kinetics of the fluorometric substrate ethidium bromide in the presence and absence of VP and NUNL02 were evaluated. The NUNL02 was found to be a more effective efflux inhibitor than VP, which is the classical inhibitor.
Conclusions: The tetrahydropyridine derivative, NUNL02, is a promising adjuvant in the treatment of infections caused by *M. abscessus*.

1. Introduction

Efflux is a common mechanism observed in both eukaryotic and prokaryotic cells, extruding several compounds by transporter proteins called efflux pumps. In bacteria, these efflux pumps can be involved in the extrusion of all classes of antimicrobials [1]. Members of the *Mycobacterium abscessus* (*M. abscessus*) group (MAG) are environmental microorganisms that are, occasionally, etiologic agents of severe infections. These infections are a substantial challenge to clinical practice because MAG members are highly resistant to antimicrobial therapy [2]. Many studies have described the role of efflux mechanisms in subclinical resistance of *Mycobacterium* spp. and highlighted the potential use of adjuvants – such as verapamil (VP), reserpine (RSP), carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and chlorpromazine (CPZ) – combined with antimicrobial therapy as a promising alternative to increase the activity of antimicrobials. Efflux inhibitors (EI) are compounds that reduce the intrinsic resistance of the bacteria by increasing the intracellular concentration of drugs [3,4,5].

Molecules such as tetrahydropyridines (THP) can act as proton transfer agents and be candidates for EI in bacteria [6]. Recently, the potential of a THP derivative as EI in vitro and in silico in *Escherichia coli* was shown. This study evaluated this THP derivative – NUNL02 – as a potential EI in *M. abscessus* [7].

2. Materials and methods

2.1. Strain and bacterial suspension

This study used one reference strain *M. abscessus* subsp. *abscessus* (ATCC 19977) and three clinical isolates (provided by the

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Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil): *M. abscessus* subsp. *abscessus* (AT 07), *M. abscessus* subsp. *bolletii* (AT 46) and *M. abscessus* subsp. *bolletii* (AT 52). The strains were previously identified by *rpoB* and *hsp65* gene sequencing. The strains were cultured on Ogawa-Kudoh medium and incubated at 37 °C for 2–15 days. The inoculum was prepared according to Clinical and Laboratory Standards Institute (CLSI) guidelines, in distilled water, and adjusted to McFarland tube no 0.5 [8].

2.2. Antimicrobials and efflux inhibitor

Amikacin (AMI), ciprofloxacin (CIP), clarithromycin (CLA), and verapamil (VP) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and solubilised according to the manufacturer's recommendations. Stock solutions were stored at −20 °C. The work solutions were prepared in Mueller-Hinton (MH) broth at the day of the experiment.

2.3. Derivative of tetrahydropyridines

The NUNL02 (molecular formula $C_{23}H_{24}F_3N_2O$) was obtained according to the method described by Zanatta et al. [9]. Fig. 1 shows NUNL02 core and radicals. The NUNL02 was solubilised in 99.5% dimethyl sulfoxide (DMSO) at a concentration of 10 mg/mL and stored at 4 °C.

2.4. Determination of minimum inhibitory concentration

Susceptibility testing was performed using a 96-well microtiter plate. The minimum inhibitory concentration (MIC) of the antimicrobials, EI and THP were determined using the broth microdilution method according to CLSI, with some modifications [8]. Serial two-fold dilutions of each antimicrobial were prepared directly in the plate containing 100 μL MH broth to which 100 μL of the inoculum, diluted 1:10, was added. The inoculated plates were incubated at 37 °C in a normal atmosphere for 48 h. Then, 30 μL of 0.02% resazurin were added to each well, and the plate was incubated overnight at 37 °C for colour modification. The resazurin was used as a cell growth and viability indicator, and bacterial growth was evidenced by a change in colour from blue (oxidized state) to pink (reduced state) [10]. The MIC was defined as the lowest concentration of the drug that inhibited bacterial growth [11]. A growth control (inoculum only, without antimicrobial) and a sterile control (only medium) were included in each tested plate. In addition, to detect CLA-induced resistance, the plates were incubated for 14 additional days. Strains were considered susceptible when the MIC was ≤2 μg/mL and resistant when MIC had higher values. Susceptible isolates at day 3 and resistant after day 5 were considered to show induced resistance [12]. All assays were carried out in triplicate.

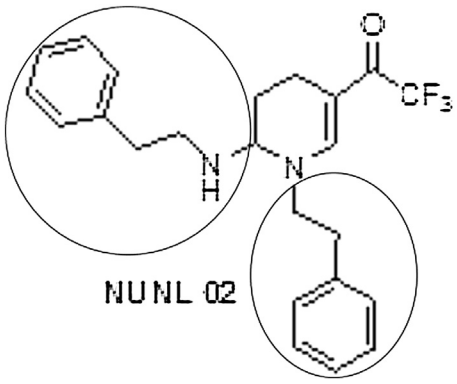

Fig. 1. Chemical struture of the Tetrahydropyridine derivate, NUNL2.

2.5. Cytotoxicity assay

The toxicity of NUNL02 was assessed using J774A.1 and MCF-7 cells. Cells were exposed to NUNL02 at concentrations ranging between 200–0.8 μg/mL for 24 h. Cell viability was evaluated at 24-h exposure by adding resazurin (0.01%) to cell cultures, which revealed after 6 h of incubation. The IC_{50} value was defined as the NUNL02 concentration at which 50% of the cells were viable relative to the control [13] The inhibition (%) of cell proliferation in each concentration was determined as follows:

inhibitory growth = (1-Abs600 treated cells/Abs600 control cells) × 100).

2.6. Modulatory effect of efflux inhibitors on antimicrobial activity

The MICs of AMI, CIP and CLA were determined in the absence and presence of a subinhibitory concentration ($^1/_2$ of MIC) of a known EI (VP) and NUNL02. The inhibitory effect was observed by a reduction in the MIC in the presence of EI relative to the control (absence of EI). The modulation factor (MF) was used to quantify the effect of EI on the MIC of AMI, CIP and CLA through the following formula:

MF = MIC antimicrobial/MIC antimicrobial + EI [14].

The MF reflects a reduction of MIC values of a given antimicrobial in the presence of the EI, being considered significant when MF ≥ 4 (four-fold reduction) [5]. These assays were carried out in triplicate.

2.7. Ethidium bromide accumulation by semi-automated fluorometric method

The method was carried out using an Infinite F200 fluorometer (Tecan), to monitor the accumulation of ethidium bromide (EtBr) at concentration of 1.0 μg/mL. The fluorescence was acquired in 60 cycles of 90 s at 37 °C. The excitation and emission wavelengths were 530 nm and 580 nm, respectively. Accumulation assays were conducted in triplicate, as previously described, with some modifications [15]. Briefly, the strains were grown in 10 mL of MH with 5% OADC supplement (oleic acid/albumin/dextrose/catalase) (Becton and Dickinson, Diagnostic Systems, Sparks, MD, USA) and with 0.05% Tween 80 at 37 °C until they reached an optical density at 600 nm ($O.D._{600}$) of 0.8. The cell washes were performed by centrifugation at 3500 rpm for 3 min; the supernatant was discarded, and the pellet washed in phosphate buffered solution (PBS) 1x. The VP and NUNL02 were tested at subinhibitory concentrations ($^1/_2$ of MIC) to assess their inhibitory ability against efflux systems present in *M. abscessus*. Control wells containing only EtBr and bacterial suspension were included [15,16]. The inhibitory activity of VP and NUNL02 was determined by calculating the relative final fluorescence (RFF) value according to the formula:

$$REF = \frac{RF_{treated} - RF_{untreated}}{RF_{untreated}}$$

The $RF_{treated}$ corresponds to the relative fluorescence at the last time point of the retention curve of EtBr in the presence of EI; $RF_{untreated}$ corresponds to the relative fluorescence at the last time point of the EtBr accumulation curve of the untreated control well. The difference between $RF_{treated}$ and $RF_{untreated}$ is dependent on the degree of accumulation of EtBr [15,16]. An index of activity above zero indicates that the cells accumulate more EtBr under the

condition used than the control (untreated cells). Negative RFF values indicate that the treated cells accumulated less EtBr than the control [17].

3. Results and discussion

The results show that the antimicrobial susceptibility of the four strains included in the study has a diverse profile (Table 1). All four were susceptible to AMI. The clinical strains were CIP-resistant, while the reference strain ATCC 19997 was CIP-susceptible. Regarding CLA, AT 52 was resistant starting at the third day; the other strains showed induced resistance, which was observed after the fifth day of incubation in AT 46, and after the seventh day in ATCC 19997 and AT 07. Induced resistance can only be detected after an extended incubation period in the presence of the drug [12]. The MIC of VP was 312.5 μg/mL to the four strains, while NUNL02 showed MIC of 200 μg/mL to ATCC 19997, 100 μg/mL to AT07 and AT46, and 50 μg/mL to AT52. Furthermore, no cytotoxicity was observed for NUNL02 ($IC_{50} \geq 200$ μg/mL).

The NUNL02 has been shown to be an inhibitor of the *Escherichia coli* (*E. coli*) AcrB efflux-pump [7]; therefore, the potential of this compound as an EI in *M. abscessus* compared with the classical inhibitor VP was also evaluated. The MIC for each tested antimicrobial was determined in the absence and presence of $^1/_2$ MIC of VP and NUNL02. It is important to note that $^1/_2$ MIC has no effect on the viability of the microorganisms, indicating that the effect of the EI on the MIC of an antimicrobial is related to efflux system interference [18]. While in the presence of VP, the MIC of the antimicrobials was reduced up to eight-fold; in the presence of NUNL02, the MIC was reduced by more than 16-fold (Table 1). The variation on the degree of MIC reduction between the different strains may be related to the variability between MAG species or to the background of the strains of the same species.

The NUNL02 proved to be a good adjuvant candidate for antimicrobials against the *M. abscessus* isolates tested. Therefore, whether the inhibition of efflux is a possible mechanism of action was investigated using a fluorometric technique in the presence of the efflux substrate EtBr. Fig. 2 shows that in the evaluated

Table 1
Interaction between the efflux inhibitors in combination with antimycobacterial drugs against *Mycobacterium abscessus* strains.

Drug	EI	Strain							
		ATCC 19977		AT07		AT 46		AT 52	
		MIC (μg/mL)	MF	MIC (μg/mL)	MF	MIC (μg/mL)	MF	MIC (μg/mL)	MF
AMI	**No EI**	**1.0**	–	**2.0**	–	**1.0**	–	**4.0**	–
	+VP	0.25	**4**	1.0	2	0.25	**4**	2.0	2
	+NUNL02	≤0.06	>**16**	1.0	2	1.0	1	2.0	2
CIP	**No EI**	**1.0**	–	**2.0**	–	**4.0**	–	**8.0**	–
	+VP	0.5	2	2.0	1	4.0	1	4.0	2
	+NUNL02	0.5	2	2.0	1	4.0	1	2.0	**4**
CLA	**No EI**	**4.0**[c]	–	**8.0**[c]	–	**32.0**[b]	–	**128.0**[a]	–
	+VP	4.0	1	2.0	**4**	4.0	**8**	64.0	2
	+NUNL02	0.25	**16**	1.0	**8**	4.0	**8**	32.0	**4**

Abbreviations: MIC, minimum inhibitory concentration; MF, modulation factor; AMI, amikacin; CIP, ciprofloxacin; CLA, clarithromycin; VP, verapamil; EI, efflux inhibitor. Bold values are modulatory factor.
[a] Value by visual reading in day 3.
[b] Value by visual reading in day 5.
[c] Value by visual reading in day 7.

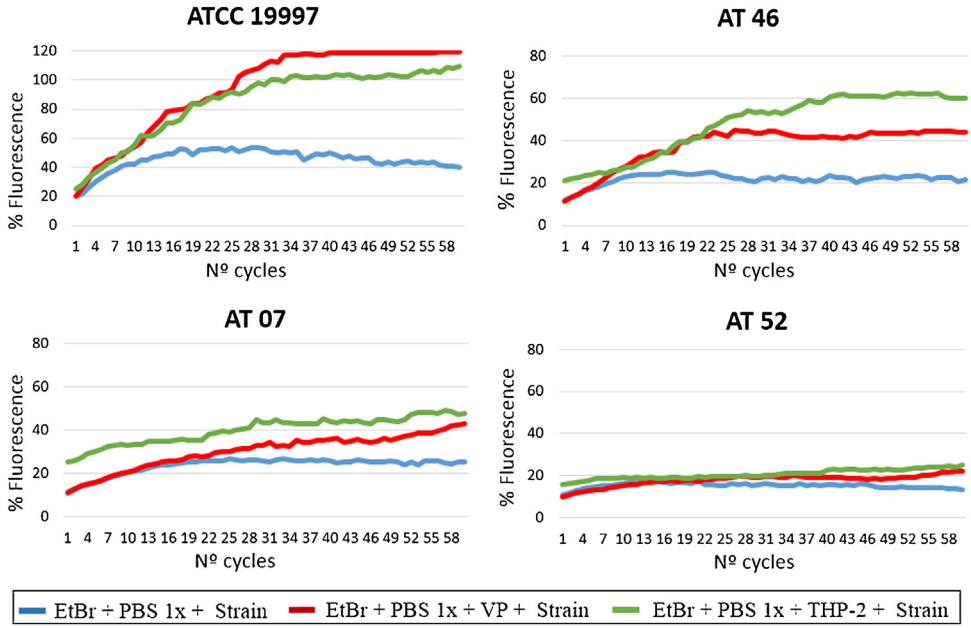

Fig. 2. Effect of the efflux inhibitors Verapamil- VP and NUNL02 on the accumulation of ethidium bromide (EtBr) in M. abscessus subsp abscessus (ATTC 19977), M. abscessus subsp abscessus (AT 07), M. abscessus subsp bolletii (AT 46) and M. abscessus subsp bolletii (AT 52).

conditions, all strains accumulated EtBr; however, the accumulation was higher in the presence of NUNL02. The RFF values of the EI tested against the *M. abscessus* clinical isolates demonstrated the efficacy of NUNL02 on the inhibition of EtBr efflux, being more efficient than VP. The RFF values were 2.1, 0.7, 1.0, and 0.7 to VP, and 1.7, 0.9, 1.8, and 0.9 to NUNL02, against ATCC 19977, AT 07, AT 46 and AT 52, respectively. In addition to these findings, Silva Jr et al. also showed that NUNL02 is effective as an EI in *E. coli* [19]. Based on docking studies, they suggest that the mechanism of action of this derivative relies on competition. Then, when the inhibitor is also a substrate, competition for the same binding sites would underlie the mechanism for efflux inhibition [19].

This study highlighted the THP derivative, NUNL02, as a potential adjuvant in the treatment of infections caused by *M. abscessus* strains and suggested efflux inhibition as a possible mechanism of action.

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Competing interests

None.

Ethical approval

Not required.

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