Original Article

Targeting Bedaquiline Mycobacterial Efflux Pump to Potentially Enhance Therapy in *Mycobacterium abscessus*

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Abstract

Background: *Mycobacterium abscessus* is notorious for being intrinsically resistant to most antibiotics. Antibiotic efflux is one of the mechanisms used by *M. abscessus* to pump out antibiotics from their cells. Inhibiting efflux pumps (EPs) can be an attractive strategy to enhance the activity of drugs. The objective of this study is to determine the activity of EP inhibitors (EPIs) to enhance the efficacy of the new drug bedaquiline against *M. abscessus* clinical isolates. **Methods:** A total of 31 phenotypically and genotypically identified *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* clinical isolates were studied. The contribution of EPs was determined by investigating the minimum inhibitory concentration (MIC) levels of bedaquiline reduction in the absence and presence of EPIs verapamil and reserpine using the resazurin microtiter assay. **Results:** The observed bedaquiline MIC reduction by verapamil was observed in 100% isolates and by reserpine in 54.8% isolates. Bedaquiline MIC was 4–32-fold using verapamil with *M. abscessus* subsp. *bolletii* showing the highest fold change and between 2- and 4-fold using reserpine. **Conclusions:** The results obtained in this study confirm that bedaquiline MIC decreased in the presence of EPIs verapamil and reserpine in clinical isolates of *M. abscessus*. Verapamil was the most effective EPI. As shown in previous studies, verapamil may have clinical potential as adjunctive therapy to enhance the effect of bedaquiline.

Keywords: Bedaquiline, efflux pump, *Mycobacterium abscessus*, mycobacteria

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Introduction

Mycobacterium abscessus is a rapid-growing mycobacterium that causes severe pulmonary and skin infections and is considered as an emerging human pathogen in cystic fibrosis (CF) patients. *M. abscessus* remain very difficult to treat because of its innate resistance to many different classes of antimicrobial drugs, thus leading to unsatisfactory treatment outcome.[1,2] Therefore, there is a significant need for search of new effective antimicrobial treatment. The introduction of new drugs could potentially improve *M. abscessus* treatment outcomes. Recently, two new antituberculosis (TB) drugs, bedaquiline and delamanid, have reached the market. Our group has shown that bedaquiline has *in vitro* activity against nontuberculous mycobacteria (NTM).[3,4] However, and worryingly, efflux-mediated bedaquiline resistance has been identified.[5] This mechanism of resistance decreases the intracellular drug concentration of bedaquiline, rendering the antibiotic treatment ineffective. Recent studies have explored strategies to reverse the resistance phenotype conferred by efflux pump (EP) activity by the addition of EP inhibitors (EPIs). *In vitro* studies shown that the EPI such as verapamil decreased the minimum inhibitory concentrations (MICs) of bedaquiline (and clofazimine) against *Mycobacterium tuberculosis* H37Rv by 4–16-fold.[6] The same studies focusing on *M. abscessus* are, however, extremely limited. *M. abscessus* forms a complex of three closely related "species." Significant differences exist between

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M. abscessus ssp. *abscessus* which is the most virulent species compared to *M. abscessus* ssp. *bolletii* and *M. abscessus* ssp. *massiliense*. Macrolides (e.g., clarithromycin) are frequently the only drug that is active *in vitro* against *M. abscessus*. However, in *M. abscessus* ssp. *abscessus* and *M. abscessus* ssp. *bolletii*, the induction of erythromycin ribosome methyltransferase gene (erm41) can lead to macrolide resistance. This inducible macrolide resistance is not found in *M. abscessus* ssp. *massiliense* due to deletions present in the *erm41* gene. EPs are, thus, now largely recognized as playing an important role in induced drug resistance in mycobacteria and emerged as a major challenge in this field of bacterial resistance.[7] However, to date, the knowledge of bedaquiline resistance mechanisms in *M. abscessus* is limited. EPI may have clinical potential as adjunctive treatment. The objective of this study is to determine the activity of EPI (verapamil and reserpine) for enhancing the efficacy of bedaquiline activity against *M. abscessus* strains isolated in our hospital, including the three subspecies.

Methods

Clinical isolates

A total of 31 clinical isolates of *M. abscessus* obtained from sputum samples from CF and non-CF patients from the University Hospital Saint-Luc, Brussels, Belgium, were included in the present study.

Efflux pump inhibitors and drug

Verapamil and reserpine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solution of verapamil was dissolved in distilled water, whereas reserpine was prepared in dimethyl sulfoxide (DMSO). Bedaquiline was kindly provided by Janssen Pharmaceutica (Beerse, Belgium) and was dissolved in DMSO.

Identification and resistance profile of isolates

Isolates were identified as *M. abscessus* by MALDI-TOF and then subtyped by the GenoType NTM-DR line probe assay version 1.0 (Hain Lifescience, Nehren, Germany) for the identification and resistance profile determination of the three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* according to the manufacturer's recommendations and as previously described.[8]

Effect of efflux pump inhibitors on the minimum inhibitory concentration levels of bedaquiline

MIC levels for bedaquiline were determined using the resazurin microtiter assay (REMA) as previously described[9] in the presence or absence of EPI (verapamil and reserpine). Final concentrations used in REMA for verapamil were 40 mg/l and for reserpine 12 mg/l. A total of 100 µl volume of Middlebrook 7H9 broth supplemented with 10% oleic acid, albumin, dextrose, and catalase and 0.5% glycerol was dispensed in the wells of a 96-well cell culture plate. Bedaquiline concentrations used ranged from 2, 1, 0.5, 0.25, 0.125, and 0.062 mg/l. *M. abscessus* freshly grown on 7H10 agar plate was taken to prepare a bacterial suspension of 0.5 McFarland standard and diluted to 1:10 in 7H9 broth. This diluted suspension (100 µl) was used to inoculate each well of the plate. Plates were sealed and incubated at 37°C for 2–3 days. After that, the resazurin dye (Sigma, USA) (0.01%, 30 µl) was added to each well and the plates were re-incubated for two more days. A change in color from blue to pink indicated the growth of bacteria, and the MIC was read as the lowest bedaquiline concentration that prevented the color change in resazurin dye.

Results

Identification of isolates

GenoType NTM-DR V.1.0 line probe assay enables *M. abscessus* subspecies identification and the simultaneous determination of antibiotic resistance to macrolides and aminoglycosides of mutations at position 28 in erm (41), position 2058/2059 in rrl, and position 1408 in rrs. Of 31 *M. abscessus* tested, the GenoType NTM-DR identified 8 *M. abscessus* subsp. *abscessus* erm (41) genotype t28, 3 *M. abscessus* subsp. abscessus erm (41) genotype c28, 10 *M. abscessus* subsp. *Bolletii,* and 10 *M. abscessus* subsp. *massiliense* [Table 1]. Table 2 shows the genotype resistance patterns to macrolides and aminoglycosides in detail according to each *M. abscessus* subsp. tested.

Minimum inhibitory concentrations of bedaquiline with and without efflux pump inhibitors verapamil and reserpine

MICs of bedaquiline determined in the absence of efflux inhibitors (verapamil or reserpine) were compared with those determined in the presence of verapamil or reserpine. Two-fold or more reduction in MIC levels was considered as an indication of the presence of EP activity in bedaquiline *M. abscessus* isolates. It was observed a presence of EP activity in all clinical isolates (100%) tested in the presence of verapamil and in 17 of 31 (54.8%) isolates in the presence of reserpine. MICs of bedaquiline and fold change in bedaquiline MIC in the presence of verapamil and reserpine are shown in Table 3. For the control, MICs of bedaquiline and efflux inhibitors alone have been tested. For verapamil, for the majority of *M. abscessus* subsp. *abscessus* isolates, 9 of 11 (81%) show 4-fold change reduction in bedaquiline MIC and 2 isolates show 8-fold change reduction. For *M. abscessus* subsp. *bolletii*, 4 of 10 isolates (40%) showed a decrease in bedaquiline MIC with 16–32-fold, 4 isolates (40%) 8-fold change reduction, and 2 isolates (20%) a 4-fold change. For *M. abscessus* subsp. *massiliense* half of the isolates, 5 of 10 (50%) show an 8-fold change and 50% a 4-fold change. For reserpine, the 17 of 31 (54.8%) isolates that show EP activity had a 4-fold change reduction in bedaquiline MIC.

Discussion

In this study, we explored the impact of EP activity on bedaquiline in *M. abscessus*. We have demonstrated verapamil and reserpine

Table 1: GenoType NTM-DR identification results

Mycobacterial isolates ($n=31$)	GenoType NTM-DR results							
	Number of isolates	erm (41)	rrl		rrs		MA resistant	AG resistant
			WT	MUT	WT	MUT		
M. abscessus subsp. *abscessus erm (41)* t28	8	t28	8	0	8	0	8	0
M. abscessus subsp. *abscessus erm (41)* c28	3	c28	3	0	3	0	0	0
M. abscessus subsp. *bolletii*	10	t28	9	1	10	0	10	0
M. abscessus subsp. *massiliense*	10	t28	10	0	10	0	0	0

MA: Macrolides, AG: Aminoglycosides, *M. abscessus*: *Mycobacterium abscessus*

Table 2: Resistance to macrolides and aminoglycosides according to *Mycobacterium abscessus* subspecies

Mycobacterial isolates	*n*	erm (41) t28, *n* (%)	erm (41) c28, *n* (%)	rrl mutation			rrs mutation			MA resistance, n (%)	AG resistance, n (%)
				MUT	WT	Percentage	MUT	WT	Percentage		
M. abscessus	11	8 (73)	3 (27)	0	0	0	0	0	0	8 (73)	0 (0)
M. bolletii	10	10 (100)	0 (0)	1	9	100	0	0	0	10 (100)	0 (0)
M. massiliense	10	10 (100)	0 (0)	0	0	0	0	0	0	0 (0)	0 (0)
Total	31	28 (90)	3 (10)	1	9	32	0	0	0	18 (58)	0 (0)

MA: Macrolides, AG: Aminoglycosides, *M. abscessus: Mycobacterium abscessus, M. bolletii: Mycobacterium bolletii, M. massiliense: Mycobacterium massiliense*

activities through a simple *in vitro* phenotypic screening test that measures the changes in the MICs of the bedaquiline in the absence and presence of EPIs. This study adds more data and confirms the previous finding on existing EPIs effective against *M. abscessus*. Moreover, this is the first observation regarding the effect of EPIs on the activity of bedaquiline against clinical isolates of *M. abscessus*, including the three subspecies isolated in our hospital, showing its potential clinical significance in the treatment. Our results show that the MICs of bedaquiline in *M. abscessus* clinical isolates were affected in the presence of verapamil and reserpine suggesting a role of EP activity in bedaquiline efficacy. Results of this study are concordant with the recently published study[10] in which verapamil improved the activity of bedaquiline against *M. abscessus* with a 4- and 8-fold reduction of the bedaquiline MIC. That study, however, did not investigate the effect of reserpine. Furthermore, in *M. tuberculosis,* previous *in vitro* studies have shown that verapamil decreases the MIC of bedaquiline by 8–16-fold.[5-11] In 2015, Srikrishna *et al*.[12] showed in a preclinical study that verapamil potentiated the activity of bedaquiline against *M. tuberculosis* reducing the dose required for cure. The study of Philley *et al*.[13] demonstrates the potential clinical and microbiologic activity of bedaquiline in patients with *Mycobacterium avium* and *M. abscessus* lung disease. Furthermore, it has been shown that bedaquiline could be an alternative in multidrug treatment regimens for severe or relapsing disease, potentially including patients with underlying CF.[14] Other studies have also shown a decrease in the MIC of isoniazid, rifampicin, streptomycin, ciprofloxacin, ofloxacin, and linezolid against *M. tuberculosis* and NTM in the presence of EPIs.[15,16] In other mycobacteria, Rodrigues *et al*. found a significant reduction of resistance to clarithromycin and erythromycin in *M. avium* ATCC 25291 in the presence of verapamil.[17]

It is unknown if the concentration of verapamil used in *in vitro* studies is attainable in patients.[18] Verapamil is a small molecule that acts as an ion channel blocker and is used in the treatment of hypertension. Verapamil is also known to have negative cardiac side effects.[19] It is, therefore, vital to further investigate EPIs as a viable treatment option by improving current EPIs. *In vivo* use of EPIs as an adjuvant to treatment regimens in *M. abscessus* has only recently been explored, and Gupta group has demonstrated the treatment shortening by inclusion of verapamil into standard treatment regimens in a mouse model of infection and its adjunctive use could help preserve bedaquiline activity on standard TB treatment.[20] However, there is a limited amount of information on model organisms for which the efficacy of EPIs was evaluated in animal models. In another mouse model of infection study, timcodar treatment resulted in 1.0 and 0.4 $\log_{10}$ reduction in bacterial burden when used in combination with rifampicin and isoniazid, respectively. This suggests its promise as an adjuvant treatment.[21] Recently, Ramis *et al*. evaluated *in silico* and *in vitro* the tetrahydropyridine compounds as efflux inhibitors in *M. abscessus* subsp. *abscessus*.[22] Based on their analysis, this compound can be a potential pharmacophore candidate for the development of a therapeutic adjuvant for *M. abscessus* infections. Studies in *M. tuberculosis* have shown that verapamil potentiates the activity of bedaquiline and ofloxacin.[23] Further studies have identified that verapamil inhibits the activity of MATE pumps.[24,25] It has a low amount of toxicity toward bacterial cells not expressing MATE EPs, suggesting specificity toward bacteria expressing these pumps and a competitive mode of inhibition. Reserpine, an antipsychotic drug extracted from the roots of *Rauwolfia serpentina*, is a promising EPI that targets EPs of the MFS and RND. The clinical application of reserpine

Table 3: Effect of efflux pump inhibitors on bedaquiline minimum inhibitory concentrations in *Mycobacterium abscessus* isolates

Isolates number (n=31)	Morphotype	Bedaquiline MIC (mg/L)		Fold change	Bedaquiline MIC (mg/L)		Fold change
		−verapamil	+ verapamil		−reserpine	+reserpine	
M. abscessus subsp. *abscessus erm (41)* t28							
28024558	R	0.5	0.125	4	0.5	0.25	2
28156067	S	0.5	0.125	4	0.5	0.25	2
27936647	R	0.5	0.062	8	0.5	0.125	4
27130762	R	0.5	0.125	4	0.5	0.125	4
26628639	R	0.5	0.125	4	0.5	0.25	2
27524729	S	1	0.25	4	1	0.5	2
27848307	R	0.5	0.125	4	0.5	0.25	2
27180065	R	0.5	0.125	4	0.5	0.25	2
M. abscessus subsp. *abscessus erm (41)* c28							
27985712	S	0.5	0.125	4	1	0.25	4
28026244	S	0.5	0.125	4	0.5	0.25	2
28184712	S	0.5	0.062	8	0.5	0.125	4
M. abscessus subsp. *bolletii erm (41)* t28							
28066288	R	0.25	0.03	8	0.25	0.125	2
28102098	R	0.5	0.125	4	0.5	0.25	2
26107772	S	1	0.25	4	1	0.25	4
26687394	R	0.125	0.0035	32	0.125	0.03	4
26392763	S	0.5	0.03	16	0.5	0.125	4
26672441	R	0.125	0.007	16	0.125	0.03	4
26820229	R	0.125	0.007	16	0.125	0.03	4
27107934	R	0.062	0.007	8	0.125	0.03	4
27792716	S	0.125	0.015	8	0.125	0.015	8
27993375	S	0.25	0.03	8	0.25	0.062	4
M. abscessus subsp. *massiliense erm (41)* t28							
27967603	R	1	0.25	4	1	0.5	2
28012928	R	0.5	0.062	8	0.5	0.25	2
28169521	S	0.25	0.03	8	0.25	0.062	4
26215870	R	0.5	0.125	4	0.5	0.25	2
27103382	R	0.5	0.125	4	0.5	0.25	2
27693918	S	0.5	0.062	8	0.5	0.125	4
27912547	S	0.5	0.062	8	0.5	0.125	4
27237226	R	0.5	0.125	4	0.5	0.25	2
27881541	S	0.5	0.062	8	0.5	0.125	4
27956700	S	0.5	0.125	4	0.5	0.125	4

MICs: Minimum inhibitory concentrations, *M. abscessus: Mycobacterium abscessus*

with clinically used antibiotics, however, has not yet been achieved due to its nephrotoxic nature.[26] Finally, to know which EPs are involved in this activity, transcriptomic study will be a necessary.

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Conflicts of interest

There are no conflicts of interest.

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