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Unraveling antibiotic resistance mechanisms in *Mycobacterium abscessus*: the potential role of efflux pumps

Saskia E. Mudde[a,*], Jodie A. Schildkraut[b], Nicole C. Ammerman[a], Corné P. de Vogel[a], Jurriaan E.M. de Steenwinkel[a], Jakko van Ingen[b], Hannelore I. Bax[a,c]

[a] Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, Rotterdam, The Netherlands
[b] Radboudumc Center for Infectious Diseases, Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands
[c] Department of Internal Medicine, Section of Infectious Diseases, Erasmus University Medical Center, Rotterdam, The Netherlands

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ABSTRACT

Objectives: *Mycobacterium abscessus* is an opportunistic respiratory pathogen in patients with underlying lung disease. It is infamously known for its low treatment success rates because of its resistance to multiple classes of antibiotics. Further insight into *M. abscessus* resistance mechanisms is needed to improve treatment options. In this in vitro study, the role of efflux pumps in reaction to antibiotic stress is explored, as well as the ability of the putative efflux inhibitors, thioridazine and verapamil, to potentiate the activity of guideline-recommended antibiotics.

Methods: To evaluate the effects of antibiotic stress on mycobacterial efflux pumps, *M. abscessus* subspecies *abscessus* was exposed to amikacin, cefoxitin, clarithromycin, clofazimine, and tigecycline for 24 hours. Transcriptomic responses were measured by RNA sequencing to gain insight into upregulation of efflux pump encoding genes. Subsequently, in time-kill kinetics assays, the above-mentioned antibiotics were combined with thioridazine and verapamil to evaluate their potentiating capacity.

Results: All five antibiotics led to a fold change of $\geq$2 Log_2 in expression of one or more genes encoding transporter systems. This effect was most pronounced for the ribosome-targeting antibiotics amikacin, clarithromycin, and tigecycline. Time-kill kinetics assays demonstrated synergy between amikacin, tigecycline, clofazimine, cefoxitin, and both thioridazine and verapamil.

Conclusion: Antibiotic stressors induce expression of efflux pump encoding genes in *M. abscessus*, especially antibiotics that target the ribosome. Putative efflux inhibitors thioridazine and verapamil show synergy with various guideline-recommended antibiotics, making them interesting candidates for the improvement of *M. abscessus* treatment.

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1. Introduction

Among the nontuberculous mycobacteria (NTM), *Mycobacterium abscessus* is an important opportunistic respiratory pathogen in patients with underlying lung disease [1]. In patients with cystic fibrosis, pulmonary infection caused by *M. abscessus* can be particularly detrimental, as it is associated with accelerated progression to end stage lung disease [2] and person-to-person transmission [3] and may jeopardize successful lung transplantation [4].

A variety of antibiotic resistance mechanisms are responsible for *M. abscessus* being infamously known as difficult to treat. This applies especially to *M. abscessus* subspecies *abscessus*, in which macrolide resistance is induced upon exposure to these drugs by the expression of the ribosome methylase-encoding *erm(41)* gene [5]. Apart from inducible macrolide resistance, acquired point mutations in the *rrl* gene may also abolish macrolide susceptibility [6]. Resistance to aminoglycosides by target modification and mutations in the *rrs* gene [7], resistance to β-lactam antibiotics by the production of a class A β-lactamase [8], and tetracycline resistance by enzymatic inactivation [9] further complicate the treatment of *M. abscessus* infections. Consequently, patients are treated for prolonged periods with multidrug therapies based on in vitro susceptibility testing, often accompanied by burdensome side effects. Despite intensive therapy, treatment success rates as low as

* Corresponding author. Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands.

E-mail address: s.e.mudde@erasmusmc.nl (S.E. Mudde).

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33.0% for pulmonary disease caused by *M. abscessus* subspecies *abscessus* have been reported [10].

Apart from the above-mentioned resistance mechanisms, there has been increasing evidence for the role of efflux pumps in antibiotic resistance in *M. abscessus*. Vianna et al. demonstrated that *M. abscessus* exposed to clarithromycin (CLR) showed overexpression of *MAB_1409* and *MAB_3142*, two efflux pump encoding genes [11]. In line, a recent study by Guo et al. showed an association between the upregulation of efflux pump genes *MAB_2355c, MAB_1409c*, and *MAB_1846* and CLR resistance, also in isolates without *rrl* mutations [12]. Furthermore, Richard et al. found clofazimine (CFZ)-resistant *M. abscessus* isolates harboring mutations in *MAB_2299c*, which were associated with the upregulation of an MmpS/MmpL efflux pump system and resulted in cross-resistance to bedaquiline [13]. Another study by Li et al. found mutations in *MAB_4384* encoding the repressor of the efflux pump MmpS5/MmpL5 in clinically isolated strains resistant to bedaquiline [14]. Lastly, enhanced expression of *MAB_0937c, MAB_1137c, MAB_4117c*, and *MAB_4237c*, all encoding efflux pumps and transporter systems, were shown to be upregulated upon exposure to amikacin (AMK) [15]. However, the exact contribution of efflux pumps to antibiotic resistance in *M. abscessus* is not fully understood.

Increasing interest in the potential role of efflux pumps as a resistance mechanism in mycobacteria has led to the exploration of efflux inhibitors as an adjunctive therapy in preclinical studies. The preclinical body of evidence for efflux inhibitors of both natural and synthetic origin as adjunctive therapies against mycobacteria is growing [16]. However, clinical studies evaluating efflux inhibitors have yet to be conducted. The current study concentrates on two putative efflux inhibitors: verapamil (VP) and thioridazine (TZ). Thioridazine, a phenothiazine, is originally an antipsychotic drug antagonizing dopamine receptor 2. It also has antiemetic, antihistaminic, and anticholinergic activities [17]. Although the efflux inhibitory activity of TZ has been observed in various mycobacterial species [16], studies with TZ and *M. abscessus* are scarce. Verapamil is a calcium channel blocker used in the treatment of cardiovascular diseases [18] and has been extensively evaluated as a potentiator of antituberculosis drugs in preclinical models [19,20]. Its capacity to inhibit drug efflux in *M. abscessus* was demonstrated by Vianna et al. [21]. Therefore, we hypothesized that TZ and VP may similarly potentiate drug activity against *M. abscessus*.

Targeting drug efflux in *M. abscessus* infections could be a strategy for the development of new and more effective treatment options. For this reason, this study evaluated the effects of several guideline-recommended antibiotics on efflux pump gene expression in *M. abscessus*, as well as the ability of TZ and VP to enhance the anti-mycobacterial killing activity of these antibiotic compounds in vitro.

2. Materials and methods

2.1. Bacterial strain and culture conditions

The *M. abscessus* subsp. *abscessus* ATCC 19977 strain was used in all experiments. For the gene expression experiments (performed at Radboud University Nijmegen Medical Centre), stock vials of *M. abscessus* were stored at −70°C in trypticase soy broth containing 40% glycerol. For time-kill kinetics assays (performed at Erasmus University Medical Center Rotterdam), *M. abscessus* was cultured in Cation Adjusted Mueller Hinton broth ([CAMHB] Becton, Dickinson and Company [BD], Sparks, MD) supplemented with 10% OADC (BD) under shaking conditions at 96 rpm and 37°C. Vials with bacterial stock suspensions in CAMHB with 10% OADC were stored at −80°C until use.

2.2. Antimicrobial compounds

Amikacin (AMK), cefoxitin (FOX), clarithromycin (CLR), clofazimine (CFZ), thioridazine (TZ), and verapamil (VP) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Tigecycline (TIG) was manufactured by Pfizer (Brussels, Belgium).

2.3. Drug susceptibility testing

Minimum inhibitory concentrations (MICs) were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (3rd edition) by broth microdilution in CAMHB incubated at 30°C for 3 to 5 days. However, CFZ was found to precipitate in CAMHB. This issue could be resolved by diluting CFZ in CAMHB with 4% dimethyl sulfoxide (DMSO). The CLSI guidelines recommend determining the MIC of CLR after 14 days because of inducible resistance by the *erm*-gene. However, in this study, experiments with CLR did not exceed 3 days, so no induced CLR resistance was expected during this period. Therefore, the MIC of CLR was determined after 3 days of incubation.

2.4. Drug stability testing

Drug stability of AMK and FOX was evaluated by assessing antimicrobial activity over time using the standard large-plate agar diffusion assay [22]. In brief, for AMK and FOX, *Escherichia coli* ATCC 25922, susceptible to both compounds, was plated onto diagnostic sensitivity test agar (Oxoid, Hampshire, UK) or Mueller Hinton agar (BD), respectively. A standard concentration series of each drug with 2-fold increasing concentrations was prepared. Test concentrations per drug were incubated at 30°C, similar to the temperature at which the time-kill kinetics assays were performed (Section 2.6.). On days 1, 3, and 7, both the standard concentrations and a sample of the test concentrations were added onto the solid agar. The concentration of AMK and FOX in the test conditions over time was determined by comparing their inhibition zones to those of the standard concentrations, representing the remaining drug concentrations. The stability of TIG was determined similarly in a previous study, using *Micrococcus luteus*, susceptible to TIG, plated onto diagnostic sensitivity test agar [22]. For CFZ, no bacterial indicator strain is available. Therefore, a pre-incubation MIC determination with *M. abscessus* was used to determine drug stability. The MIC was determined by broth microdilution assay according to the CLSI guidelines. The CFZ concentrations were either freshly prepared or pre-incubated for 1 week at 30°C before determining the MIC. In this assay, equal MICs indicated that the compound was stable over the time course of a time-kill kinetics assay.

2.5. Gene expression

RNA sequencing was performed as described previously [23]. Bacterial inocula were grown in CAMHB with 0.05% Tween 80 until early log-phase. Subsequently, antibiotics were added to the culture (AMK 8 mg/L, FOX 8 mg/L, CLR 4 mg/L, CFZ 1 mg/L, and TIG 0.5 mg/L), which was further incubated for 24 hrs prior to RNA isolation. All conditions were completed in biological triplicate.

Following lysis with bead beating, RNA isolation was performed using the Nucleospin RNA kit (Machery Nagel, Düren, Germany). Following isolation, RNA integrity was determined, rRNA was depleted, and the mRNA library was constructed and sequenced on a NextSeq 500 (Illumina, San Diego, CA).

All obtained reads were mapped to the *M. abscessus* ATCC 17799 genome (NCBI reference sequence: NC_010397.1) using STAR (v2.7.0). Differential expression analysis of putative efflux transporter genes was performed in R (v3.3.3) using the DESeq2 pack-

age, and the cut-off for gene upregulation was defined as a Log2 fold change ≥2; a *P* value (≤0.05) corrected for multiple guessing.

2.6. Time-kill kinetics assay

The concentration-dependent and time-dependent killing activity of AMK, TIG, FOX, and CFZ, alone or in combination with TZ or VP, was assessed as previously described [22]. In brief, 25 mL cultures of *M. abscessus*, with a starting inoculum of ~5,5 × 10^5 colony-forming units (CFU)/mL in CAMHB supplemented with 10% OADC, were exposed to AMK, FOX, TIG, or CFZ at concentrations of 1/4x or 1x the MIC with or without TZ or VP. For TZ and VP, concentrations of 1/2x or 1/8x MICs were used, as 1/2x MIC was previously shown to inhibit ethidium bromide efflux in mycobacteria [24]. Thus, the concentrations tested for each drug were as follows: AMK 4 and 16 mg/L; FOX 8 and 32 mg/L; TIG 1 and 4 mg/L; CFZ 0.125 and 0.5 mg/L; TZ 8 and 32 mg/L; and VP 128 and 512 mg/L. To prevent precipitation of CFZ in CAMHB with 10% OADC, 0.4% DMSO was added in time-kill kinetics assays with CFZ. Because CFZ at 1x MIC did not inhibit bacterial growth in the time-kill kinetic assay, MIC determination of CFZ was repeated in CAMHB supplemented with 10% OADC to better reflect the experimental conditions in the time-kill kinetics assay. As a 4-fold higher MIC of 2 mg/L was observed in the presence of 10% OADC, 4x MIC (2 mg/L) was included for CFZ. The cultures were incubated at 30°C under shaking conditions. In a previous study, an incubation temperature of 35°C was used [22]. However, growth of *M. abscessus* at 30°C was found to be more optimal in comparison with growth at 35°C (data not shown). Also, the temperature of 30°C is in line with the CLSI (3rd edition) recommendations for MIC determinations. Based on the results of drug stability experiments (see Section 3.2.), losses in drug stability were compensated for in the case of FOX and TIG by daily addition of minimal volumes (100 μL) of drug concentrations to the cultures. After 1, 3, and 7 days of drug exposure, the cultures were sampled for mycobacterial load determination (CFU/mL). Samples were centrifuged at 14.000 g for 10 min, the supernatant was discarded, and the pellet was suspended in phosphate buffered saline. This washing procedure was performed twice to prevent drug carry-over onto the agar plates. The number of CFU per mL in the samples was determined by preparing 10-fold serial dilutions up to 10^{-7} and plating 200 μl per dilution onto Mueller Hinton agar supplemented with 10% OADC. CFU were counted after incubating the plates for 5 to 7 days at 30°C. CFU counts were $\log_{10}$ transformed. The lower limit of detection was 0.7 $\log_{10}$ CFU/mL. All experiments were performed in duplicate.

2.7. Synergy in the time-kill kinetics assays

Activity between antimycobacterial compounds and efflux inhibitors was considered to be synergistic when it conformed to either of the following definitions: i) a ≥100-fold (a difference of 2 $\log_{10}$) increase in mycobacterial killing with the 2-drug combination compared with the most active single drug, or ii) when a drug combination achieved elimination of *M. abscessus* that was not achieved by exposure to a single drug [25]. The definition of synergy was only met when at least one of these criteria was fulfilled in both (duplicate) experiments.

3. Results and discussion

3.1. Minimum inhibitory concentrations

The MICs established for the *M. abscessus* ATCC 19977 strain were as follows: 16 mg/L for AMK, 32 mg/L for FOX, 4 mg/L for CLR, 4 mg/L for TIG, and 0.5 mg/L for CFZ. The MIC established for TZ was 64 mg/L and that for VP was 1024 mg/L.

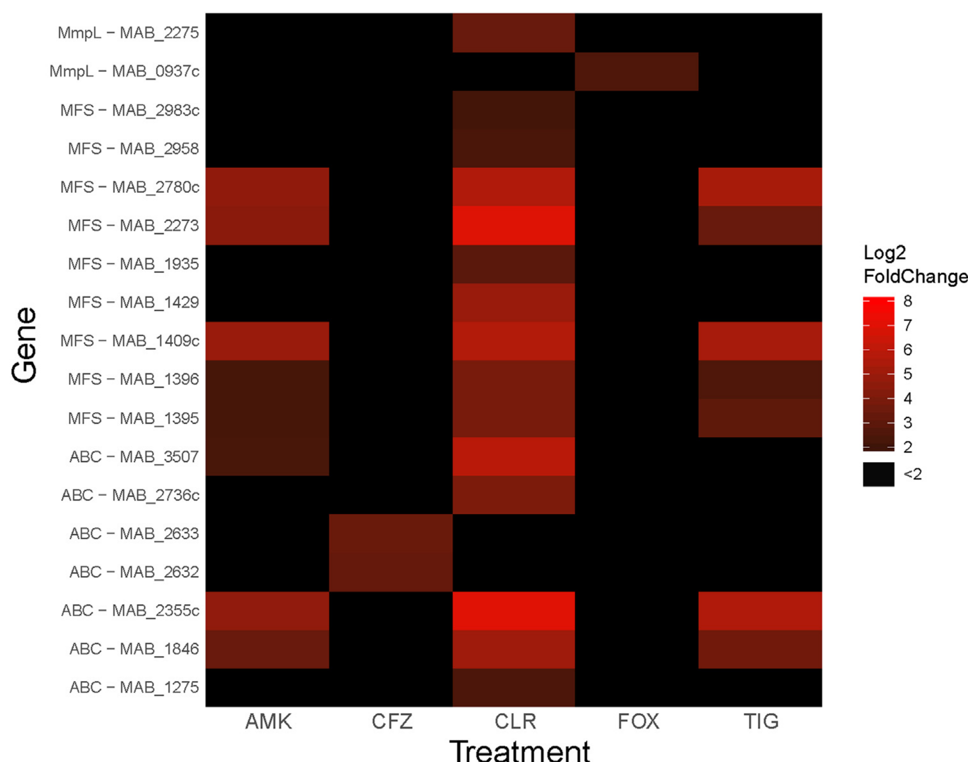

Fig. 1. Differentially expressed transporter systems. Heat map illustrating transporter systems that had a fold change of ≥2 Log_2 and a *P* value of <0.05. AMK = amikacin; CFZ = clofazimine; CLR = clarithromycin; FOX = cefoxitin; TIG = tigecycline.

3.2. Drug stability

AMK was shown to be stable, but FOX showed a 33% decline daily. For TIG, an 80% decline daily was previously shown [22]. Both freshly prepared and pre-incubated CFZ concentrations demonstrated the same MIC for *M. abscessus*, indicating that CFZ was stable over the course of the time-kill kinetics assay.

3.3. Gene expression

Exposure to subinhibitory concentrations of AMK, CFZ, CLR, FOX, and TIG resulted in an increased expression of a wide range of putative efflux transporter genes in M. *abscessus* (Fig. 1). The largest number of upregulated efflux pump encoding genes was seen following CLR exposure (15), followed by AMK (8), and TIG (7). All genes upregulated following AMK and TIG exposure were also upregulated as a result of CLR exposure. In contrast, CFZ and FOX only induced a small set of efflux genes. CFZ induced the expression of *MAB_2632* and *MAB_2633*, encoding the CydC/CydD ABC transporter in the mycobacterial respiratory chain, while FOX induced specific expression of *MAB_0837c*, encoding a putative MmpL protein.

3.4. Time-kill kinetics assays

The time-dependent killing activities of AMK, TIG, FOX, and CFZ with or without TZ are presented in Fig. 2 and Table A.1, and those with or without VP are presented in Fig. 3 and Table A.2.

For the 1/2x MIC concentration of TZ, synergy was found with AMK at 1x MIC, TIG at 1/4x and 1x MIC, FOX at 1/4x and 1x MIC, and CFZ at 4x the MIC. When TZ at 1/2x MIC was combined with TIG at 1x MIC, no CFUs were retrieved at days 3 and 7. TZ at 1/8x MIC did not show synergy with any of the antibiotics tested. Of notice, TZ at 1/2x MIC showed substantial activity against *M. abscessus* on its own (Fig. 2, Table A.1).

Similar results were found for VP. Synergy was observed between VP at 1/2x MIC and AMK, and TIG and FOX at 1/4x and 1x MIC. CFZ at 1x and 4x MIC showed synergy with VP at 1/2x MIC, as well. Like TZ, the 1/8x MIC concentration of VP did not demonstrate synergy with any of the antibiotics (Fig. 3, Table A.2).

4. Discussion

While all five antibiotics were found to increase transcription of one or more genes encoding transporter systems, this effect was more pronounced for AMK, CLR, and TIG, as compared with CFZ and FOX. Time-kill kinetics assays showed synergistic activity between AMK, TIG, CFZ, FOX, and both TZ and VP. To our knowledge, these drug combinations have not been evaluated before against *M. abscessus* in vitro [26].

Although the precise functions of the proteins encoded by the upregulated genes are not yet fully understood, these genes have been linked to different families of efflux pumps and other transporter systems [12,15,27–29]. As such, the findings in this study strengthen the growing body of evidence for efflux mediated antibiotic resistance mechanisms in *M. abscessus* [11–13,15].

Interestingly, compared with CFZ and FOX, gene upregulation for efflux transporters was more pronounced upon exposure to AMK, CLR, and TIG, all three being ribosome-targeting antibiotics. FOX, on the other hand, targets cell wall synthesis, and CFZ is thought to interfere with the mycobacterial respiratory chain [23]. Although one might speculate that ribosome-targeting antibiotics are more potent inducers of efflux pump systems specifically, it is good to interpret these results in a broader context of gene upregulation. In fact, it was recently shown by Schildkraut et al. that AMK, CLR, and TIG led to more extensive elevated transcriptional responses, in general, in comparison with FOX and CFZ [23]. One of the common transcriptomic responses shared by AMK, CLR, and TIG is the upregulation of the *whiB7* regulon, a transcriptional regulator controlling genes involved in intrinsic antibiotic resistance [23,28]. In line, in the current study, 9 of 14 efflux pump genes upregulated by exposure to AMK, CLR, and TIG are described as within the *M. abscessus whiB7* regulon [28,29]. Induction of *whiB7* by ribosome-targeting antibiotics could perhaps explain why upregulation of efflux pump encoding genes was most explicit for AMK, CLR, and TIG. On the other hand, AMK has been considered as a weak *whiB7*-inducer [28,29]; however, it led to substantial induction of multiple transporter genes in the current study.

Apart from a possible indirect link between efflux pumps and ribosome-targeting antibiotics via *whiB7*, there might also be a more direct link. The function of efflux pumps extends beyond efflux of antibiotics, as they contribute to cell homeostasis by secreting waste products, as well as specific substrates that assist in cell-to-cell communication, biofilm formation, and nutrient acquisition [30]. Additionally, there is increasing evidence that efflux pumps might be involved in ribosomal protection. A subfamily of ATP-binding cassette (ABC) transporters, the cytosolic ABC-F proteins, are capable of displacing antibiotics from the ribosome, thereby providing protection against ribosome targeting antibiotics [31]. More specific to *M. abscessus*, a recent study by Guo et al. found that *MAB_2355c*, a putative ABC transporter with homology to ABC-F proteins, is strongly induced by macrolides. Using *Escherichia coli* S30, erythromycin-inhibited ribosomal translation was reversed upon addition of purified MAB_2355c in a dose-dependent manner, suggesting a protective role towards the ribosome for this *M. abscessus* transporter [27]. Indeed, in the current study, *MAB_2355c* was strongly induced by ribosome-targeting AMK, CLR, and TIG, but not by CFZ and FOX. So far, *MAB_2355c* is the only transporter in *M. abscessus* directly linked to ribosomal protection. Additional research is needed to shed light on functions of efflux pumps beyond antibiotic efflux in *M. abscessus*.

CFZ and FOX exposure resulted only in a modest induction of efflux pump genes. FOX exposure induced specific expression of *MAB_0937c*, encoding a putative member protein of the MmpL family that is highly homologous to *mmpL10* in *Mycobacterium tuberculosis* [15,32]. MmpL10 is thought to be involved in the transport of cell wall components [32], and may have been induced to restore the damage caused by FOX. CFZ induced expression of *MAB_2632* and *MAB_2633*, encoding the CydC/CydD ABC transporter. In *M. tuberculosis*, this transporter is thought to be essential for the function of the bd-type ubiquinol oxidoreductase [33]. This is in line with the finding that CFZ exposure induces bd-type ubiquinol oxidoreductase, which probably aids in the defense against CFZ-related oxidative stress [23].

Based on the more extended gene upregulation upon exposure to AMK, CLR, and TIG compared with CFZ and FOX, higher levels of synergy were expected between AMK, CLR, and TIG and the putative efflux inhibitors TZ and VP. Nevertheless, equal levels of synergy with TZ and VP were observed for CFZ and FOX and for the ribosome-targeting antibiotics. Apparently, the degree of efflux pump gene expression did not correlate with the level of synergy between antibiotics and efflux inhibitors. These findings do not elucidate the precise mechanisms underlying the potentiating activity of TZ and VP but do indicate that mechanisms other than efflux inhibition might drive the synergistic interactions observed here. In fact, in the time-kill kinetics assays, TZ demonstrated considerable activity on its own. In the context of *M. tuberculosis*, TZ inhibits growth by interfering with the aerobic respiratory chain [34]. One might hypothesize that the metabolic and

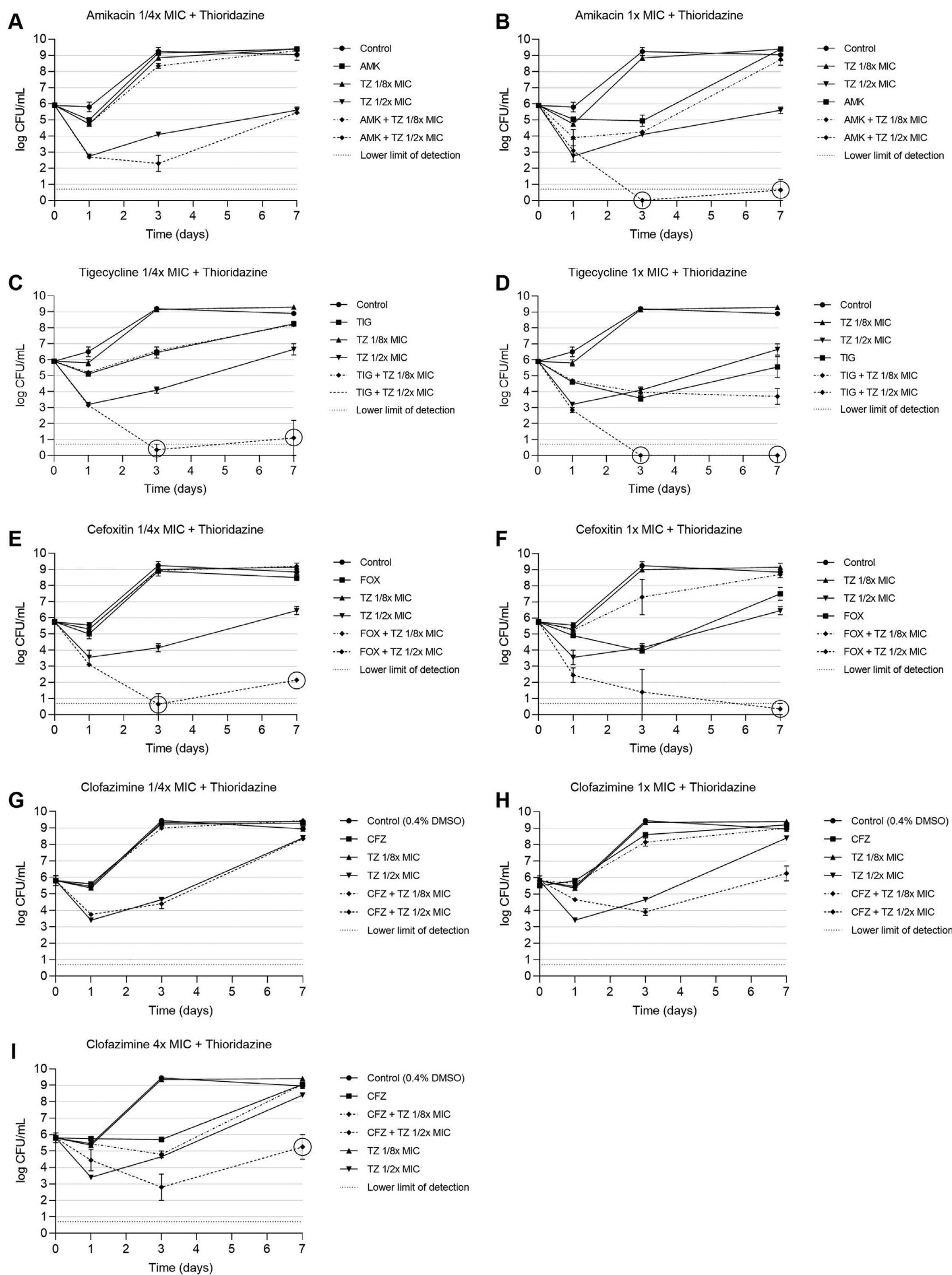

Fig. 2. Concentration-dependent and time-dependent bactericidal activity of thioridazine (TZ) at 1/8x MIC (8 mg/L) or 1/2x MIC (32 mg/L) combined with amikacin (AMK) at 1/4x MIC (4 mg/L) (A) or 1x MIC (16 mg/L) (B); tigecycline (TGC) at 1/4x MIC (1 mg/L) (C) or 1x MIC (4 mg/L) (D); cefoxitin (FOX) at 1/4x MIC (8 mg/L) (E) or 1x MIC (32 mg/L) (F); clofazimine (CFX) at 1/4x MIC (0.125 mg/L) (G); and 1x MIC (0.5 mg/L) (H) or 4x MIC (2 mg/L) (I) against *M. abscessus* subspecies *abscessus*. MIC values are based on the Clinical and Laboratory Standards Institute method. Mycobacterial cultures were exposed to the antibiotics with or without TZ for 7 days at 30°C under shaking conditions. On days 1, 3, and 7, the mycobacterial load, expressed as colony forming units (CFU), was determined by sampling the cultures and plating them onto antibiotic-free solid Mueller Hinton agar supplemented with 10% oleic acid-albumin-dextrose-catalase. The experiments were performed in duplicate. Results are expressed as the mean CFU (+/– the range). Circles indicate combinations showing synergy. The dashed horizontal line indicates the lower limit of detection of CFU.

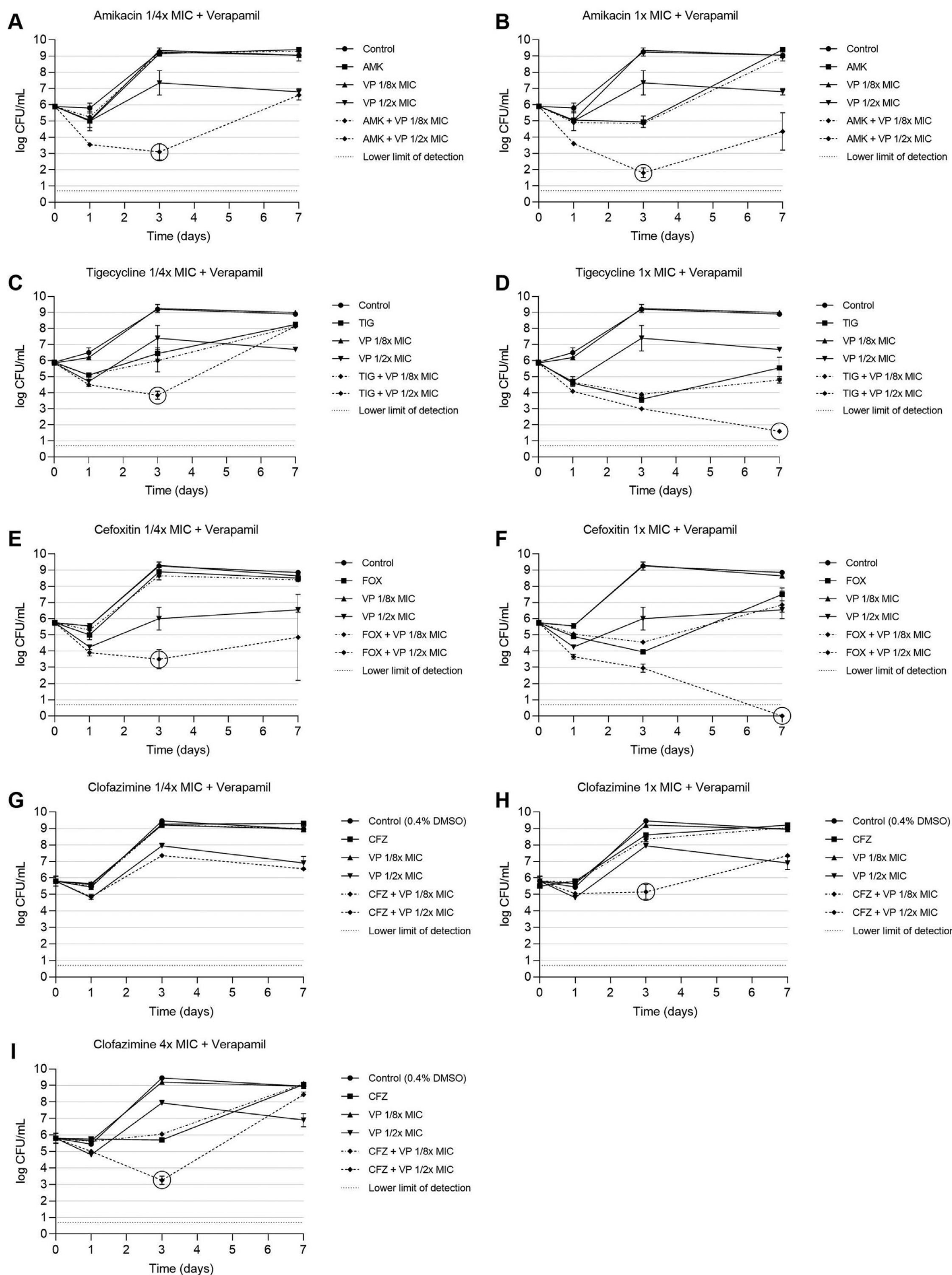

Fig. 3. Concentration-dependent and time-dependent bactericidal activity of verapamil (VP) at 1/8x MIC (128 mg/L) or 1/2x MIC (512 mg/L) combined with amikacin (AMK) at 1/4x MIC (4 mg/L) (A) or 1x MIC (16 mg/L) (B); tigecycline (TGC) at 1/4x MIC (1 mg/L) (C) or 1x MIC (4 mg/L) (D); cefoxitin (FOX) at 1/4x MIC (8 mg/L) (E) or 1x MIC (32 mg/L) (F); and clofazimine (CFX) at 1/4x MIC (0.125 mg/L) (G), 1x MIC (0.5 mg/L) (H), or 4x MIC (2 mg/L) (I) against *M. abscessus* subspecies *abscessus*. MIC values are based on the Clinical and Laboratory Standards Institute method. Mycobacterial cultures were exposed to the antibiotics with or without VP for 7 days at 30°C under shaking conditions. On days 1, 3, and 7, the mycobacterial load, expressed as colony forming units (CFU), was determined by sampling the cultures and plating them onto antibiotic-free solid Mueller Hinton agar supplemented with 10% oleic acid-albumin-dextrose-catalase. The experiments were performed in duplicate. Results are expressed as the mean CFU (+/– the range). Circles indicate combinations showing synergy. The dashed horizontal line indicates the lower limit of detection of CFU.

respiratory shifts in *M. abscessus* following exposure to commonly prescribed antibiotics [23] are hampered by TZ's effect on the respiratory chain, thus leading to non-efflux based synergistic activity. Regarding VP, Chen et al. demonstrated that VP did not increase intracellular drug concentrations in *M. tuberculosis*, contradicting its efflux inhibitory function [35]. This could be a consequence of redundancy in efflux pumps [35]. On the other hand, it has been suggested that VP inhibits drug efflux in *M. tuberculosis* more indirectly by interfering with the proton motive force, thereby affecting various transport processes in the cell [16]. It could be that such a mechanism, or yet to be identified mechanisms, also underlie the potentiating activity of VP towards antibiotics in *M. abscessus*.

A couple of limitations to this study need to be considered. First, the experiments were conducted with planktonic mycobacteria, whereas in vivo *M. abscessus* also resides intracellularly in macrophages. This is relevant, considering that VP can act on mammalian transporters, as well [36]. Whether the activity of the studied antibiotics and TZ and VP is also present against *M. abscessus* within macrophages is an important topic for further study. Secondly, experiments were only performed with a reference *M. abscessus* subspecies *abscessus* strain. Differential drug activity against the reference strain and clinical isolates of *M. abscessus* should be assessed in future experiments. Also, stability testing of TZ and VP, as well as gene expression following TZ and VP exposure, were not tested. Not having this information is a clear limitation that should be addressed in future work. Lastly, the concentrations of TZ and VP used in the time-kill kinetics assays were extremely high and not clinically feasible, considering the toxicity accompanying these drugs. In a clinical study, steady-state TZ serum concentrations ranged between 0.4 to 8.8 mg/L after a median dose of 150 mg/day [37], which is barely equal to the concentration of 1/8x the MIC of TZ (8 mg/L). TZ at this concentration showed no (synergistic) activity in the time-kill kinetics assays. The same issue applies to VP, as serum concentrations in humans above 0.9 mg/L are considered toxic [38]. High MICs, together with the unfavorable toxicity profiles of TZ and VP, complicate the potential use of these compounds in clinical *M. abscessus* treatment. Nonetheless, the synergistic activity of TZ and VP with guideline-recommended antibiotics shows encouraging proof-of-principle results that may inform the development of safer derivatives and targeted drug administration. For example, norverapamil has demonstrated preserved activity but has a more favorable toxicity profile [19]. Also, inhalation-based therapies with TZ and VP are currently being developed and aim to establish high local drug concentrations while reducing systemic exposure and accompanying toxicity [39,40].

In conclusion, induction of efflux pump encoding genes is an important response in *M. abscessus* triggered by antibiotic stressors, especially ribosome-targeting antibiotics. Putative efflux inhibitors, such as TZ and VP, show synergy with antibiotics that are currently recommended and may be promising leads for the improvement of *M. abscessus* treatment options.

Declaration of Competing Interest

None declared.

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Ethical approval

Not required.

Appendix

Table A.1
Summary of interactions between amikacin (AMK), tigecycline (TIG), cefoxitin (FOX), and clofazimine (CFZ) and the efflux inhibitor thioridazine (TZ) based on the time-kill kinetics assays. Synergistic activity is indicated by '+'. MIC values are based on the CLSI method.

		Day 3		Day 7	
		1/8x MIC TZ	1/2x MIC TZ	1/8x MIC TZ	1/2x MIC TZ
AMK	1/4x MIC				
	1x MIC		+		+
TIG	1/4x MIC		+		+
	1x MIC		+		+
FOX	1/4x MIC		+		+
	1x MIC				+
CFZ	1/4x MIC				
	1x MIC				
	4x MIC				+

Table A.2
Summary of interactions between amikacin (AMK), tigecycline (TIG), cefoxitin (FOX), and clofazimine (CFZ) and the efflux inhibitor verapamil (VP) based on the time-kill kinetics assays. Synergistic activity is indicated by '+'. MIC values are based on the CLSI method.

		Day 3		Day 7	
		1/8x MIC VP	1/2x MIC VP	1/8x MIC VP	1/2x MIC VP
AMK	1/4x MIC		+		
	1x MIC		+		
TIG	1/4x MIC		+		
	1x MIC				+
FOX	1/4x MIC		+		
	1x MIC				+
CFZ	1/4x MIC				
	1x MIC		+		
	4x MIC		+		

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