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Increased Vancomycin Susceptibility in Mycobacteria: a New Approach To Identify Synergistic Activity against Multidrug-Resistant Mycobacteria

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***Mycobacterium tuberculosis* is wrapped in complex waxes, impermeable to most antibiotics. Comparing *Mycobacterium bovis* BCG and *M. tuberculosis* mutants that lack phthiocerol dimycocerosates (PDIM) and/or phenolic glycolipids with wild-type strains, we observed that glycopeptides strongly inhibited PDIM-deprived mycobacteria. Vancomycin together with a drug targeting lipid synthesis inhibited multidrug-resistant (MDR) and extensively drug-resistant (XDR) clinical isolates. Our study puts glycopeptides in the pipeline of potential antituberculosis (TB) agents and might provide a new antimycobacterial drug-screening strategy.**

Mycobacterium tuberculosis remains a leading cause of morbidity, tuberculosis (TB), and mortality in the world. *M. tuberculosis* is intrinsically resistant to most classical antibiotics, partly because of its impermeable cell wall (1–6). Due to selective mutations in *M. tuberculosis*, almost one-third of new TB patients are now infected with first-line drug-resistant strains, monoresistant strains, or multidrug-resistant (MDR) strains. Consequently, second-line therapies are often implemented, leading to the appearance of extensively drug-resistant (XDR) strains (7, 8). There is therefore a growing urgency in the need for new antimycobacterial therapies.

The mycobacterial cell wall is composed of peptidoglycan covalently attached to arabinogalactan, which are in turn esterified by very-long-chain mycolic acids. Various noncovalently attached lipids are embedded at the outer surface and necessary for capsule formation. Among these lipids, two related waxes, phthiocerol dimycocerosates (PDIM) and phenolic glycolipids (PGL), are involved in virulence (9–11). PDIM and PGL are only or mostly, respectively, found in pathogenic mycobacteria, but their roles in antibiotic resistance remain unclear (12–16). In *Mycobacterium marinum*, a mild (2- to 10-fold) increase in antibiotic susceptibility was observed in PDIM- and PGL-deficient strains (14, 15). In contrast, in PDIM- and PGL-deficient *M. tuberculosis*, no change was detected (13).

The present study aimed to understand how mycobacteria can become susceptible to glycopeptides. Using PDIM-negative and/or PGL-negative strains of *Mycobacterium bovis* BCG and *M. tuberculosis*, we investigated the correlation between the absence of PDIM and the glycopeptide susceptibility. Subsequently, we investigated whether vancomycin could synergistically inhibit MDR and XDR strains with a mycobacterial lipids synthesis inhibitor.

We recently reported that the chaperonin Cpn60.1/GroEL-1/Hsp60-1 of *M. bovis* BCG was necessary for the integrity of the cell wall as the Δ*cpn60.1* strain showed an abnormal mycobacterial cell wall with a lack of PDIM and mycolates with two more carbon atoms (17). We investigated the susceptibility of the wild-type (WT), Δ*cpn60.1*, and complemented Δ*cpn60.1 M. bovis* BCG (GL2 strain) strains to several antituberculosis drugs. We used the NCCLS agar proportion method (18) to determine the MIC scale range of each antibiotic. We inoculated equal quantities of several dilutions of a 3 McFarland standard inoculum on 7H11 agar supplemented with oleic acid-albumin-dextrose (Difco Laboratories) with or without drug (10-fold dilution assays). The BacT/Alert MP (mycobacteria process) system was used to determine the MIC more accurately. BacT/Alert MP bottles (11 ml) supplemented with 0.5 ml restoring fluid were inoculated with 0.1 ml water or drug solution and 0.4 ml of mycobacterial suspension (0.5 McFarland standard in 7H9 medium, 0.05% Tween 80, 10% albumin-dextrose). A 100-fold diluted bacterial inoculum was injected in a drug-free vial, as a 1/100 proportional growth control. The concentration of the antibiotic in a bottle flagged positive in the same amount of time as the 1/100 control bottle was considered the MIC (19).

The WT *M. bovis* BCG strain and the Δ*cpn60.1* mutant were susceptible to all antituberculosis drugs (Table 1), but the Δ*cpn60.1* mutant showed a MIC 5 times lower for rifampin. This increase in susceptibility was totally abolished by reintroducing expression of Cpn60.1. We unexpectedly also observed that the Δ*cpn60.1* mutant showed 100-fold higher susceptibility to glycopeptides (teicoplanin and vancomycin), usually not used in the

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TABLE 1 Antibiotic susceptibility for the three *M. bovis* BCG strains and H37Rv *M. tuberculosis*

Antibiotic	MIC (μg/ml)[a]			
	WT BCG	Δ*cpn60.1*	Δ*cpn60.1*Comp	H37Rv
Isoniazid	0.1	0.1	0.1	0.1
Rifampin	0.05	0.01	0.05	1
Ethambutol	1	1	1	5
Streptomycin	0.2	0.2	0.2	1
Ethionamide	4	4	4	5
Ciprofloxacin	0.25	0.25	0.25	1
Moxifloxacin	0.05	0.05	0.05	0.25
Teicoplanin	>1,000	17.5	>1,000	100
Vancomycin	>500	5	>500	65
Cerulenin	0.75	0.75	0.75	2.5

[a] The BacT/Alert MP system was used to determine the MIC more accurately. Δ*cpn60.1* Comp, the complemented Δ*cpn60.1* strain.

treatment of tuberculosis or Gram-negative bacterial infection because of their outer membranes. This gain in susceptibility was totally abolished in the complemented strain, suggesting that loss of Cpn60.1 conferred an unusual and very high susceptibility to this class of antibiotic.

To assess if the glycopeptide susceptibility of the Δ*cpn60.1 M. bovis* BCG mutant was linked to a PGL deficiency in addition to the PDIM deficiency, as previously reported (17), we analyzed its lipid composition in more detail (see the supplemental material). The mass spectra of the lipid extract of the Δ*cpn60.1*, complemented, and WT strains confirmed the longer chain lengths in α-mycolates (2 extra carbons) and the absence of intact PDIM (see Fig. S1A to C in the supplemental material) and showed the absence of intact PGL in the Δ*cpn60.1* mutant (see Fig. S1A, B, D, and E).

To assess the impact of either PDIM or PGL deficiency on vancomycin susceptibility, we determined the vancomycin susceptibility of the wild type (BCG Pasteur 1173P2), the PMM50 mutant (BCG Δ*ppsE*, PDIM$^-$ and PGL$^-$), and the PMM137 mutant (BCG Δ*fadD26*, PDIM$^-$ and PGL$^+$) (20, 21). The wild-type BCG Pasteur 1173P2 was resistant to vancomycin, like the related GL2 strain, with a MIC of approximatively 100 μg/ml (data not shown). All *M. bovis* BCG mutants defective in the PDIM component, regardless of the presence or absence of PGL, presented a MIC of around 5 μg/ml to vancomycin (Fig. 1A and B) as observed for the mutant Δ*cpn60.1* (Table 1). To assess the impact of PDIM in *M. tuberculosis*, the vancomycin susceptibilities of two *M. tuberculosis* strains, wild-type H37Rv (naturally deficient for PGL) or PMM56 (H37Rv Δ*ppsE*, PDIM$^-$), were compared (22). The wild-type *M. tuberculosis* H37Rv was resistant to vancomycin, with a MIC of approximatively 65 μg/ml (Table 1). The *M. tuberculosis* PMM56 mutant (Δ*ppsE*) showed a MIC of between 0.5 and 1 μg/ml to vancomycin (Fig. 1C), allowing us to make the same association between PDIM deficiency and the increase in vancomycin susceptibility in *M. tuberculosis*.

The potential clinical use of vancomycin in combination with a cell wall-targeting drug was investigated as a proof of concept. The combination of vancomycin and cerulenin, a potent long-chain lipid synthesis inhibitor (23, 24), was used at a sub-MIC on *M. tuberculosis* H37Rv and on MDR and XDR *M. tuberculosis* clinical isolates. Thirteen clinically unrelated isolates were selected from a *M. tuberculosis* collection (Tuberculosis Center, Public Health Research Institute [PHRI], NJ) (25). A synergistic effect was evaluated in the BacT/Alert MP system by the *x*/*y* methodology (26–29). A Δ*x*/Δ*y* quotient of <0.5 indicates enhanced drug action, with *x* being the growth index (GI) value obtained for the vial with the combination of drugs and *y* being the lowest GI value obtained with any of the single drugs used within the combination tested. A combination of vancomycin (5 μg/ml) and cerulenin (0.5 μg/ml) inhibited 99% of the H37Rv *M. tuberculosis* growth (data not

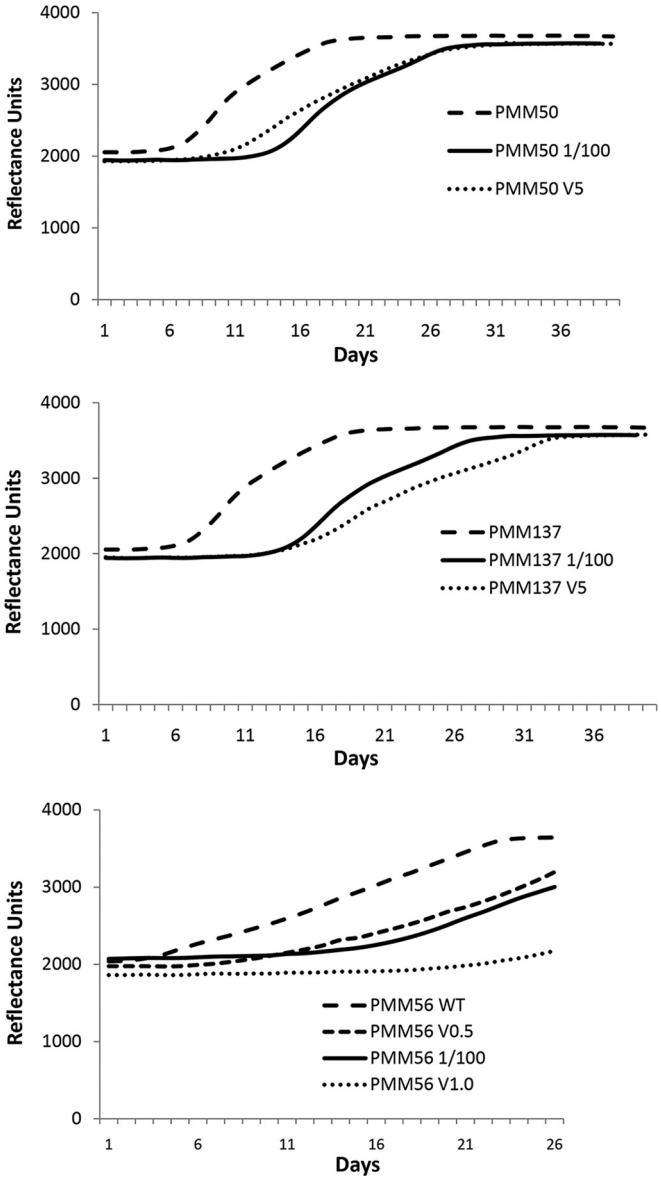

FIG 1 The lack of PDIM in mycobacteria is associated with glycopeptide susceptibility. Typical fluorometric reflectance results showing mycobacterial cell growth in the absence and presence of 5 (V5), 1 (V1.0), and 0.5 (V0.5) μg/ml vancomycin. (A) Representative growth curves of PMM50 *M. bovis* BCG (PDIM and PGL deficient) diluted (1/100) or not diluted. The MIC corresponds to a concentration of 5 μg/ml vancomycin. (B) Representative growth curves of PMM137 *M. bovis* BCG (PDIM deficient, PGL positive) diluted (1/100) or not diluted. The MIC corresponds to a concentration of 5 μg/ml vancomycin. (C) Representative growth curves of PMM56 *M. tuberculosis* (PDIM deficient) diluted (1/100) or not diluted. The MIC is between 0.5 and 1.0 μg/ml vancomycin.

TABLE 2 Synergic effect of vancomycin and cerulenin in *M. tuberculosis* multidrug-resistant clinical isolates

Isolate[a]	FP[b]	TN no.[c]	Resistance profile[d]		Synergic effect[e]	MIC[f] to vancomycin (μg/ml)
			First-line drugs	Second-line drugs		
1	OO1	16054	INH, RIF, EMB, STR	RMC, PAS	+ (0.30)	Between 50 and 75
2	BE	17182	INH, RIF	ETH, PAS	++ (0.10) ($V_5C_{0.5}$)[f]	Between 50 and 75
3	W283	14178	INH, RIF, EMB, PZA, STR	KAN, CAP, RFB, PAS	+ (0.43) ($V_5C_{0.5}$)[f]	>200
4	OO1	18048	INH, RIF, PZA, STR	RFB, RMC, PAS	+ (0.32)	>200
5	P	16442	INH, RIF, PZA, STR	RMC, PAS	+ (0.20)	Between 75 and 100
6	P23	16906	INH, RIF, EMB, PZA, STR	ETH, RMC, PAS	+ (0.25)	20
7	BE	18460	INH, RIF, EMB, STR	ETH, CYC, CIP, KAN, CAP, RFB, RMC, PAS	+++ (0.05)	>200
8	W	2550	INH, RIF, EMB, PZA, STR	ETH, OFX, KAN, CYC, PAS	+ (0.28)	>200
9	HD15	18985	INH, RIF, EMB, PZA, STR	CYC, CIP, OFX, KAN, AMI, CAP, RFB, RMC, PAS	+ (0.13)	200
10	W	14614	INH, RIF, EMB, STR	ETH, AMI, KAN, RFB, RIP	>0.5	200
11	W12	15183	INH, RIF, EMB, STR	ETH, AMI, KAN	>0.5	>200
12	W	14003	INH, RIF, EMB, STR	ETH, RFB, RMC, PAS	>0.5	>200
13	W148	13438	INH, RIF, EMB, PZA, STR	KAN, AMI, RFB, RIP	>0.5	>200

[a] Isolates 7 to 9 are XDR strains.
[b] FP, fingerprint name based on IS*6110* typing and PHRI nomenclature.
[c] TN no., tracking number, a PHRI unique identifier for each isolate.
[d] Abbreviations: AMI, amikacin; CAP, capreomycin; CIP, ciprofloxacin; CYC, cycloserin; EMB, ethambutol; ETH, ethionamide; INH, isoniazid; KAN, kanamycin; OFX, ofloxacin; PAS, *para*-aminosalicylic acid; PZA, pyrazinamide; RFB, rifabutin; RIF, rifampin; RIP, rifapentine; RMC, rifamycin; STR, streptomycin.
[e] Synergy between vancomycin (6 μg/ml) and cerulenin (1 μg/ml). Values in parentheses show $\Delta x/\Delta y$ quotients. A $\Delta x/\Delta y$ quotient of <0.5 in the case of a two-drug combination indicates a synergic effect of the drug action. The respective $\Delta x/\Delta y$ quotients are illustrated as follows: +, <0.5; ++, <0.1; +++, <0.05.
[f] These values were obtained from tests with 5 μg/ml vancomycin (V_5) and 0.5 μg/ml cerulenin ($C_{0.5}$). The BacT/Alert MP system was used to determine the MIC more accurately.

shown). Interestingly, the combination of vancomycin (6 μg/ml) with cerulenin (1 μg/ml) was synergistically effective, inhibiting the growth of 6 MDR and 3 XDR out of 10 MDR and 3 XDR *M. tuberculosis* clinical isolates (Table 2).

Vancomycin is a large (molecular weight [MW] of 1,449) hydrophilic molecule able to form a hydrogen bond with the terminal D-alanyl-D-alanine moieties during peptidoglycan biosynthesis, thereby preventing bacterial cell wall backbone synthesis. The target of vancomycin, ubiquitous in bacteria, is thus only easily reachable on bacteria with thin cell walls or without an outer lipid membrane (6) or without PDIM, as suggested by our results either using various *M. bovis* BCG and *M. tuberculosis* mutants, or co-treated by a drug targeting long-chain lipid synthesis, such as cerulenin.

Interestingly, Arain et al. had already reported in 1994 that some *M. tuberculosis* strains were potentially inhibited *in vitro* by the coadministration of teicoplanin with ethambutol (30). Despite the fact that the route of administration of glycopeptides restricts their use in ambulatory care, our results suggest that it might be interesting to investigate if these antibiotics might be useful for treating multidrug-resistant (MDR) and extensively drug-resistant (XDR) infections, an important issue as these strains are emerging all over the world (8).

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