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Reappraising the use of β -lactams to treat tuberculosis

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Abstract

The emergence of multidrug-resistant and extensively drug-resistant tuberculosis calls for novel approaches to treatment. Recent studies have shown that BlaC, the β -lactamase of *Mycobacterium tuberculosis*, is the major determinant of β -lactam resistance. This review invites the reader to explore evidence in order to answer the questions: can β -lactam and β -lactamase inhibitors adequately treat *M. tuberculosis* infection and are they a viable option in the management of resistant tuberculosis today?

Keywords

β -lactam; β -lactamase; ampicillin/clavulanate; clavulanic acid; ^{L,D} transpeptidases; *Mycobacterium tuberculosis*; penicillin-binding protein

Historical review

With the discovery of streptomycin by Waksman and Smith in 1943, the first naturally derived ‘antibiotic’ was introduced into clinical medicine. Streptomycin was a major breakthrough in the treatment of bacterial infections, as this discovery offered hope against pulmonary tuberculosis (TB), a major scourge of society [1]. At the same time, the penicillins, the prototype of cell wall-targeting antibiotics, were also being developed [2]. Unlike streptomycin, penicillins did not offer sustained benefit in the treatment of TB. Although early studies showed that penicillin G and several β -lactam antibiotics were able to inhibit growth of *Mycobacterium tuberculosis* cultures [3,4], their clinical effectiveness was never firmly established. Many believed that the inability of β -lactam antibiotics to reach therapeutic concentrations at the intracellular target was due to the fairly impermeable mycobacterial cell wall, and their lack of effect on slowly dividing bacteria would make them unsuitable for effective treatment. Subsequently, *M. tuberculosis* was also found to have a penicillinase, with the capacity to effectively hydrolyze penicillins [5]. This was

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followed years later by some clinical reports and small case series that used amoxicillin and clavulanate, a β -lactam and β -lactamase inhibitor combination, as an adjunct to treat resistant TB [6,7]. More recently, the growing concern about drug-resistant TB has matched up with advances in the field of β -lactamase research and drug development to revisit the question: can the combination of β -lactam and β -lactamase inhibitors effectively treat *M. tuberculosis*. The following discussion sections provide information about why β -lactams should work in *M. tuberculosis*, why they may not work, the identified culprit, the currently available solutions and options for the future.

Mechanism of action of β -lactam antibiotics: cell wall synthesis, penicillin-binding proteins & alternative L_D transpeptidases

The mycobacterial cell wall is far more complex than that of other bacteria and consists of several layers [8]. The basic structure of it is represented in Figure 1. The most distinct feature is the double layer of lipids: the outer layer contains a complex array of proteins and saccharides [9], and among them, trehalosedimycolates, formerly known as ‘cord factor’, are present. This structure is noncovalently attached to an inner layer of mycolic acids, which give the cell wall its thickness and are responsible for the distinct ‘acid-fast’ staining pattern. Mycolic acids are anchored to a layer of the mixed polysaccharide arabinogalactan, which is covalently attached to the peptidoglycan, the principal cell wall component common to most bacteria. In mycobacteria, peptidoglycan is a complex and intricate mesh of glycan strands, cross-linked by short peptides. Cross-linking of peptidoglycan is performed by the D,D -transpeptidases, which cleave the D -Ala⁴- D -Ala⁵ of a pentapeptide donor and link the carbonyl of D -Ala⁴ to the side chain amine at the third position of an acceptor stem (4 $\rightarrow$ 3 cross-links). β -lactams resemble the distinctive D -Ala- D -Ala C-terminal dipeptide of the stem pentapeptide and are therefore recognized as a suicide substrate by the cross-linking enzymes, the penicillin-binding proteins (PBPs) [10]. PBPs are globular proteins located on the exterior of the plasma membrane. According to convention, PBPs are categorized into high- and low-molecular weight PBPs, the former being the major target of penicillins. Four PBPs were identified in *M. tuberculosis*, and it was shown that β -lactam binding to three high-molecular weight PBPs is necessary to exhibit an antibacterial effect [11].

Subsequently, several groups discovered that some bacteria are able to synthesize an alternative form of murein, which consists of glycan strands cross-linked in a nonclassical 3 $\rightarrow$ 3 fashion; among them are *Mycobacterium smegmatis* and *M. tuberculosis* bacillus Calmette–Guérin Pasteur strain (see [12,13] for review). This alternative cross-linking is catalyzed by L_D transpeptidases, which are resistant to inactivation by penicillins [14,15]. The dual nature of the peptidoglycan synthesis machinery has gained recent attention in *M. tuberculosis*: The nonclassically cross-linked peptidoglycan was found to be the predominant form in nondividing cells (‘persistors’) of *M. tuberculosis* [16]. Five paralogues of L_D transpeptidase were subsequently identified in *M. tuberculosis*, and two of them were further characterized: Ldt_{MT1} was found to be upregulated during the dormant state [17], which is in accordance with the abovementioned observation. Ldt_{MT2} was found to play a major role in sustained growth, as well as in the persistence of infection in a mouse model. Furthermore, the Ldt_{MT2}-defect strains exhibited enhanced susceptibility to amoxicillin in the presence of clavulanate. This highlights that the L_D transpeptidase and PBP activities act synergistically in synthesis and maintenance of the peptidoglycan layer, especially in the nondividing state [18]. As detailed later, this has important implications for the use of β -lactam antibiotics and opens a novel target for drug development.

Biochemical characterization of β -lactam resistance mechanisms in *M. tuberculosis*

The ability to traverse the complex layers of the *M. tuberculosis* cell wall is one of the major prerequisites for agents to be effective against this pathogen. More than being a passive diffusion barrier, this cell wall contains an active 'traffic control system', which consists of porin proteins and efflux pumps that are regulated on a transcriptional level [19]. To illustrate, even isoniazid, a small nonpolar molecule at physiologic pH that migrates readily through lipid layers of *M. tuberculosis* [20], is subject to active efflux transport (e.g., the *iniA* gene participates in the development of tolerance to isoniazid and encodes an efflux pump component) [21]. This also applies to the β -lactams; they must traverse the complex lipid bilayer. However, as of today, the cell wall compounds of *M. tuberculosis* that determine their permeability are not fully characterized. In *M. smegmatis*, for example, the deletion of the porin MspA was associated with a 16-fold increase in ampicillin resistance [22].

Yet, resistance to β -lactam combinations can arise by multiple mechanisms, starting from the entry into the cell. In order to be effective, β -lactam antibiotics have to reach a therapeutic concentration at the infected tissue (i.e., the lung), penetrate monocyte cell membranes to reach intracellular mycobacteria in the early phase of the infection, penetrate mycobacterial cell walls, resist degradation by β -lactamase enzymes and exert their inhibitory effect on the target protein, the PBPs, while maintaining clinically effective concentrations.

Which of these is the main factor contributing to resistance? A systematic analysis of the contribution of all of these five factors was conducted by Chambers *et al.* [11]. Using quantitative cultures of *M. tuberculosis* ingested by peritoneal mouse macrophages, the penetration of amoxicillin into monocytes with and without β -lactamase inhibitor was assessed. These experiments showed that there is a significant reduction in mycobacterial burden after incubation with amoxicillin- β -lactamase inhibitor combinations. Moreover, although the permeability of the mycobacterial cell wall for β -lactams was about 100-times less compared with that of *Escherichia coli*, one should recall that similar rates of penetration are observed with *Pseudomonas aeruginosa*. In addition, mycobacteria have a longer generation time than other bacteria (8–24 h compared with 20 min, respectively), during which a significant increase in intracellular drug levels can be expected, despite diffusion limitations.

Are the PBPs of *M. tuberculosis* susceptible to β -lactam inactivation at clinically achievable concentrations? To answer this question, four PBPs of *M. tuberculosis* were purified from membrane preparations in order to determine their affinity for different β -lactams. Simultaneous binding to three PBPs (94-, 82- and 52-kDa proteins) was necessary to have an antibacterial effect; this was seen with the aminopenicillins, some cephalosporins (notably, ceftriaxone and cephaloridine) and imipenem [11]. On the contrary, nafcillin, which has good affinity to 94- and 82-kDa proteins but not to the 52-kDa protein, and cefoperazone with good affinity to the 52-kDa PBP only, had no effect on growth. Finally, the systematic reduction in MICs during coinubation with clavulanate, which was observed for a wide array of tested penicillins and cephalosporins, demonstrated that β -lactamase activity is the major resistance determinant and that it can be overcome.

Succeeding studies identified one major β -lactamase in *M. tuberculosis*, now termed BlaC [23,24]. Earlier reports found additional enzymes in purified β -lactamase preparations of the avirulent laboratory strain H37Ra [24]. However, a BlaC deletion mutant of the virulent strain H37Rv did not show any residual β -lactamase activity [25].

Table 1 presents a summary of susceptibility data of different β -lactam antibiotics against three clinical strain collections of *M. tuberculosis*, all of which include multidrug resistant (MDR) and extensively drug-resistant (XDR) strains [11,26,27]. The data of Flores *et al.*, which show the remarkable difference in susceptibility pattern of the parent laboratory strain H37Rv and the BlaC deletion mutant, have been included [25]. Here, one sees that susceptibility testing of β -lactams against mycobacteria is far from being standardized. One particular challenge arises from the long incubation time of the assay, which is in stark contrast to the instability of several compounds in solution, for example, the carbapenems. Although there are differences in absolute MIC values among different studies, susceptibility studies uniformly show that the addition of β -lactamase inhibitors improves the activity of β -lactams. This holds true for otherwise resistant strains. In an interesting study to strengthen this point, Dincer *et al.* determined the proportion of MDR and drug-susceptible strains that would be susceptible to different β -lactam–clavulanate combinations if the breakpoints for Gram-negative bacilli were applied. The cefazolin–clavulanate combination achieved the highest susceptibility rate of 74%, followed by meropenem–clavulanate (72%), cefuroxime clavulanate (68%) and amoxicillin–clavulanate (62%) [28].

The *M. tuberculosis* β -lactamase enzyme

β -lactamases are a group of periplasmic proteins capable of hydrolyzing the β -lactam ring of penicillins, cephalosporins and, to some extent, carbapenems. Figure 2 shows a typical two-step hydrolysis reaction. After initial substrate binding, the highly constrained four-membered β -lactam ring is subjected to a nucleophilic attack by the hydroxyl group of Serine 70. This attack results in a covalent acyl-enzyme. In the subsequent hydrolysis reaction, the cleaved β -lactam molecule is released from the enzyme (see [29] for review).

Based on their primary sequences, β -lactamases can be grouped into four classes: A–D (we will use the Ambler classification system in this discussion) [30]. Class A β -lactamases are serine-hydrolases that are usually susceptible to the clinically used β -lactamase inhibitors (clavulanate, tazobactam, and sulbactam). Typical members of this class are the TEM and SHV enzymes found in *E. coli* and *Klebsiella* spp., respectively. Clinically important members of class A that have challenged our β -lactam armamentarium are the extended spectrum β -lactamases and the carbapenemases such as KPC [31–34]. Class B enzymes are metallo-enzymes with a very broad substrate (β -lactam) profile, and they currently pose a major threat to the use of all β -lactam antibiotics. Examples are VIM and the recently emerged NDM enzyme [35–37]. Class C β -lactamases are primarily cephalosporinases; representatives are the AmpC of *Pseudomonas aeruginosa* and P99 of *Enterobacter* spp. Class C enzymes are not usually susceptible to inhibitors [38]. Class D β -lactamases are serine oxacillinases that are found principally in *P. aeruginosa* and *Acinetobacter baumannii*. Oxacillinases demonstrate a variety of resistance profiles, including extended spectrum β -lactamase and carbapenemase substrate profiles, and are usually resistant to β -lactamase inhibitors [39].

BlaC (Figure 3), the β -lactamase of *M. tuberculosis*, belongs to Ambler class A and is accordingly susceptible to inactivation by the commercially available β -lactamase inhibitors. Wang *et al.* provided one of the first systematic biochemical and structural characterizations of BlaC [40]. BlaC shares a 40% sequence homology with other known class A enzymes and displays highly conserved motifs. However, there are some notable differences: although BlaC has a broad substrate profile, including penicillins and cephalosporins, the overall catalytic activity ($k_{\text{cat}}/K_{\text{m}}$) is rather low compared with SHV-1 or TEM-1 [41,42]. An examination of the crystal structure of BlaC reveals a wider binding pocket, which is able to accommodate different substrate side chains. Interestingly, β -lactamase inhibitors

such as clavulanic acid are also less effective against BlaC compared with their efficacy against SHV-1 or TEM-1 [41,42].

Available inhibitors & their clinical use

As detailed earlier, effective inhibition of BlaC is crucial for the clinical implementation of β -lactams against *M. tuberculosis* infection. Currently available β -lactamase inhibitors interact with class A enzymes as if they were substrates; hence, they are called 'suicide inhibitors'. This 'molecular mimicry' leads to a stable, covalently bound acyl enzyme, followed by subsequent reactive intermediates, which occupy the active center for a prolonged period of time (e.g., clavulanate) (Figure 4). The mechanism of inhibition of BlaC by clavulanate, the most effective available inhibitor, was studied in detail [43,44]. Following substrate-binding and formation of the acyl-enzyme, which results in opening of the β -lactam ring, the cyclic enol group functions as a strong leaving group, which leads to second ring opening and formation of reactive imine intermediate. The reactive imine rearranges to form an enamine, which undergoes decarboxylation to form an aldehyde and hydrated aldehyde (as demonstrated in [21,45]).

Carbapenems are among the most potent β -lactam antibiotics that resist hydrolysis by class A β -lactamases. Biochemically, carbapenems are zwitter ionic compounds that act as 'slow substrates' with a rapid acylation step, followed by a very slow deacylation (in some ways, very similar to a β -lactamase inhibitor). The formation of the acyl enzyme with opening of the β -lactam ring allows for a tautomeric change, which leads to impairment of the second hydrolysis step necessary for release of the product and liberation of the free enzyme [45–47]. This was demonstrated for the interaction of BlaC with meropenem [27], doripenem and ertapenem [48].

The combination of clavulanic acid and meropenem was found to be very effective against cultures of clinical isolates, including XDR strains [27,48]. This held true even in bacteriostatic culture conditions, which are thought to resemble the dormant state of latent TB. As mentioned earlier, the nonclassically cross-linked peptidoglycan predominates in nondividing bacteria. Therefore, the sustained efficacy may involve inactivation of the earlier-mentioned L_D transpeptidase enzymes, which are not inactivated by β -lactams with the notable exception of carbapenems [14,16,49].

Despite these very encouraging experimental data, there are only few clinical reports available. As mentioned at the beginning, clinicians have used amoxicillin–clavulanate as an adjunct in the treatment of resistant TB [6]; although one study demonstrated early bactericidal activity of this combination in patients with sputum-positive TB [50], this was not confirmed by a second study [51]. These inconsistent results may partly be explained by different dosing protocols, which highlight the importance of pharmacokinetic aspects of β -lactam therapy. Chambers *et al.* described a clinical case series of ten patients with MDR-TB, who received a combination that included imipenem for over a period of 4–9 months. Negative cultures were achieved in eight patients during therapy, and seven remained culture-negative off-treatment. Of note, the isolate of one nonresponder was found to have acquired resistance to imipenem [52]. Following the report that XDR strains are susceptible to the combination of meropenem and clavulanate [27], a clinical case report proved this combination to be effective as part of a multidrug regimen [53].

Clinical challenge of drug-resistant TB

With the development of XDR- and MDR-TB, new targets for development of effective therapy are urgently sought. Novel β -lactamase and β -lactam combinations were recently reconsidered as potential treatment options, and the limited clinical reports of their use are

promising. However, many challenges and unanswered questions remain. Carbapenems, the most active agents, are currently only available as injectable antibiotics. Although optimal drug target levels are not known, the rapid clearance demands frequent administration. Ertapenem has the longest half-life among clinically available carbapenems and may therefore be an attractive choice. However, in a recent mouse-model, which compared the efficacy of several carbapenem–clavulanate combinations against infection with a standard laboratory strain (H37Rv), ertapenem–clavulanate was inferior to imipenem–clavulanate and meropenem–clavulanate in both lowering MICs into the therapeutic range and outcome [54]. While the optimal duration of treatment is not known, we suggest that that treatment would require several months. As a result, the skills and resources limited to centers that are specialized in the treatment of MDR and XDR TB are needed. The historic lesson of TB treatment is the following: *M. tuberculosis* will eventually develop resistance to β -lactam and β -lactamase inhibitor combinations, and maybe even to carbapenems. Potential mechanisms responsible for this are plenty, beginning with impaired permeability or active efflux, alteration in PBPs, upregulation or structural alteration of BlaC and upregulation of yet-uncharacterized mechanisms involving the cell wall architecture. And because current β -lactams have a broad range of activity, resistance development in other bacteria may become a major problem.

We believe that these challenges are not insurmountable: The time has come for well-controlled prospective clinical trials to determine optimal combinations of currently available carbapenems and β -lactam inhibitors to define their role in a multidrug regimen, their dosing and duration of therapy, and to monitor for side effects that may arise during prolonged treatment courses. Accumulating clinical data may serve as an impetus to develop new agents with improved pharmacokinetic properties that target specifically the β -lactamase BlaC, the PBPs and the L,D transpeptidases of *M. tuberculosis*.

Expert commentary

The challenge of treating MDR- and XDR-TB is ever present and is likely to accelerate as the population of immune-compromised patients increases. The armamentarium of available therapies for MDR- and XDR-TB includes drugs that have particular limitations, especially in patients who have failed chemotherapy due to drug resistance or toxicities. β -lactam– β -lactamase inhibitor combinations present an unexplored and attractive opportunity. On a mechanistic basis, the inhibition of the β -lactamase of TB and the inactivation of the mycobacterial PBPs allow the clinician to use a treatment modality that possesses the potential to synergize with other drugs and to produce more permanent cures. The advances in protein crystallography allow for the opportunity to design even more effective β -lactamase inhibitors.

Five-year view

In five years, clinicians will be completing clinical trials and implementing β -lactam and β -lactamase inhibitor therapy against pulmonary TB. The primary use will be part of combination chemotherapy. We also anticipate that novel and more potent β -lactam and β -lactamase inhibitor combination will be tried. An attractive use of β -lactam and β -lactamase inhibitor therapy will be cases that have proven refractory to standard chemotherapy.

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Key issues

- New treatments effective against multidrug-resistant and extensively drug-resistant tuberculosis are urgently sought.
- The β -lactamase of *Mycobacterium tuberculosis*, BlaC, has been identified as the major resistance determinant against β -lactam antibiotics, and effective inhibition restores susceptibility.
- The combination of carbapenems and clavulanate was found to be effective against otherwise pan-resistant strains of *M. tuberculosis in vitro*.
- Clinical trials are needed to define optimal dosing regimens and to determine clinical effectiveness.
- The recent advances in our understanding of β -lactam resistance on the molecular level allows for the design of even more effective β -lactamase inhibitors.

Figure 1. Mycobacterial cell wall
PBP: Penicillin-binding protein.

Figure 2. Hydrolysis reaction of a β -lactam

Note the stepwise mechanism with formation of a covalent acyl-enzyme followed by hydrolysis. [TS1] and [TS2] refer to high-energetic transitional states. The β -lactam ring is highlighted in red.

Figure 3. Crystal structure of the BlaC β -lactamase
(A) BlaC with a meropenem molecule trapped in the active center (PDB3 DWZ). (B) BlaC with meropenem (close up): The β -lactam ring has opened to form an acyl-enzyme (the green residue is Ser 70).

Figure 4. β -lactamase inhibition by mechanism-based inhibitors, exemplified by clavulanate
Effective inhibition starts with binding of clavulanate as a substrate and continues through formation of the acyl-enzyme, which undergoes subsequent rearrangements (highlighted in blue) that lead to a stable form of the acyl-enzyme.

Table 1

Available MIC data for clinical isolates of *Mycobacterium tuberculosis*, as well as of the laboratory strain H37Rv and its BlaC deletion mutant for several β -lactams.

β -lactam	MDR and susceptible strains (total 35) [55]		Five MDR isolates[11]		13 XDR strains [27]		Laboratory strain and BlaC deletion [25]	
	- clav.	+ clav. (2:1)	- clav.	+ clav. (8 μ g/ml)	- clav.	+ clav. (2.5 μ g/ml)	H37Rv	BlaC deletion
Ampicillin			16		> 10	1.25 to >10		
Amoxicillin	64 to >256	16–128	16	2	10	0.32–10	>256	16
Carbenicillin	128 to >256	8–64					>960	60
Nafcillin			>16	>32				
Cephalothin			>16		5 to>10	0.47–3.75		
Cefoxitin			>16	4–8			960	30
Cefotaxime	16–128	4–32						
Ceftriaxone	64–256	2–32	16	2–4			480	60
Cefoperazone			>16					
Imipenem			2–4		2.5 to >10	0.32–10		
Meropenem					1.25–7.5	0.23–1.25		
Aztreonam	>64	>64						

All MIC values are in μ g/ml. Note the change in MICs in presence of clavulanate (+ clav., concentration in parenthesis), clav.: Clavulanate; MDR: Multidrug-resistant; XDR: Extensively drug-resistant.