International Journal of Antimicrobial Agents 49 (2017) 302–307

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Activity of moxifloxacin and linezolid against *Mycobacterium tuberculosis* in combination with potentiator drugs verapamil, timcodar, colistin and SQ109

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ARTICLE INFO

Article history:
Received 4 July 2016
Accepted 25 November 2016

Keywords:
Mycobacterium tuberculosis
Moxifloxacin
Linezolid
Verapamil
Colistin
SQ109

ABSTRACT

Current treatment for tuberculosis (TB) is complicated by the emergence of multidrug resistant TB (MDR-TB). As a result, there is an urgent need for new powerful anti-TB regimens and novel strategies. In this study, we aimed to potentiate a moxifloxacin + linezolid backbone as treatment for MDR-TB with the efflux pump inhibitors verapamil and timcodar as well as with drugs that act on mycobacterial cell wall stability such as colistin and SQ109. Using a time–kill kinetics assay, the activities of moxifloxacin, linezolid, verapamil, timcodar, colistin and SQ109 as single drugs against *Mycobacterium tuberculosis* were evaluated. In addition, the activity of the moxifloxacin + linezolid backbone in combination with one of the potentiator drugs was assessed. As little as 0.125 mg/L moxifloxacin achieved 99% killing of *M. tuberculosis* after 6 days of exposure. Linezolid showed moderate killing but 99% killing was not achieved. Verapamil, timcodar and colistin only resulted in killing with the highest concentrations tested but 99% killing was not achieved. SQ109 resulted in complete elimination after 1 day of exposure to 256 mg/L and in 99% elimination after 6 days of exposure to 1 mg/L. Furthermore, colistin added to the moxifloxacin + linezolid backbone resulted in increased elimination, whereas verapamil, timcodar and SQ109 showed no added value to the backbone. This finding that colistin potentiates the activity of the moxifloxacin + linezolid backbone against *M. tuberculosis* suggests its potential role in further studies on the applicability of a moxifloxacin + linezolid treatment of MDR-TB.

1. Introduction

Tuberculosis (TB) is the leading cause of death due to an infectious disease worldwide [1,2]. Treatment of this dreadful disease is complicated by the emergence of multidrug-resistant TB (MDR-TB), occurring in approximately half a million new TB cases annually [2]. The current treatment of MDR-TB is long and complex, with several drugs used in combination, resulting in patient non-compliance, inadequate response in 50% of patients, and the emergence of further drug resistance [2]. Therefore, every effort should be made to improve MDR-TB treatment.

Moxifloxacin, one of the most potent fluoroquinolones, is the cornerstone of MDR-TB treatment in many countries, together with levofloxacin in others. In addition, linezolid, one of the oxazolidinones, shows excellent efficacy but also toxicity in MDR-TB treatment [3]. The potential of oxazolidinones is underlined by the ongoing development of structural analogues to linezolid as anti-TB drugs [4,5].

Regarding adjuvant strategies in MDR-TB treatment, accumulating evidence shows that there is a significant role for mycobacterial efflux pumps in the extrusion of anti-TB drugs and the emergence of resistance towards these drugs [6], including fluoroquinolones and oxazolidinones [7]. Inactivation of these mycobacterial efflux pumps with efflux pump inhibitors (EPIs) may increase the intrabacterial concentration of anti-TB drugs, thereby increasing efficacy while avoiding high systemic exposures associated with adverse effects. This concept may even prevent or reverse resistance [8,9]. Recently, a combination of moxifloxacin, linezolid and the EPI thioridazine resulted in a favourable response in patients with extensively-drug resistant TB (XDR-TB), suggesting the potential of EPIs as treatment adjuncts for the treatment of *M. tuberculosis* infections [10].

Verapamil is a calcium channel antagonist. It is recognised to inhibit bacterial efflux pumps and has been shown to accelerate mycobacterial killing in mice infected with *M. tuberculosis* and to decrease relapse rates with shortened treatment regimens [11–13].

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http://dx.doi.org/10.1016/j.ijantimicag.2016.11.027
0924-8579/

It also reduced the mycobacterial drug tolerance induced in the intracellular department in macrophages as well as in zebrafish granuloma-like lesions [14].

Timcodar has also been described as a bacterial EPI [15]. Timcodar, used as a single agent, has a moderate effect *in vitro* against *M. tuberculosis* in liquid cultures [16]. However, this effect is more pronounced in an *M. tuberculosis*-infected macrophage assay [16]. In addition, timcodar added to rifampicin consistently improved the clearance of *M. tuberculosis* in infected mice [16].

A different strategy to increase intrabacterial drug concentrations and to improve the effect of anti-TB drugs is to target the stability of the mycobacterial cell wall. The old antimicrobial colistin is part of the polymyxin family acting primarily on the cell wall by causing changes in the permeability of the plasma membrane of Gram-negative bacteria [17]. Recently it has been shown that colistin potentiated the activity of isoniazid and amikacin against *M. tuberculosis in vitro* and also prevented the emergence of isoniazid resistance [18].

SQ109 is an anti-TB drug candidate that was identified by creating more potent analogues of ethambutol, but it demonstrated a different mode of action compared with ethambutol [19]. SQ109 decreases the incorporation of mycolic acids into the mycobacterial cell wall, thereby targeting the mycobacterial cell wall [20]. SQ109 is also an EPI, at least to human efflux pumps [21]. It demonstrated synergistic interactions with isoniazid and rifampicin as well as with moxifloxacin [20].

In this study, we focused on combining moxifloxacin and linezolid as a proposed fluoroquinolone/oxazolidinone backbone for the treatment of MDR-TB. We aimed to further potentiate the antimycobacterial effect of this backbone by using the potentiator drugs verapamil and timcodar as EPIs, and by colistin and SQ109, drugs that target the stability of the mycobacterial cell wall.

2. Materials and methods

2.1. Materials

Moxifloxacin, linezolid and verapamil were all purchased from Sigma Chemical Co. (St Louis, MO) and were dissolved in water. Colistin sulphate (Hospital Pharmacy, Rotterdam, The Netherlands) was dissolved in water. Timcodar (Vertex Pharmaceuticals Inc., Boston, MA) was dissolved in dimethyl sulphoxide (DMSO). SQ109 (Sequella Inc., Rockville, MD) was dissolved in water.

M. tuberculosis suspensions for the *in vitro* assays were cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 10% oleic acid–albumin–dextrose–catalase enrichment (OADC) (Baltimore Biological Laboratories, Baltimore, MD), 0.5% glycerol (Scharlau Chemie S.A., Sentmenat, Spain) and 0.02% Tween 20 (Sigma Chemical Co.) under shaking conditions at 96 rpm at 37 °C. All cultures on solid medium were grown on Middlebrook 7H10 agar (Difco Laboratories) supplemented with 10% OADC and 0.5% glycerol for 28 days at 37 °C with 5% CO_2.

2.2. Bacterial strain

The *M. tuberculosis* genotype strain Beijing VN 2002-1585 (BE-1585) with a minimum inhibitory concentration (MIC) of 0.25 mg/L for moxifloxacin and 1 mg/L for linezolid was used [22,23]. MICs were determined according to Clinical and Laboratory Standards Institute (CLSI) standards [24].

2.3. In vitro *time–kill kinetics*

The activities of moxifloxacin, linezolid, verapamil, timcodar, colistin and SQ109 were first determined as single compounds in liquid medium as described previously [25]. In short, cultures of actively replicating (exponential-phase) BE-1585 were exposed to increasing concentrations of the drugs at 37 °C at 96 rpm. Concentrations of moxifloxacin and linezolid tested were based on the maximum concentration of unbound drug that can be achieved in patients (fC_{max}). Ranges were 4× to 1/256× the fC_{max}. Samples (200 μL) were taken on days 1, 2, 3 and 6 provided that the mycobacterial suspension did not show visible aggregation. Samples were centrifuged at 14,000 × *g*, were washed with phosphate-buffered saline to avoid drug carry-over and were subcultured on drug-free solid medium. After 28 days of incubation at 37 °C with 5% CO_2, the number of CFU was counted. To assess selection of moxifloxacin-resistant or linezolid-resistant bacteria, samples were subcultured on drug-containing solid medium. The drug concentrations in the subculture plates were four-fold the critical concentrations, i.e. 2 mg/L of moxifloxacin or 8 mg/L of linezolid as reported by the CLSI [24].

Next, the time–kill kinetics of drug combinations with exponential-phase BE-1585 were determined both for moxifloxacin and linezolid as single drugs in combination with the potentiator drugs verapamil, timcodar, colistin or SQ109 and for the moxifloxacin + linezolid backbone in combination with the potentiator drugs. A concentration of moxifloxacin of 0.031 mg/L (1/64× fC_{max}) and a linezolid concentration of 0.069 mg/L (1/16× fC_{max}) were selected based on results in the single-drug experiments. These concentrations resulted in a mycobacterial load on day 6 that deviated by no more than 1.5 log compared with day 0. Similarly, the concentrations of potentiator drugs selected for these experiments resulted in a limited change in mycobacterial load on day 6 compared with day 0 in the single-drug experiments. Verapamil concentrations of 16, 64 and 256 mg/L, timcodar concentrations of 4, 16 and 64 mg/L, colistin concentrations of 16, 64 and 256 mg/L and SQ109 concentrations of 0.063, 0.25 and 1 mg/L were added to the moxifloxacin + linezolid backbone.

All time–kill kinetic assays were performed in duplicate as independent experiments. The lower limit of quantification was 5 CFU (log 0.7). Emergence of resistance to moxifloxacin or linezolid was tested by subculturing samples on drug-containing solid medium.

2.4. Endpoints for assessment of antituberculous drug combinations

The two endpoints of this experiment were drug synergy and the prevention of emergence of drug resistance. Synergistic activity of a two- or three-drug combination was determined after 6 days of exposure and was defined as a ≥100-fold (Δlog 2) decrease in mycobacterial load in the combination compared with the mycobacterial load achieved with the potentiator drug as a single agent and moxifloxacin or linezolid as a single agent in the case of a two-drug combination and compared with the backbone of moxifloxacin + linezolid in the case of a three-drug combination. The definition of synergy was also met when a two- or three-drug combination achieved mycobacterial elimination that was not achieved during exposure to moxifloxacin or linezolid as single agents or to the backbone of moxifloxacin + linezolid [8,18,26].

3. Results

3.1. Single-drug exposure of moxifloxacin and linezolid

The bactericidal activity of moxifloxacin as single-drug exposure is illustrated in Fig. 1A. Moxifloxacin showed a concentration-dependent killing against BE-1585. A concentration of 0.125 mg/L moxifloxacin was needed to achieve 99% killing. However, there appeared to be an optimum concentration (0.5 mg/L) beyond which an increase in concentration did not result in more efficient killing of *M. tuberculosis*. A concentration of 8 mg/L moxifloxacin even