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Article

Efficacy of linezolid on *Treponema pallidum,* the syphilis agent: A preclinical study

Austin M. Haynes[a,†], Lorenzo Giacani[a,b,*,†], Marti Vall Mayans[c], Maria Ubals[c,d], Carles Nieto[e], Clara Pérez-Mañá[f,g], Llorenç Quintó[h,i,1], Emily Romeis[a], Oriol Mitjà[c,j,†,*]

[a] Department of Medicine, Division of Allergy and Infectious Diseases, University of Washington, Seattle, WA, USA
[b] Department of Global Health, University of Washington, Seattle, WA, USA
[c] Fight Aids and Infectious Diseases Foundation, Hospital Universitari Germans Trias i Pujol, Barcelona, Spain
[d] Facultat de Medicina, Universitat de Barcelona, Barcelona, Spain
[e] Laboratorio Reig Jofre, Barcelona, Spain
[f] Clinical Pharmacology Unit, Hospital, Universitari Germans Trias i Pujol, Institut de Recerca Germans Trias i Pujol (HUGTiP-IGTP), Badalona, Spain
[g] Department of Pharmacology, Therapeutics and Toxicology, Universitat Autònoma de Barcelona, Barcelona, Spain
[h] Barcelona Institute for Global Health, Hospital Clínic-Universitat de Barcelona, Barcelona, Spain
[i] Manhiça Health Research Institute (CISM), Maputo, Mozambique
[j] Lihir Medical Centre-International SOS, Lihir Island, Papua New Guinea

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ABSTRACT

Background: Penicillin G, the current standard treatment for syphilis, has important drawbacks, but virtually no preclinical or clinical studies have been performed to identify viable alternatives. We tested, both *in vitro* and *in vivo,* three marketed antibiotics with adequate pharmacological properties to treat syphilis.

Methods: We used an *in vitro* culturing system of *T. pallidum* to perform drug susceptibility testing and applied quantitative PCR targeting the *tp0574* gene to measure bacterial growth. To confirm *in vivo* efficacy, fifteen rabbits were infected intradermally with *T. pallidum* at eight sites each and randomly allocated to an experimental treatment (linezolid, moxifloxacin, clofazimine) or a control arm (benzathine penicillin G [BPG], untreated). The primary outcome was treatment efficacy defined as the time to lesion healing measured from the date of treatment start. Secondary outcomes were absence of treponemes or treponemal mRNA in injection sites, absence of seroconversion, and cerebrospinal fluid (CSF) abnormalities and negative rabbit infectivity tests (RIT).

Findings: Linezolid showed *in vitro* bactericidal activity at concentrations of 0.5 μg/mL or higher. When administered orally to experimentally infected rabbits, it induced healing of early lesions at a time similar to BPG (hazard ratio 3.84; 95% CI 2.05−7.17; $p < 0.0001$ compared to untreated controls). In linezolid-treated animals, dark-field microscopy and qPCR assessment showed no presence of treponemes after day 3 post-treatment start, serologic test did not convert to positive, CSF had no abnormalities, and RIT was negative. Moxifloxacin and clofazimine failed to inhibit bacterial growth *in vitro* and could not cure the infection in the rabbit model.

Interpretation: Linezolid, a low-cost oxazolidinone, has *in vitro* and *in vivo* activity against *T. pallidum*, with efficacy similar to BPG in treating treponemal lesions in the animal model. Our findings warrant further research to assess the efficacy of linezolid as an alternative to penicillin G to treat syphilis in human clinical trials.

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Introduction

Syphilis, a multi-stage, chronic, sexually transmitted infection caused by the spirochete *Treponema pallidum* subsp. *pallidum* (*T. pallidum*) still represents a significant global health problem. The infection has been steadily resurgent in many high-income nations in

* Corresponding author.
  E-mail addresses: giacal@uw.edu (L. Giacani), omitja@flsida.org (O. Mitjà).
† These authors contributed equally
1 Please direct any inquiries to Lorenzo Giacani: Department of Medicine, Division of Allergy and Infectious Diseases, University of Washington, Seattle, WA, USA.

Research in context

Evidence before this study

In December 2020, we searched PubMed database for articles reporting on preclinical studies about antibiotics to treat syphilis. Search terms included "syphilis", "*Treponema pallidum*", "antimicrobial treatment", and "susceptibility testing". The drug susceptibility profile of *Treponema pallidum* (*T.p.*) was unknown because the microorganism could not be grown in culture until very recently. In animal models, penicillin, some cephalosporins, aztreonam, and macrolides had shown curative results, while clindamycin and ofloxacin did not cure syphilis. Strains of *T.p.* resistant to azithromycin had appeared quite rapidly after its introduction, therefore current therapeutic options for syphilis are restricted to parenteral penicillin (preferred drug) and doxycycline or ceftriaxone (second-line drugs).

Added values of this study

For the first time, we have screened multiple antibiotics to treat treponemal disease using a novel approach based on a tissue culture system that supports long-term multiplication of *T.p.* In addition, we have confirmed drug effectivity in the rabbit model, which is a mandatory step because the clinical relevance of *in vitro* susceptibility testing is uncertain. In addition, we have employed sensitive molecular methods to evaluate the burden of live treponemal cells in animals following treatment, rather than more archaic approaches that are prone to error.

Implications of all the available evidence

Our preclinical efficacy studies provide strong evidence on the clinical promise of linezolid to treat syphilis. Oral linezolid, if shown efficacious to treat neurosyphilis and ocular syphilis in human clinical trials, would overcome the need for 10-to-14 days hospital admission to using intravenous aqueous crystalline penicillin every 4 hours.

North America and Europe during the last two decades [1−4]. Globally, most syphilis cases occur in Sub-Saharan Africa, South East Asia, and South America, where congenital syphilis accounts for up to 50% of stillbirths [1−4]. In adults, the bacteria disseminates to virtually every organ and affects the patient's cardiovascular and central nervous systems (CNS) [5]. Hence, the choice of treatment for syphilis needs to consider the outstanding invasiveness of this pathogen and its ability to colonize so many microenvironments.

Parenteral penicillin G is currently the recommended treatment for syphilis [6,7]. Although the *T. pallidum* genome encodes for at least one enzyme with β-lactamase activity (i.e., the lipoprotein Tp0574), no genetic resistance to this antibiotic has ever been observed [8]. Nonetheless, the use of penicillin G has important limitations. First and foremost, because penicillin has low penetration to the CNS, [9,10] treatment of neurosyphilis and ocular syphilis requires patient admission for the administration of intravenous (IV) aqueous crystalline penicillin every four hours, for 10−14 days. Second, benzathine penicillin G (BPG), used to treat uncomplicated syphilis, must be delivered by trained personnel via an intramuscular (IM) injection. Third, penicillin-allergic patients need to be either desensitized before treatment or treated with second-line drugs [7,11]. Finally, the supply of BPG is being compromised by product shortages (currently experienced by 39 countries, according to the WHO) [12,13].

Studies to support the use of alternatives for treating syphilis are overall limited. In the preclinical setting, cephalosporins, [14−16] aztreonam, [17] and macrolides [18,19] have shown anti-syphilis activity. However, only three randomized clinical trials have compared penicillin to other antibiotics (i.e., ceftriaxone and azithromycin) [20−22]. Conversely, the efficacy of doxycycline, the second-line treatment for syphilis, is mainly supported by two prospective studies [23,24] and other small retrospective studies [25−27].

Overall, additional effort is needed to uncover alternative treatment options for syphilis that allow overcoming the limitations of penicillin G. The ideal compound to treat syphilis infections should achieve effective tissue concentrations in all anatomical regions, including the CNS, be able to be delivered orally, and not have reported shortages. In this study, we used an *in vitro* cultivation system for *T. pallidum* [28,29] and the rabbit model of syphilis to evaluate the efficacy of linezolid (a protein synthesis inhibitor), moxifloxacin (a DNA gyrase inhibitor), and clofazimine (a bacterial respiratory chain blocker) to treat syphilis, compared to penicillin G (*in vitro*) and BPG (*in vivo*) as positive controls.

Methods

Compounds selection and dosage

Candidate molecules for the treatment of syphilis were screened from FDA-approved antibiotics based on their pharmacological properties and activity against other pathogenic spirochetes. We used genome-based prediction of resistance mechanisms to choose drugs against which *T. pallidum* was unlikely to develop resistance. The dose for experiments on rabbits was established as the human equivalent systemic exposure based on bodyweight dosing. Pharmacokinetic parameters (AUC, C_{max}, and $t_{1/2}$) were obtained from the literature; [30−33] the variation in metabolic systems, including metabolic rates and enzymatic inhibition, were also considered.

In vitro *assessments*

The SS14 strain of *T. pallidum* used to inoculate culture plates was obtained from a frozen stock of treponemes propagated intratesticularly in New Zealand White (NZW) rabbits [34]. We tailored the culturing system of *T. pallidum* [28] to perform the susceptibility tests in our lab (Supplementary Methods, Appendix). Briefly, treponemes were sub-cultured into three 96-well cell culture plates to allow a total of nine replicates for each antibiotic concentration to be tested.

Tissue-culture grade antibiotics were obtained from Sigma-Aldrich (St. Louis, MO). Linezolid and clofazimine were solubilized in DMSO, while moxifloxacin and penicillin G were dissolved in sterile water. Final DMSO concentration in the wells was 1% (v/v). Wells without antibiotic and wells containing only solvent were included as negative controls. After seeding the treponemes and adding the antibiotics, the culture plates were incubated at 34°C for seven days in the microaerophilic incubator. Two of the three plates were then used to determine treponemal growth after a week of exposure to each antibiotic concentration, while one of the three plates was used to reseed another plate containing antibiotic-free media to assess the viability of treponemes and confirm treponemocidal activity of the tested drugs. The plates were processed for DNA extraction using a Quick DNA-96 kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol, and we used a qPCR approach targeting the *tp0574* gene [34].

Rabbit infection and treatment

Adult male NZW rabbits, an established animal model for syphilis [35,36], were used for experimental infections for antibiotic testing (Appendix).

For evaluation of antibiotic efficacy, 15 animals were infected intradermally on the same day in eight sites/animal on their shaved backs. One million (10^6) viable *T. pallidum* cells were inoculated at

each injection site. Following intradermal inoculation, animals were randomly assigned to one of the five treatment arms, including three experimental arms (groups 1-3) and two control arms (groups 4-5). Group 1 received oral linezolid (75 mg/Kg) every 8 h for 5 days; group 2 received oral moxifloxacin (40 mg/Kg) every 12 h for 5 days, group 3 received oral clofazimine (75 mg/Kg) once daily for three days, followed by a reduced dose of 25 mg/Kg once daily for four additional days. Group 4 (positive control) received a single IM injection of BPG (200,000 units, equivalent w/w to 2.4 million units for humans), while group 5 (negative control) was left untreated. Rabbits belonging to different treatment groups were housed in different racks in the room to avoid confounders. Study arms were blinded to experimenters and analysts. Antibiotic treatment was initiated when at least two injection sites per animal were found to harbour treponemes upon analysis of needle aspirates by Dark Field Microscopy (DFM).

Ethics

Animal care was provided in accordance with the Guide for the Care and Use of Laboratory Animals, and all experimental procedures were conducted under protocol #4243-01 (PI: Lorenzo Giacani), approved by the University of Washington (UW) IACUC. All animal procedures were conducted in compliance with the ARRIVE guidelines. Details on animal care are provided in Appendix 1.

Clinical monitoring and sample collection

Intradermally-infected rabbits were shaved daily until day 40 post-inoculation to allow monitoring of lesion progression and facilitate collection of needle aspirates to evaluate treponemal burden by DFM and collection of lesion biopsies to evaluate burden by qPCR. In all animals, challenge sites were monitored by recording lesion progression and healing. Both the diameter of induration or ulceration and the diameter of erythema were measured using a calliper every day for 40 days post-intradermal inoculation. Lesion aspirates and lesion biopsies were collected at days 3, 8, 12, and 16 post-treatment initiation. Aspirates were examined by DFM and *T. pallidum* cells were counted blindly in a total of 200 fields. Biopsies were collected, minced using sterile scalpel and forceps, resuspended in 400 μL of TRIzol reagent, used for RNA extraction, and tested by qPCR targeting the *tp0574* mRNA [34,37].

Serum was also obtained from each animal every week to perform Venereal Disease Research laboratory (VDRL) and fluorescent treponemal antibody absorption (FTA-ABS) tests to monitor the development of humoral immunity in response to infection. VDRL data were collected for each week, while FTA-ABS tests were performed only up to when sera became reactive. Nine weeks post-treatment initiation, animals underwent spinal tap to obtain CSF and were subsequently euthanized. Approximately 900 μL of CSF were sent to Phoenix Laboratories (Mukilteo, WA) to be evaluated for red blood cell contamination, presence of nucleated cells, glucose, and total protein, while 100 μL were used to perform CSF-VDRL in our laboratory.

To conduct the rabbit infectivity test (RIT), naïve recipient rabbits were injected intratesticularly with minced popliteal lymph nodes from untreated, BPG-treated and linezolid-treated donor rabbits. Serum was collected monthly to assess seroconversion of these recipient animals via VDRL testing (Appendix 1).

Statistics

Results regarding the molecular assessment of *T. pallidum in vitro* growth were described as the mean and standard error (SE). Differences in growth between no treatment wells and each antibiotic dose were compared using one-way ANOVA.

For the *in vivo* study, no sample size estimate was conducted because this was a proof-of-concept study. A sample size of 12 in each arm should suffice for pilot studies; [38] our sample size amounted to 120 lesions (15 rabbits, 8 lesions each). The effect of treatment on rabbits was described using the mean and SE of the diameter of the lesions and the *T. pallidum* lesion burden, determined by DFM analysis and by qPCR. The primary endpoint for the *in vivo* assessment was treatment efficacy, defined as the time to disease-free event (lesion healing), measured from the date of treatment start (day 7) or the day of the appearance of a skin lesion (whatever occurred last) to lesion healing. The primary analysis was performed for two definitions of lesions: (1) lesions that were either indurated or ulcerated, with a measurable diameter, and (2) lesions in the broadest sense, including also erythema observed in the days preceding or following induration/ulceration. The cumulative incidence of treatment success among treatment groups was estimated using Fine and Gray competing risk regression models for clustered data and considering biopsies as the competing events; results were reported as the Sub-Hazard Ratios (HR) and their 95% Confidence Intervals (CI) [39,40]. The equivalence of the cumulative incidence functions between treatment groups was assessed by the weighted log-rank test [39]. The significance threshold was set at a two-sided alpha value of 0.05 for all analyses. Descriptive and comparative analyses were performed using Prism 8 (GraphPad Software, San Diego, CA), whereas time-to-event analyses were performed using Stata version 16 (StataCorp. 2019. Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC).

Role of funders

The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit it for publication.

Results

Antimicrobial drug selection

Based on the screening of antimicrobial drugs, we identified two drugs, linezolid [41] and moxifloxacin [42] that met the predefined pharmacokinetic criteria [43] (Table S2, Appendix) and were active against other spirochetes [44−46]. CNS penetration of linezolid (75%) and moxifloxacin (45%) is higher than penicillin G (5%) [22]. The possibility of linezolid resistance occurring as a result of point mutation of 23S ribosomal RNA in *T. pallidum* was deemed to warrant consideration [47]. *T. pallidum* lacked putative genes encoding for intrinsic resistance mechanisms for moxifloxacin. We also included clofazimine [48], despite its inability to cross the blood-brain barrier, because of its long half-life, low cost, and strong safety profile [49]. Additional drugs that were not prioritized will be tested in a second round of experiments, including cefixime, isoniazid, pyrazinamide, dalbavancin, and zoliflodacin.

In-vitro effects

Based on clinically relevant concentrations of antibiotics (i.e., maximum plasma concentration achievable in humans), we tested the anti-treponemal activity of linezolid at a concentration range of 0.25 to 4.0 μg/mL, and clofazimine and moxifloxacin at 0.06 to 2.0 μg/mL. Penicillin G was tested at a concentration range of 0.1 to 60.ng/mL (Table S1).

Linezolid inhibited treponemal replication at all concentrations tested, except 0.25 μg/mL (Fig. 1a). Clofazimine failed to completely inhibit treponemal replication at all concentrations tested, except 2.0
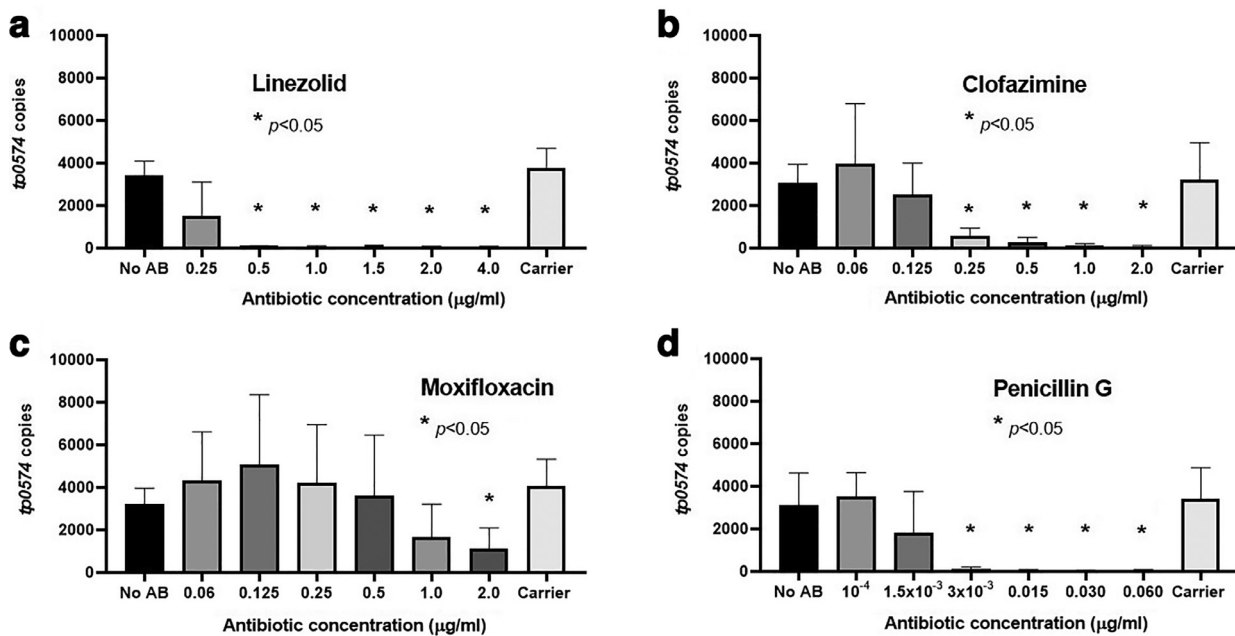
Fig. 1. qPCR amplification of the *tp0574* gene in treated and control treponema cultures.

Sample size was 192 wells distributed in four antibiotic groups, each group having eight samples (with six different drug concentrations, and two controls − no antibiotic and no solvent), and each sample having six biological replicates (Table S1). Following DNA extraction from each replicate, treponemal burden was evaluated for each replicate in triplicate using a qPCR approach targeting the *tp0574* gene to obtain a total of 18 data points (6 replicates x 3 qPCR reading) for each antibiotic concentration tested. An absolute quantification protocol using an external standard was used to quantify the *tp0574* gene copy number at the time of sample harvest.

Bar height is the mean of 18 data points for each antibiotic concentration tested, and error bars show the standard error. Asterisks indicates significance ($p \leq 0.05$) calculated using one-way ANOVA in comparison to the No AB (No antibiotic) data. Carrier indicate wells containing antibiotic carrier only (water or DMSO). Panels **a-d** are Linezolid, Clofazimine, Moxifloxacin, and Penicillin G, respectively.

μg/mL, although doses from 0.25 to 1.0 μg/mL were associated with a significantly lower growth than untreated wells (Fig. 1b). Moxifloxacin only showed capacity to significantly reduce growth compared with untreated wells at 2.0 μg/mL (Fig. 1c), whereas penicillin G significantly inhibited growth at concentrations of 0.003 μg/mL (approximately 0.06 U/mL) or higher (Fig. 1d).

After re-seeding treated treponemes into an antibiotic-free 96-well plate, treponemes from wells without antibiotic grew but those that had been exposed to either penicillin G or linezolid at any concentration did not, indicating a bactericidal effect (Fig. S1). Growth after re-seeding was observed in all treponemes that had been treated with moxifloxacin and those treated with clofazimine at a concentration of 0.125 μg/mL or lower.

Treatment effects on the animal model

After intradermal infection with the treponemal inoculum of 15 rabbits, all animals developed clinical signs of a lesion in each of their eight challenge sites; pre-treatment needle aspirates on day 7 showed the presence of treponemes. The mean lesion diameter of rabbits treated with BPG and linezolid decreased from one and two days after treatment start, respectively (Fig. 2a); no induration could be measured in the linezolid group at day 12 (or later) post-treatment initiation. Macroscopically, there were no differences between BPG- and linezolid-treated animals at day 15 post-treatment (Fig. 2b). Lesions of untreated rabbits and those treated with moxifloxacin and clofazimine progressively increased along the week following treatment start. In clofazimine-treated animals, the mean lesion diameter was significantly smaller than that of untreated controls between days 17−26 post-infection (Fig. 2a). Moxifloxacin-treated animals ulcerated and presented a lesion size similar to untreated controls over the study period.

In the primary analysis for time to lesion healing using a restrictive definition of the lesion (i.e., indurated or ulcerated, with a measurable diameter), treatments with BPG and linezolid

were significantly associated with a shorter time to healing compared with untreated rabbits (Table S3, Figure S2): HR for healing was 5.94 (95% CI 2.92 − 12.08) and 3.84 (2.05 − 7.17) for BPG and linezolid, respectively ($p < 0.001$ for both molecules). This effect was observed for neither moxifloxacin (HR 1.19 [95% CI 0.97 − 1.46]; $p = 0.102$) nor clofazimine (0.90 [0.78 − 1.05]; $p = 0.198$). The corresponding analysis based on the expanded definition of lesion (i.e., including erythema) showed a similar trend, with HRs of 10.60 (95% CI 3.75 − 29.94) and 3.60 (2.03 − 6.41) for BPG and linezolid, respectively ($p < 0.001$ for both molecules), and 1.05 (0.89 − 1.24; $p = 0.563$) and 0.97 (0.79 − 1.19; $p = 0.796$) for moxifloxacin and clofazimine, respectively.

Dark-field microscopy assessment showed absence of treponemes in lesion aspirates from animals treated with BPG at all time-points; treponemes could be found in animals in the linezolid group at day 3 post-treatment start, but not after this time point (Fig. 3a). The quantification of the *tp0574* mRNA of *T. pallidum* in lesion biopsies, used as a surrogate of the burden of viable treponemal cells, showed no evidence of treponemal growth at any time point in animals treated with BPG. In animals treated with linezolid, treponemal growth was only identified at day 3 post-treatment start (Fig. 3b). Amplification of *tp0574* was detected in untreated animals and those treated with moxifloxacin and clofazimine at all time points.

Serology and CSF involvement

Serologic nontreponemal VDRL tests, performed weekly for nine weeks after treatment start, showed no seroconversion of rabbits treated with linezolid and BPG at any time point (Fig. 4). All rabbits from the remaining groups (untreated controls, and moxifloxacin- and clofazimine-treated animals) became VDRL-positive between weeks 2 and 3 post-inoculation. Similarly, linezolid- and BPG-treated animals did not become positive for the FTA-ABS test throughout the whole 9-week observation period, while sera from untreated
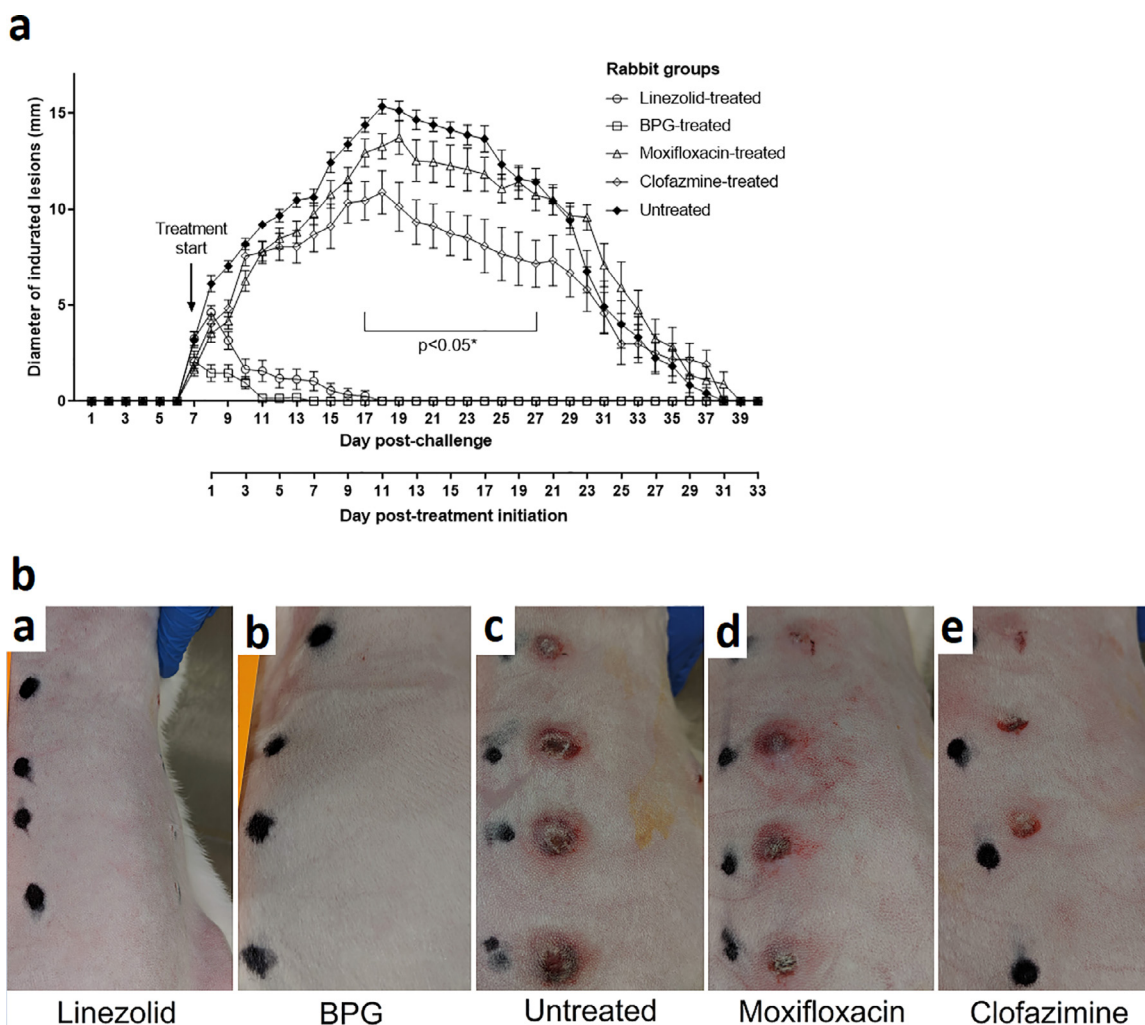
a

b

Fig. 2. Monitoring of cutaneous lesion development in experimentally infected rabbits.

Sample size was 120 lesional sites distributed in five treatment arms, each arm having three rabbits with eight injection sites each. Animals were infected by intradermal injection of *T. pallidum* (Nichols stain) at eight sites on their shaved backs. A total of 10^6 *T. pallidum* viable cells were inoculated at each challenge site.

(a) Dots represent the mean diameter (mm) of 21 indurated/ulcerated lesions (3 rabbits x 8 lesions sites) and error bars represent the standard error. The lesion diameter in clofazimine-treated animals was significantly lower ($p < 0.05$) compared to the untreated controls between day 17-27 post-infection.

(b) Pictures of the rabbit right-side backs taken at day 23 post-infection (day 16 post-treatment initiation) in treated and control animals. One rabbit per group is shown. Black dots were used to mark the skin about 2 cm below the injection site. Subpanel **a, b, d, e** are pictures taken from linezolid-, BPG-, moxifloxacin-, and clofazimine- treated animals, respectively, while subpanel **c** is a picture from one of the untreated controls. The left side of the rabbit backs is not pictured because it was used to obtain lesion biopsies for mRNA quantification studies.

controls, clofazimine- and moxifloxacin-treated animals matched the level of reactivity of the provided control sera within week 3 or 4 post-infection.

The cellular and biochemical analysis of the CSF revealed a significantly higher protein content in the CSF of untreated animals than linezolid- or BPG-treated ones (Figure S3). Rabbits treated with clofazimine and moxifloxacin had lower concentration of proteins in the CSF than controls but higher than those treated with linezolid or BPG (Fig. S3 a). No differences in nucleated cell count or CSF glucose concentration were seen across all groups (Fig. S3 b-c), and CSF-VDRL tests were negative in all animals.

In the RIT, only the recipient of the lymph nodes from the untreated control group developed orchitis and became VDRL positive (1:32) approximately a month following inoculation. None of the recipients from BPG treated and linezolid treated groups developed orchitis or seroconverted. RIT was deemed unnecessary, and hence not performed, using lymph nodes from clofazimine- and moxifloxacin-treated animals because these antibiotics were shown to be ineffective against *T. pallidum* based on lesion development, serology, and treponemal burden data.

Discussion

In this study, we sequentially used *in vitro* and *in vivo* approaches for repurposing marketed antibiotics with the potential for being a therapeutic alternative to penicillin G to treat syphilis. We sought out molecules that could be administered orally, preferentially as a short-course regimen (e.g., 5 days) to improve treatment acceptance and compliance, had no reported shortage or allergic reactions, and had reported at least *in vitro* activity against pathogenic spirochetes. Of the three selected molecules currently marketed that met the established criteria, only linezolid showed a significant *in vitro* and *in vivo* efficacy. Remarkably, the bactericidal effect of linezolid on *T. pallidum* cultures and its efficacy to cure treponemal lesions in model animals was similar to that of penicillin G. Furthermore, analyses of the lymph nodes (through RIT) and CSF of treated animals revealed the ability of linezolid to prevent treponemal dissemination to these tissues. To our knowledge, no antibiotics of the oxazolidinone class, of which linezolid is the lead compound, have been tested against *T. pallidum* in animal models or clinical studies of syphilis. Our in-silico investigation ruled out the potential of *T. pallidum* harbouring efflux
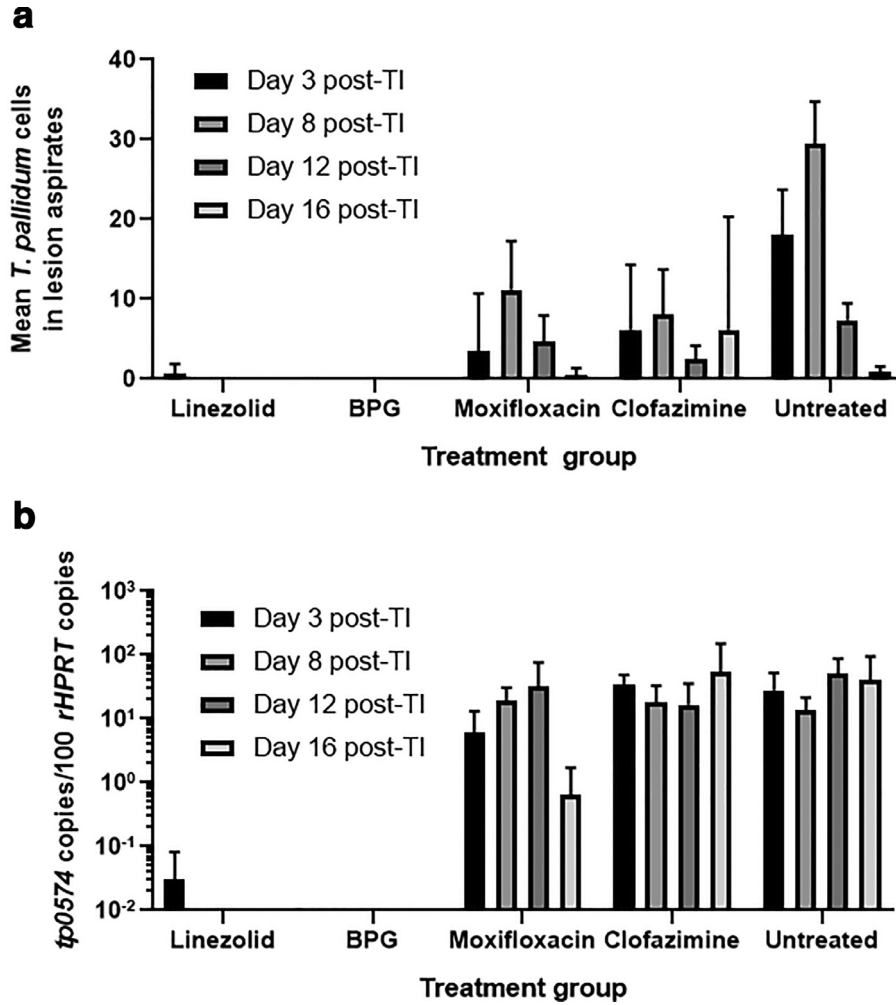
Fig. 3. Assessment of treponemal burden within lesions of experimentally infected rabbits.

Sample size was 120 lesional sites distributed in five treatment arms, each arm having three rabbits with eight injection sites each. Lesion aspirates were collected from all sites on days 3, 8, 12, and 16 post-treatment initiation.

(a) Treponemal burdens in lesions from treated and control rabbits post-infection challenge were measured by dark-field microscopy (DFM) of lesion aspirates. Bar height is the mean of 24 data points (day 3 post-TI), 21 data points (day 8 post-TI), 18 data points (day 12 post-TI), and 16 data point (day 16 post-TI) for each antibiotic tested; error bars show the standard error. The number of data points decreased over time because once an injection site is biopsied, it is no longer sampled for DFM.

(b) Treponemal burden measured by qPCR targeting *T. pallidum tp0574* gene of lesion biopsies. Message quantification was performed using a qPCR approach targeting the message for the treponemal 47 kDa lipoprotein (encoded by the *tp0574* gene) that normalizes the *tp0574* signal to the message for the rabbit hypoxanthine-guanine phosphoribosyl transferase housekeeping gene (*rHPRT*). Bar height is the mean of 3 data points for each antibiotic tested, and error bars show the standard error. One biopsy was taken for each rabbit at each time point (three rabbits each arm).
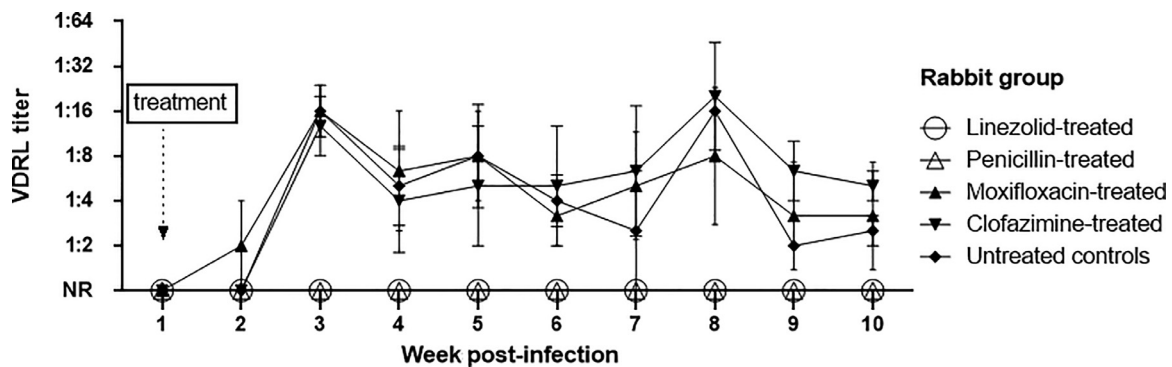

Fig. 4. VDRL titers in experimentally infected rabbits.

Sample size was 15 rabbits distributed in five treatment arms, each arm having three rabbits. Mean ($\pm$ standard error [SE]) serum VDRL titers in linezolid-, BPG-, moxifloxacin-, and clofazimine-treated rabbits, as well as in untreated controls. The data represent 3 rabbits per treatment group, and mean serum VDRL titres are shown through 10 weeks post-infection, and at least 8 weeks after initiation of treatment. For calculating mean $\pm$ SE, titres were converted to $\log_2$ with nonreactive=0; R(1:2)= 1, and R(1:4) = 2, and so on. Antilog of mean $\pm$ SE of titres are shown in this figure.

pump genes typically associated with antibiotic resistance in gram-negative bacteria. We cannot exclude that, like reported for other bacteria [47], *T. pallidum* might develop resistance to linezolid through selection of mutations in the 23S rRNA gene. To evaluate the likelihood of this event, we cultivated *T. pallidum* SS14 strain for 10 weeks in sub-therapeutic concentration of linezolid (~30 ng/mL). At the end of the experiment, we demonstrated that the linezolid minimum inhibitory concentration (MIC) was not changed in these treponemes, suggesting that no selection of a resistant strain occurred. Future work will aim at characterizing these treponemes at the molecular level and will provide a comprehensive overview of any genomic and transcriptomics changes that might have occurred during propagation in low linezolid concentration. We found that linezolid had MIC values of 0.5 μg/mL for *T. pallidum* cells. Assuming that the 24 h Area Under the Curve (AUC24) of linezolid is 155 μg $\times$ h/mL and that efficacy is driven by AUC24/MIC $>50-80$, we estimate that a single-dose can achieve effective drug concentration in the human infected tissue over 24 h; therefore, a once-a-day (QD) dosing is expected to be effective. Our in-silico analysis suggested that *T. pallidum* might also be susceptible to the fluoroquinolone moxifloxacin. However, *in vitro* and *in vivo* experiments showed a very limited capacity of the molecule to inhibit treponemal growth. Fluroquinolones target two bacterial proteins, the gyrase (GyrA) and topoisomerase IV, with the latter not being encoded by *T. pallidum* genome [50]. Hence, the selection of mutations with substantial resistance phenotypes might occur readily in this pathogen. Whether mutational alteration in target enzymes is the reason for resistance in treponema should be further investigated, particularly given that new-generation fluoroquinolones have shown good inhibitory effects on cultures of the other pathogenic spirochetes like *Borrelia sp.* [51].

Our study has some caveats intrinsically related to conducting research in syphilis. First, drug susceptibility testing in syphilis is remarkably challenged by the difficulties of propagating the infectious agent *T. pallidum* in laboratory cultures. In this regard, our adaptation of the cultivation method described by Edmonson et al., [28] which was tailored for testing antibiotic efficacy against *T. pallidum*, entails significant progress in the field. Owing to the inability to use traditional methods for estimating the MIC, we developed a qPCR-based assay in which *tp0574* gene was used to identify treponemal growth. The consistency of the DFM and qPCR findings, obtained after incubation in the presence of antibiotics and after re-seeding for an additional week-long incubation in antibiotic-free media, allowed us to confirm robustly which antibiotic concentrations were treponemicidal. The *in vitro* results were also in line with subsequent findings in model animals. Second, preclinical studies on model animals are typically limited in statistical power because of the small sample size (in our case, three rabbits per group with eight challenges site each). Furthermore, quantitative methods for measuring the clinical progression of treponemal lesions are scant. Lesion measurement was done using a calliper with an accuracy of about ±0.02 mm. Precision errors in measurement would affect equally both treatment arms because of randomization and blinding; hence, the difference between treatment arms is unlikely to be affected. In addition to the time-to-healing of the lesions, assessed by naked-eye examination, we analysed the treponemal burden by treponema cell count on DFM and transcription levels of the *tp0574* gene by qPCR. Like in the *in vitro* approach, the findings from the different approaches for measuring lesion progression were consistent regarding the ability of linezolid to cure treponemal lesions with efficacy similar to penicillin G.

In summary, the results presented in our study indicate the therapeutic potential of linezolid against syphilis and provide a significant insight for both mechanistic and translational research to establish new therapeutic approaches to halt the alarming resurgence of syphilis in many countries. Our findings warrant further research to assess the efficacy of linezolid as an alternative to penicillin G to treat syphilis in human clinical trials. Low-cost generic linezolid was added to the WHO list of prequalified medicinal products in 2018. The current price range of linezolid is 44.2 − 75.0 USD (ex-works price) for 100 tablets blister; therefore, the cost range of a once-daily 5-day regimen is 2.2 − 3.75 USD. Linezolid, as short-course chemotherapy, is overall safe and generally very well tolerated. A drawback is that it is in US-FDA pregnancy category C, meaning there have been no adequate studies of its safety when used in pregnant women. Our preclinical efficacy studies provide strong evidence on clinical promise of linezolid to treat syphilis. If shown efficacious in treating neurosyphilis and ocular syphilis it might overcome the need for 10-to-14 days hospital admission for intravenous aqueous crystalline penicillin G every 4 hours.

Contributors

L.G. and O.M. conceived of the study. C.N., C.P.M and O.M. did the literature search, drug selection, and dose calculations. L.G, A.M.H., and E. R. performed the experimental procedures, organized the data and contributed to data analysis. M.V.M., M.U.C., and LQ performed statistical analyses. All authors contributed to manuscript preparation and critically reviewed and approved the manuscript before submission.

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Data sharing statement

The data that support the findings of this study are available from the following repository at Harvard Dataverse: https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/6HE7IU

Declaration of Competing Interests

The authors have nothing to disclose.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103281.

References

[1] Gerbase AC, Rowley JT, Mertens TE. Global epidemiology of sexually transmitted diseases. Lancet 1998:351.
[2] WHO. Report on global sexually transmitted infection surveillance 2015. *WHO Doc Prod Serv Geneva, Switzerland*2016.
[3] Newman L, Rowley J, Vander Hoorn S, et al. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. PLoS One 2015;10:e0143304.
[4] Collaborators. GBD 2017 D and II and P. Global, regional, and national disability-adjusted life-years (DALYs) for 359 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990-2017: a systematic analysis for the global burden of disease study 2017. Lancet 2018;392:1859–922.
[5] Radolf JD, Lukehart SA. Pathogenic treponema: molecular and cellular biology. Norfolk, England: Caister Academic Press; 2006.

[6] Idsoe O, Guthe T, Willcox RR. Penicillin in the treatment of syphilis. The experience of three decades. Bull World Heal Organ 1972;47:1–68 **Suppl**.

[7] Workowski KA, Bolan GACDC. Sexually transmitted diseases treatment guidelines. MMWR Recomm Rep 2015.

[8] Cha JY, Ishiwata A, Mobashery S. A novel beta-lactamase activity from a penicillin-binding protein of Treponema pallidum and why syphilis is still treatable with penicillin. J Biol Chem 2004;279:14917–21.

[9] Berry CD, Hooton TM, Collier AC, Lukehart SA. Neurologic relapse after benzathine penicillin therapy for secondary syphilis in a patient with HIV infection. N Engl J Med 1987;316:1587–9.

[10] Marra CM, Critchlow CW, Hook 3rd EW, Collier AC, Lukehart SA. Cerebrospinal fluid treponemal antibodies in untreated early syphilis. Arch Neurol 1995;52:68–72.

[11] Janier M, Hegyi V, Dupin N, et al. 2014 European guideline on the management of syphilis. J Eur Acad Dermatology Venereol 2014;28:1581–93.

[12] WHO. Shortages of benzathine penicillin. How big is the problem? And why it matters. https://www.who.int/reproductivehealth/shortages-benzathine-penicillin/en/ 2019.

[13] Nurse-Findlay S, Taylor MM, Savage M, et al. Shortages of benzathine penicillin for prevention of mother-to-child transmission of syphilis: an evaluation from multi-country surveys and stakeholder interviews. PLoS Med 2017;14:e1002473.

[14] Johnson RC, Bey RF, Wolgamot SJ. Comparison of the activities of ceftriaxone and penicillin G against experimentally induced syphilis in rabbits. Antimicrob Agents Chemother 1982;21:984–9.

[15] Baker-Zander SA, Lukehart SA. Efficacy of cefmetazole in the treatment of active syphilis in the rabbit model. Antimicrob Agents Chemother 1989;33:1465–9.

[16] Korting HC, Haag R, Walter D, Riethmüller U, Meurer M. Efficacy of ceftizoxime in the treatment of incubating syphilis in rabbits. Chemotherapy 1993;39:331–5.

[17] Lukehart SA, Baker-Zander SA, Holmes KK. Efficacy of aztreonam in treatment of experimental syphilis in rabbits. Antimicrob Agents Chemother 1984;25:390–1.

[18] Lukehart SA, Fohn MJ, Baker-Zander SA. Efficacy of azithromycin for therapy of active syphilis in the rabbit model. J Antimicrob Chemother 1990(25 Suppl A):91–9.

[19] Alder J, Jarvis K, Mitten M, Shipkowitz NL, Gupta P, Clement J. Clarithromycin therapy of experimental Treponema pallidum infections in hamsters. Antimicrob Agents Chemother 1993;37:864–7.

[20] Riedner G, Rusizoka M, Todd J, et al. Single-dose azithromycin versus penicillin G benzathine for the treatment of early syphilis. N Engl J Med 2005;353:1236–44.

[21] Hook 3rd EW, Behets F, Van Damme K, et al. A phase III equivalence trial of azithromycin versus benzathine penicillin for treatment of early syphilis. J Infect Dis 2010;201:1729–35.

[22] Cao Y, Su X, Wang Q, et al. A multicenter study evaluating ceftriaxone and benzathine penicillin G as treatment agents for early syphilis in Jiangsu, China. Clin Infect Dis 2017;65:1683–8.

[23] Bolan RK, Beymer MR, Weiss RE, Flynn RP, Leibowitz AA, Klausner JD. Doxycycline prophylaxis to reduce incident syphilis among HIV-infected men who have sex with men who continue to engage in high-risk sex: a randomized, controlled pilot study. Sex Transm Dis 2015;42:98–103.

[24] Long CM, Klausner JD, Leon S, et al. Syphilis treatment and HIV infection in a population-based study of persons at high risk for sexually transmitted disease/HIV infection in Lima, Peru. Sex Transm Dis 2006;33:151–5.

[25] Antonio MB, Cuba GT, Vasconcelos RP, Alves APPS, da Silva BO, VI Avelino-Silva. Natural experiment of syphilis treatment with doxycycline or benzathine penicillin in HIV-infected patients. AIDS 2019;33:77–81.

[26] Xiao H, Liu D, Li Z, et al. Comparison of doxycycline and benzathine penicillin G for the treatment of early syphilis. Acta Dermatovenerol Croat 2017;25:107–11.

[27] Dai T, Qu R, Liu J, Zhou P, Wang Q. Efficacy of doxycycline in the treatment of syphilis. Antimicrob Agents Chemother 2017;61. doi: 10.1128/aac.01092-16.

[28] Edmonson DG, Hu B, Norris SJ. Long-term in vitro culture of the syphilis spirochete treponema pallidum subsp. pallidum. MBio 2018;9. doi: 10.1128/mBio.01153-18.

[29] Cox DL, Moeckli RA, Fieldsteel AH. Cultivation of pathogenic treponema in tissue cultures of SflEp cells. In Vitro 1984;20:879–83.

[30] Swanson R V, Adamson J, Moodley C, et al. Pharmacokinetics and pharmacodynamics of clofazimine in a mouse model of tuberculosis. Antimicrob Agents Chemother 2015;59:3042–51.

[31] Strahilevitz J, Lev A, Levi I, Fridman E, Rubinstein E. Experimental pneumococcal pleural empyema model: the effect of moxifloxacin. J Antimicrob Chemother 2003;51:665–9.

[32] Kjellsson MC, Via LE, Goh A, et al. Pharmacokinetic evaluation of the penetration of antituberculosis agents in rabbit pulmonary lesions. Antimicrob Agents Chemother 2012;56:446–57.

[33] Dailey CF, Dileto-Fang CL, Buchanan L V, et al. Efficacy of linezolid in treatment of experimental endocarditis caused by methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother 2001;45:2304–8.

[34] Giacani L, Molini B, Godornes C, et al. Quantitative analysis of tpr gene expression in Treponema pallidum isolates: differences among isolates and correlation with T-cell responsiveness in experimental syphilis. Infect Immun 2007;75:104–12.

[35] Esteves PJ, Abrantes J, Baldauf H-M, et al. The wide utility of rabbits as models of human diseases. Exp Mol Med 2018;50:1–10.

[36] Turner TB, Hollander DH. Biology of the treponematoses. Geneva: World Health Organization; 1957.

[37] Šmajs D, McKevitt M, Howell JK, et al. Transcriptome of Treponema pallidum: gene expression profile during experimental rabbit infection. J Bacteriol 2005;187:1866–74.

[38] Julious SA. Sample size of 12 per group rule of thumb for a pilot study. Pharm Stat J Appl Stat Pharm Ind 2005;4:287–91.

[39] Gray RJ. A class of K-sample tests for comparing the cumulative incidence of a competing risk. Ann Stat 1988:1141–54.

[40] Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc 1999;94:496–509.

[41] FDA. ZYVOX® (Linezolid). SmPC.

[42] FDA. AVELOX (Moxifloxacin hydrochloride). Highlights Prescr. Inf.

[43] Gilbert DN, Moellering Jr RC, Eliopoulos GM, Sande MA. The sanford guide to antimicrobial therapy. 34 Hyde Park, VT: Antimicrobial Therapy; 2004.

[44] Wuthiekanun V, Amornchai P, Langla S, et al. Antimicrobial disk susceptibility testing of leptospira spp. Using leptospira vanaporn wuthiekanun (LVW) agar. Am J Trop Med Hyg 2015;93:241–3.

[45] Dever LL, Torigian C V, Barbour AG. In vitro activities of the everninomicin SCH 27899 and other newer antimicrobial agents against Borrelia burgdorferi. Antimicrob Agents Chemother 1999;43:1773–5.

[46] Pothineni VR, Wagh D, Babar MM, et al. Identification of new drug candidates against Borrelia burgdorferi using high-throughput screening. Drug Des Devel Ther 2016;10:1307–22.

[47] Long KS, Vester B. Resistance to linezolid caused by modifications at its binding site on the ribosome. Antimicrob Agents Chemother 2012;56:603–12.

[48] FDA. LAMPRENE (Clofazimine). Highlights Prescr. Inf.

[49] Holdiness MR. Clinical pharmacokinetics of clofazimine. A review. Clin Pharmacokinet 1989;16:74–85.

[50] Fraser CM. Complete genome sequence of treponema pallidum. Science (80-) 1998;281:375.

[51] Kraiczy P, Weigand J, Wichelhaus TA, et al. In vitro activities of fluoroquinolones against the spirochete Borrelia burgdorferi. Antimicrob Agents Chemother 2001;45:2486–94.