Drug Resistance Updates 80 (2025) 101213

Contents lists available at ScienceDirect

Drug Resistance Updates

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

Sanfetrinem, an oral β-lactam antibiotic repurposed for the treatment of tuberculosis

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ARTICLE INFO

Keywords:
Sanfetrinem
Repurposing
Tuberculosis
β-lactams
Intracellular

ABSTRACT

Tuberculosis (TB) is historically the world's deadliest infectious disease. New TB drugs that can avoid pre-existing resistance are desperately needed. The β-lactams are the oldest and most widely used class of antibiotics to treat bacterial infections but, for a variety of reasons, they were largely ignored until recently as a potential treatment option for TB. Recently, a growing body of evidence indicates that later-generation carbapenems in the presence of β-lactamase inhibitors could play a role in TB treatment. However, most of these drugs can only be administered intravenously in the clinic. We performed a screening of β-lactams against intracellular *Mycobacterium tuberculosis* (*Mtb*) and identified sanfetrinem cilexetil as a promising oral β-lactam candidate. Preclinical *in vitro* and *in vivo* studies demonstrated that: (i) media composition impacts the activity of sanfetrinem against *Mtb*, being more potent in the presence of physiologically relevant cholesterol as the only carbon source, compared to the standard broth media; (ii) sanfetrinem shows broad spectrum activity against *Mtb* clinical isolates, including MDR/XDR strains; (iii) sanfetrinem is rapidly bactericidal *in vitro* against *Mtb* despite being poorly stable in the assay media; (iv) there are strong *in vitro* synergistic interactions with amoxicillin, ethambutol, rifampicin and rifapentine and, (v) sanfetrinem cilexetil is active in an *in vivo* model of infection. These data, together with robust pre-clinical and clinical studies of broad-spectrum carbapenem antibiotics carried out in the 1990s by GSK, identified sanfetrinem as having potential for treating TB and catalyzed a repurposing proof-of-concept Phase 2a clinical study (NCT05388448) in South Africa.

1. Introduction

Tuberculosis (TB) is historically the world's deadliest infectious disease and the COVID-19 pandemic has caused a reversal in the gains made in diagnosing and treating TB globally (Bagcchi, 2023). Adding to these challenges, drug resistant forms of TB continue to spread, including strains resistant to bedaquiline, a drug approved just over a decade ago that is now the backbone for most MDR treatment regimens (Ismail et al., 2022). Therefore, new TB drugs that can avoid pre-existing resistance are desperately needed to combat this deadly disease.

TB is caused by a bacterium, *Mycobacterium tuberculosis* (*Mtb*). Thus, it is at first surprising to realize that the oldest and most widely used class of antibiotics for bacterial infections, the β-lactams, was largely ignored for decades as a potential treatment option for TB (Gold et al.,

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https://doi.org/10.1016/j.drup.2025.101213
Received 17 October 2024; Received in revised form 7 February 2025; Accepted 8 February 2025
Available online 15 February 2025

2022). A multitude of reasons for this historical trend exist, including: (i) *Mtb* constitutively expresses a β-lactamase, BlaC, that efficiently hydrolyzes most β-lactam drugs rendering them inactive (Iland, 1946; Fleming, 1944; Hackbarth et al., 1997); (ii) early clinical trials that showed only modest results even in the presence of clavulanic acid (Hackbarth et al., 1997); and (iii) the fact that the most efficacious β-lactam drugs showing antitubercular activity are not orally bioavailable making them non-optimal for treating TB (Smith et al., 2018).

However, since the early 2000's, a growing body of evidence has emerged indicating that later-generation carbapenems demonstrate potent activity against *Mtb* by targeting the peptidoglycan through inhibition of L,D-transpeptidases (Dubée et al., 2012). This activity is particularly evident in the presence of a β-lactamase inhibitor (Hugonnet et al., 2009; Gupta et al., 2010; Sotgiu et al., 2016; Jaganath et al., 2016), such as clavulanic acid, which irreversible inhibits *Mtb* beta-lactamase (Hugonnet and Blanchard, 2007). These results helped inspire a Phase 2a Early Bactericidal Activity (EBA) clinical trial of meropenem with Augmentin (amoxicillin/clavulanate) that demonstrated a robust reduction in sputum CFU counts, similar to the standard therapy (RHZE), definitively proving that β-lactams are active against *Mtb* in human lungs (Diacon et al., 2016). Additional studies further supported this observation (Tiberi et al., 2016). Unfortunately, meropenem suffers from two of the major drawbacks mentioned above: it requires co-dosing with a β-lactamase inhibitor and can only be administered by intravenous injection. With these limitations, the clinical use of meropenem for TB is largely limited to rescue therapy for hospitalized M/XDR patients (Payen et al., 2012). Therefore, an alternative carbapenem drug that could overcome these issues would represent a significant advance.

To this end, and in parallel to other approaches (Gold et al., 2022), we set out to screen β-lactams for activity in an intramacrophage *Mtb* assay, hypothesizing that compounds able to penetrate and kill mycobacteria within macrophages would have an inherent advantage for TB. This screen identified sanfetrinem and its ester prodrug, sanfetrinem cilexetil, as promising candidates. These compounds were discovered and developed by Glaxo Wellcome in the 1990s as potential broad-spectrum antibiotics (Géhanne et al., 1996; Wise et al., 1996; Tranquillini et al., 1996). Development was terminated following successful Phase 2 trials primarily due to commercial considerations, meaning that sanfetrinem cilexetil could offer an attractive re-purposing opportunity of a clinically validated asset. Here, we present the identification and pre-clinical characterization of this drug against *M. tuberculosis*.

2. Material and methods

2.1. Bacterial strains, general growth conditions and reagents

Mycobacterium strains were routinely propagated at 37°C in Middlebrook 7H9 broth (Difco) supplemented with 10 % Middlebrook albumin-dextrose-catalase (ADC)(Difco), 0.2 % glycerol and 0.05 % (vol/vol) tyloxapol (complete 7H9) or on Middlebrook 7H10 agar plates (Difco) supplemented with 10 % (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) (Difco). Hygromycin B (Sigma) was added to the medium (50 μg/mL) to ensure plasmid maintenance when propagating the *M. tuberculosis* H37Rv-*luc*, *M. tuberculosis* H37Rv-*gfp* and *M. bovis* BCG-*gfp* strains. *M. tuberculosis* H37Rv-*luc* constitutively expressed the luciferase *luc* gene from *Photinus pyralis* (GenBank Accession Number M15077) cloned in a mycobacterial shuttle plasmid derived from pACE-1 (Sorrentino et al., 2016), while *M. tuberculosis* H37Rv-*gfp* and *M. bovis* BCG-*gfp* strains harbored the pFPV27 plasmid (Alonso et al., 2011). *M. tuberculosis* clinical isolates were provided by the Vall d'Hebron Hospital, Barcelona, Spain and the Tuberculosis Research Section, NIH, Bethesda, USA. Reagents used, including sanfetrinem, other β-lactams and anti-TB drugs, were provided by GSK. Potassium clavulanate was obtained from Fluka (Ref. 33454). Due to compound instability, β-lactams were freshly dissolved from solid stocks on the same day of the experiment.

2.2. Extracellular susceptibility assays

Stock solutions of compounds were prepared fresh on the same day of plate inoculation, dissolved in DMSO and dispensed using a HP D3000 Digital Dispenser and HP T8 Dispenserhead Cassettes (Ref No. CV081A) in two-fold serial dilutions in a 384-well plate format. Dose-response assays were performed essentially as previously described (Ramón-García et al., 2016). Briefly, 7H9-based broth medium was supplemented with 0.2 % glycerol and 10 % ADC without detergent (i.e., tween 80 or tyloxapol). Glucose medium was prepared from 7H9 salts with a final concentration of 55 mM glucose. When needed, bovine serum albumin (BSA) was added at 0.5 % from a 5 mg/mL solution. Mycobacterial cells were grown to an OD_{600}= 0.5–0.8 and stocks were frozen at −80℃. Upon thawing, cells were diluted in assay medium to a final concentration of 10^5 cells/mL (or 10^6 cells/mL for the cholesterol assays) and 50 μL/well dispensed. Plates were placed in sealed boxes to prevent from drying of the perimetric wells and incubated without shaking at 37°C for 6 days before addition of a mix solution of 20 % tween 80 plus MTT [3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Stock 5 mg/mL, Acros Organics, Ref. 15224654) or the Bactiter-Glo Luciferase Assay System (Promega, Madison, WI), both used as cell growth indicators. For the MTT read-out, the optical density at 580 nm (OD_{580}) was measured in a Spectramax M5 (Molecular Devices) reader using black 384-microclear plate (Ref. 781091, Greiner). For the Bactiter-Glo system, ATP production was measured by luminescence (according to manufacturer indications) in an Envision Multilabel Plate Reader (PerkinElmer) using a white opaque 384-plate (Ref. 781075, Greiner) with Ultra-Sensitive luminescence mode and a measurement time of 50 ms/well. Dose response curves were plotted as percentage of growth compared to untreated internal controls (i.e., wells with no drug added/DMSO control). Moxifloxacin was used as a dose response compound control with 2-fold dilutions starting at 1 μg/mL. For the cholesterol assays, cholesterol was brought into solution (100 mM) by frequent vortexing and heating at 65°C in ethanol-tyloxapol (1:1 v/v). A 1/1000 dilution was then added to 7H9-based broth medium to give a final concentration of 0.1 mM cholesterol. Then, susceptibility assays on cholesterol as the sole carbon source were performed as previously described (Ramón-García et al., 2016, 2015).

2.3. Intracellular susceptibility assays

Two independent readouts were used to study the intracellular activity of test compounds: luminescence and CFU enumeration.

(i) Luminescence. The assay was performed essentially as previously described (Sorrentino et al., 2016). Frozen stocks of macrophage THP1 cells (ATCC TIB-202) were thawed in RPMI-1640 medium (Sigma) supplemented with 10 % fetal bovine serum (Gibco), 2 mM L-glutamine (Sigma) and 1 mM sodium pyruvate (Sigma). THP1 cells were passaged only 5 times without antibiotics and maintained between 2 and 10×10^5 cells/mL at 37°C in a humidified, 5 % CO_2 atmosphere. THP1 cells (3×10^8) were simultaneously differentiated with phorbol-myristate acetate (PMA, 40 ng/mL, Sigma) and infected for 4 hours at a multiplicity of infection (MOI) of 1:1 with a single cell suspension of *M. tuberculosis* H37Rv-*luc* cells. After incubation, infected cells were washed four times to remove extracellular bacilli and resuspended in fresh RPMI medium. Infected cells were finally resuspended (2×10^5 cells/mL) in RPMI medium supplemented with 10 % fetal bovine serum (Hyclone), 2 mM L-glutamine and pyruvate and dispensed in white, flat bottom 384-well plates (Greiner) at a concentration of ca. 10,000 cells per well in a final volume of 50 μL (max. 0.5 % DMSO). Plates were incubated for 5 days at 37°C under 5 % CO_2 atmosphere, 80 % relative humidity, before growth assessment using the Bright-Glo Luciferase

Assay System (Promega, Madison, WI) as above described. Internal wells containing drug-free medium with and without infected macrophages established maximum and minimal luminescence production, respectively. A 90 % reduction in light production was considered growth inhibition (IC_{90}). Every drug or drug combination was assayed in at least three independent experiments.

(ii) CFU enumeration. THP1 cells were treated and infected with *M. tuberculosis* H37Rv cells as above described at an MOI of 1:3 and seeded (50,000 cells/well) onto 24-well plates. Dose range concentrations of sanfetrinem were added at days 1 and 4 post-infection. Macrophages were lysed at days 1, 4 and 6 by osmotic pressure in a V_F= 500 μL. Then, 100 μL were 10-fold serially diluted in 1x PBS buffer with 0.1 % tyloxapol and plated in 7H10 plates. Agar plates were incubated at 37C for 14–21 days and CFUs enumerated. Plates were checked again at 4 weeks of incubation to count late growers. Cell density was reported as $\log_{10}$CFU/mL. Human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol.

2.4. Drug interaction assays

Checkerboard extracellular assays were used to identify pairwise interaction profiles of sanfetrinem and meropenem against a panel of clinically approved antibiotics and other antimicrobials with known mode of action. Drug activity was determined in 384-well plate format using the MTT or ATP assay, as described above. The fractional inhibitory concentration (FIC) for each compound was calculated as previously described (Ramón-García et al., 2011). FICI values indicated the degree of interaction: synergy, FICI$\leq$ 0.5; antagonism FICI $>$ 4.0; and no interaction FICI from 0.5 to 4.0.

2.5. Post-antibiotic effect assays

Exponential grown cultures (OD_{600}= 0.5–1.0) were diluted to a final cell density of 1.0×10^5 cells/mL in 10 mL supplemented 7H9-based broth medium (i.e., total $1x10^6$ cells) and treated with the compounds for 2 hours in roller bottles (5 rpm) at 37C. After the treatment period, compounds were washed out by three centrifugation steps (3500 rpm), supernatants removed and cells resuspended in 1 mL of fresh supplemented 7H9-based broth medium plus tyloxapol (final cell density 10^6 cells/mL). CFU counting was performed for every sample to determine whether cell killing had occurred during the 2 hours of treatment. From this cell suspension, 100 μL were then added to BD BBL™ Mycobacteria Growth Indicator tubes (MGIT™) (total of 1.0×10^5 cells/tube) in duplicates and the Time-To-Positivity (TTP, hours) measured in a BACTEC MGIT instrument. Positivity was defined as a Growth Index higher than 75 (GI $>$ 75).

2.6. Time kill assay

Frozen stocks of *M. tuberculosis* H37Rv were inoculated in 7H9-based broth supplemented with glycerol and ADC, without tyloxapol. Cultures were incubated at 37C for three days to allow for bacterial recovery and exponential growth phase. Then 10-mL were inoculated in 25 cm^2 tissue culture flasks (or roller bottles) to a typical final cell density of 10^5 cells/mL and drugs added at the designated times, concentrations and combinations depending on the type of assay, i.e., dose-response or replenishment. At every time point, cultures were thoroughly mixed, samples (100 μL) 10-fold serially dilute in 1x PBS buffer with 0.1 % tyloxapol and 100 μL plated on 7H10 agar plates supplemented with 10 % OADC. Agar plates were then processed as described above.

2.7. MIC determination against M. tuberculosis clinical isolates

The Minimum Inhibitory Concentration (MIC) of each tested compound against *M. tuberculosis* clinical isolates was determined in 96-well flat-bottom, polystyrene microtiter plates in Middlebrook 7H9 broth base supplemented with 10 % ADC and 0.05 % tyloxapol. Ten two-fold serial dilutions of the test compounds dissolved in DMSO were performed from column 1–10. Moxifloxacin was used as a dose response compound control starting at 1 μg/mL in column 11. Column 12 was used for positive (cells, no drug) and negative (no cells, no drug) controls. Similar plates with the same layout were also prepared including in the 7H9 medium 4–6 μg/mL of potassium clavulanate in order to test the potential MIC shift in the presence of this β-lactamase inhibitor. *M. tuberculosis* cells were grown to exponential phase (ca. OD_{600}= 0.2–0.3) and standardized to 1×10^7 CFU/mL (ca. OD_{600}= 0.125). The pre-inoculum was then further diluted (1:200) in the assay medium and 200 μL added to each well (final cell density ca. 5×10^4 CFU/mL). Plates were then incubated in sealed boxes to prevent drying of the perimetric wells and incubated without shaking at 37°C for 6 days (REMA readout) or 2 weeks (visual inspection readout). For the REMA readout (Coban et al., 2014), a resazurin solution was prepared by dissolving one tablet of Resazurin (Resazurin Tablets for Milk Testing; Ref. 330884Y VWR International Ltd) in 30 mL of sterile PBS (phosphate buffered saline). Of this solution, 25 μL were added to each well and the fluorescence measured after 48 hours in a Spectramax M5 (Molecular Devices) reader (λ_{Ex}= 530 nm, λ_{Ex}= 590 nm, cut-off 570 nm) to determine the MIC value. For the visual inspection readout, at weeks 1 and 2, plates were read with inverted enlarging mirror plate reader and individual wells grade as either growth or no growth by visual inspection. Regardless of the readout method used, the MIC value was defined as the lowest concentration that inhibited growth compared to untreated controls. The MIC_{90} value was considered the concentration that inhibited the growth of 90 % of the clinical isolates.

2.8. Single-cell time-lapse microscopy

Experiments were performed as previously described (Manina et al., 2015). Briefly, sanfetrinem was always prepared fresh in water solution (5 mg/mL), filter-sterilized and added to 50 mL of 7H9 broth at tests concentrations. A *M. bovis* BCG-*gfp* primary culture was started from a frozen stock in 10 mL of complete 7H9 supplemented with 50 μg/mL of Hygromycin B and allow to grow at 37C in a 25 cm^2 tissue culture flask until mid-exponential phase (OD_{600}= ca. 0.5). To prepare a single cell suspension, cells (2×1 mL) were pelleted, resuspended in 200 μL of complete 7H9 without tyloxapol and hygromycin B, and passed through a 5 μm syringe filter. This suspension (4 μL) was seeded onto a microfluidic device, which was assembled and transported to the microscope (Deltavision Elite Imaging System, Ref: 53–852339–002) for imaging capture. For every experiment, 110 points were labeled and acquired every 4 hours for 15 days using two microscopy channels: contrast phase (Pol, Blank, 32 %T 0.01 seg) and fluorescence (GFP, 100 %T 0.01 seg). A CoolSnap HQ camera was used with a 10x gain, 100x objective and autofocus system on contrast phase channel (7 μm on Z axis/0.2 μm steps). Sanfetrinem was infused into the microfluidic devise with a medium flow rate of 20 μL/min. Sanfetrinem-containing 7H9 media solution was refreshed every 24 hours to account for thermal drug degradation at room temperature; as such, we can assume that sanfetrinem concentration was maintained constant throughout the experiments. After a 72 hours growth phase, sanfetrinem was infused at a constant concentration of 20 μM (6xMIC; $MIC_{M.\ bovis\ BCG}$= 3.2 μM) and maintained for 144 hours (6 days), after which, sanfetrinem pressure was removed and cells allowed to recover for 3 additional days; a staining solution of propidium iodide (PI) was then added to differentiate death/alive cells.

2.9. In vivo assays in murine models of M. tuberculosis infection

The *in vivo* anti-tubercular activity of sanfetrinem, and other β-lactams, was evaluated following an established experimental design using DHP-1 knockout (Dpep-1 KO) mice in 129sv background, instead of the

previously described C57Bl/6 background (Rullas et al., 2015). In brief, pathogen-free, 8–10 weeks-old female 129sv DHP-1 KO mice were purchased from either Envigo or Taconic DK and allowed to acclimate for one week. Mice were intra-tracheal infected with approximately 10^5 CFU/mouse (*M. tuberculosis* H37Rv). Treatment was started nine days after infection and administered twice a day (bid) from day 9 to day 14 after infection at the following doses [mg/kg bw]: amoxicillin [200], clavulanate [100], faropenem [500], meropenem [300], sanfetrinem sodium salt [200] and sanfetrinem cilexetil [400] (it is estimated that a dose of sanfetrinem cilexetil 400 mg/kg bw equals to a dose of sanfetrinem 250 mg/kg bw). Meropenem and sanfetrinem sodium salt were administered subcutaneously while all other β-lactams were administered by oral gavage. Lungs were harvested on days 9 and 15 and lung lobes aseptically removed, homogenized and frozen. Homogenates were thaw, plated in 10 % OADC-7H11 medium + 0.4 % activated charcoal and CFU enumerated after 18 days of incubation at 37C. Differences in bacterial lung burden [$\log_{10}$ CFU/mouse (lungs)] of treated mice versus untreated controls (Day 15 after infection) were analyzed by the one factor ANOVA, Dunnet's posttest and Bonferroni's multiple comparisons tests using GraphPad Prism 7.0. For the pharmacokinetic studies of sanfetrinem and sanfetrinem cilexetil, animals were dosed orally at 200 mg/kg and 400 mg/kg, respectively, and blood samples taken and analyzed at 10, 60, 180 and 360 minutes post dosing. All animal studies were ethically reviewed by the internal Ethics Committee of GSK (as described in the internal protocol PACT AP32489) and by the external agency of the Comunidad de Madrid, Spain (as described in protocol PROEX 63/14). Studies were carried out in accordance with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and Treatment of Animals.

2.10. Drug quantification

Ultra-Performance Liquid Chromatography-Mass Spectrometry/Mass Spectrometry Analysis (UPLC-MS/MS) was used to quantify the amount of sanfetrinem in 7H9 medium and blood samples. The UPLC-MS/MS system consisted of an Acquity UPLC series (Waters Corporation, Madison, USA) coupled with a Sciex API 4000 instrument (AB Sciex, Toronto, Canada). Fifteen microliters of every sample were added to 190 μL of protein precipitant buffer (acetonitrile /methanol 80:20 v/v containing the internal standard) and filtered through a 0.45 μm pore size filter. From each sample, 150 μL were evaporated and re-suspended with 150 μL of milli-Q water before LC-MS/MS analysis. Samples were then loaded into an Acquity UPLC HSS T3 50 × 2.1 mm, 1.8 μm column (Waters Corporation, Madison, USA) and eluted by gradient with 10 mM ammonium formate plus 0.1 % formic acid and acetonitrile at a flow rate of 0.4 mL/min. The MS/MS system was operated in MRM negative mode with 280.1/236.3 transition.

3. Results

3.1. An intracellular screening of β-lactams identifies the anti-mycobacterial activity of sanfetrinem

A GSK in-house library of 1973 unique β-lactams was single-shot (50 μM) screened for activity against *M. tuberculosis* in infected THP1 macrophage cells; 105 active hits were identified with ca. 70 % inhibition cutoff (Fig. 1**A**). Forty-five compounds were confirmed active in successive, more stringent intracellular dose-response studies (90 % inhibition cut-off), yielding a hit discovery rate of 2.28 % (**Table S1**). Dose response studies were also performed under extracellular (7H9) conditions and IC_{90} values compared versus the intracellular (THP1) activity. Three main classes of β-lactams were identified based on their activity profile (cut-off 20 μM): active only intracellular, active only extracellular or with dual activity. Sanfetrinem cilexetil (GV118819) was identified with dual activity (Fig. 1**B**), which was also confirmed for the parent sanfetrinem compound (GV104326). Both compounds displayed slightly increased activity under intracellular conditions compared to extracellular conditions (Fig. 1**C**). The intracellular activity of sanfetrinem was comparable to that of the known intracellularly active cefdinir (Srivastava et al., 2021), and higher than other clinically approved β-lactams (Fig. 2).

3.2. Media composition impacts the activity of sanfetrinem against *M. tuberculosis*.

Dose response studies were performed under different media conditions to assess their impact on sanfetrinem's activity, i.e., standard media including dextrose and glycerol *vs.* glucose *vs.* cholesterol, detergents typically used in the assay medium, bovine serum albumin (BSA) and the presence of β-lactamase inhibitors (Fig. 2). Cholesterol is one of the main carbon sources available to mycobacteria at the site of infection (Van der Geize et al., 2007). To test the activity of sanfetrinem with cholesterol as the only carbon source the detergent tyloxapol needs to be added in order to solubilize it into the assay media, which is known to alter the cell wall structure and improve the activity of some cell wall targeting compounds, such as ethambutol (Piddock et al., 2000). In fact, the activity was improved in the presence of tyloxapol for some of the β-lactams used in this study, i.e., cefadroxil, cefdinir and faropenem, but not for amoxicillin or meropenem. Similarly, the activity of sanfetrinem was not affected by the presence of tyloxapol, which provides confidence in the observed improved activity (4-fold) in the presence of cholesterol as the only carbon source, a more physiologically relevant condition than the standard broth media supplemented with dextrose and glycerol or simply supplemented with glucose. In this case, the presence of BSA in the assay media had a significant impact, decreasing sanfetrinem's activity 4-fold, thus suggesting protein binding as a key

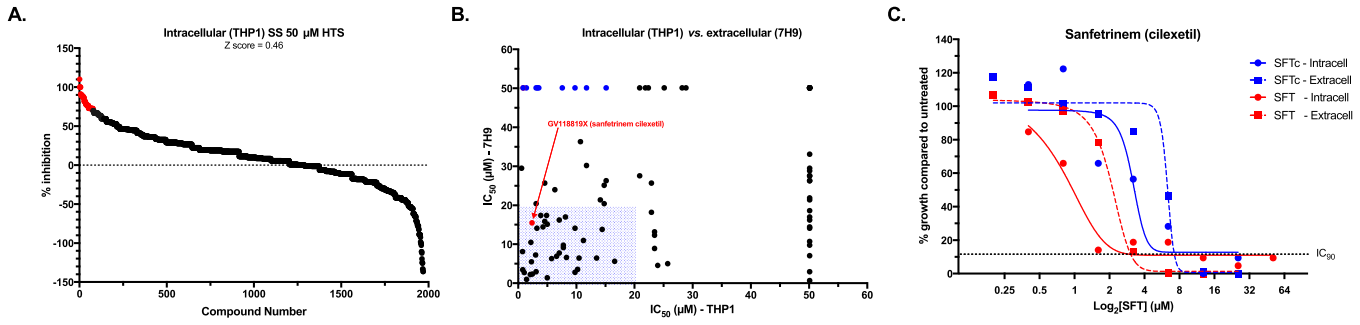


Fig. 1. Identification of sanfetrinem in a β-lactam intracellular screening assay. A library of 1973 unique β-lactams was screened for activity against *M. tuberculosis* in infected THP1 macrophage cells. **(A)** Single shot (50 μM) assays identified 105 hits with at least 70 % intracellular growth inhibition compared to untreated (red dots); **(B)** Dose response studies calculated the IC_{50} values of the 100 selected hits in both intracellular (THP1 cells) and extracellular (7H9 medium) conditions. Some β-lactam hits displayed exclusive intracellular activity (blue dots) while sanfetrinem cilexetil (red dot) displayed dual activity (cutoff 20 μM, dashed blue box); **(C)** Sanfetrinem cilexetil (SFTc) and sanfetrinem (SFT) in intracellular (THP1 cells) and extracellular (7H9 medium) dose response studies.

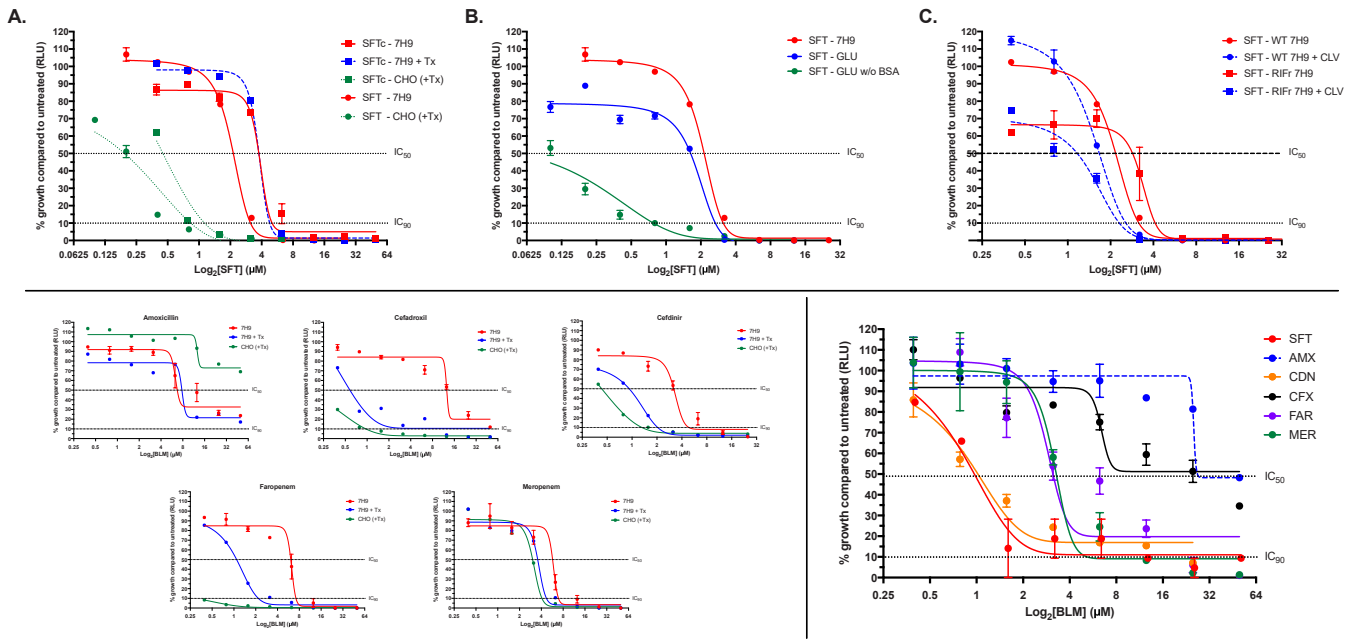


Fig. 2. ***In vitro*** **extracellular and intracellular characterization of sanfetrinem and other beta-lactams against** ***M. tuberculosis.*** **(Upper panel)** Dose response extracellular studies of sanfetrinem in (**A**) standard 7H9 medium (7H9), 7H9 plus the detergent tyloxapol (7H9 + Tx), 7H9 salts containing cholesterol as the only carbon source (CHO+Tx); (**B**) 7H9 salts containing glucose as the only carbon source with (GLU) and without BSA (GLU w/o BSA) and; (**C**) standard 7H9 medium plus the addition of clavulanic acid (7H9 + CLV). **(Lower left panel)** Dose response studies of amoxicillin, cefadroxil, cefdinir, faropenem and meropenem in standard 7H9 medium (7H9), 7H9 plus the detergent tyloxapol (7H9 + Tx), and 7H9 salts containing cholesterol as the only carbon source (CHO+Tx). **(Lower right panel)** Intracellular dose response assays of sanfetrinem and clinically approved beta-lactams. AMX, amoxicillin; BLM, β-lactam; CDN, cefdinir; CFX, cefadroxil; FAR, faropenem; MER, meropenem; RIFr, rifampicin-resistant *M. tuberculosis* H37Rv H526D strain; RLU, relative light units; SFT, sanfetrinem; SFTc, sanfetrinem cilexetil; WT, wild-type *M. tuberculosis* H37Rv strain. Unless otherwise specified, experiments were performed with the wild-type strain.

parameter to be considered for efficacy projections in pre-clinical and clinical development. Finally, the addition of clavulanate to the assay medium had a minor impact on sanfetrinem's activity against the laboratory strain *M. tuberculosis* H37Rv and a slight 2-fold improvement in a rifampicin resistant (H526D) *M. tuberculosis* H37Rv strain, although with this set of experiments the actual contribution of clavulanic acid to the *in vitro* activity of sanfetrinem could not be fully discerned (Fig. 2).

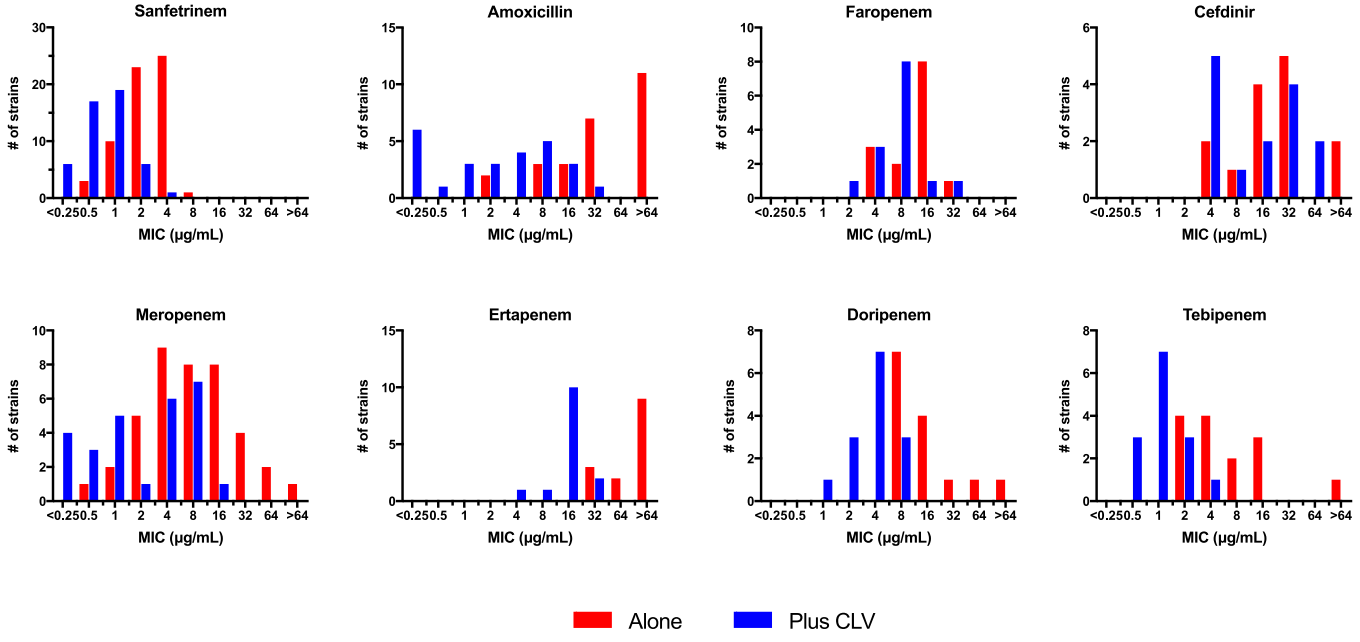


Fig. 3. Antimicrobial ***in vitro*** **activity of sanfetrinem and other β-lactams against** ***M. tuberculosis*** **clinical isolates.** The activity of several β-lactams was tested against a panel of *M. tuberculosis* drug-susceptible, multi-drug resistant and extensively drug resistant clinical isolates, together with laboratory strains alone and in combination with clavulanic acid (Plus CLV). The number of strains tested for the compound alone or in the presence of clavulanic acid were, respectively: sanfetrinem (62/49), meropenem (40/27), amoxicillin (26/26) and tebipenem, ertapenem, doripenem, faropenem and cefdinir (14/14). Detailed MIC values and resistant profiles of the clinical isolates are described in Table 1. CLV, clavulanic acid.

3.3. Broad spectrum activity of sanfetrinem against M. tuberculosis clinical isolates

In order to assess the potential for global implementation of sanfetrinem as a new anti-TB drug and the role clavulanate might play in a combined therapy, its anti-tuberculosis activity was tested against a panel of *M. tuberculosis* strains (n = 60), including drug-susceptible, MDR and XDR clinical isolates from different geographical locations alone and in the presence of clavulanate. The activity of sanfetrinem was compared to that of the clinically proven meropenem, among other clinically used β-lactams. Sanfetrinem was the most active β-lactam with an MIC_{90} value of 2–4 μg/mL, while meropenem displayed an MIC_{90} of 4–16 μg/mL, with an up to 16-fold increase in activity in the presence of clavulanate. Amoxicillin was less active ($MIC_{90\ (AMX)} > 32$ μg/mL); however, its activity was strongly enhanced up to 32-fold in the presence of clavulanate ($MIC_{90\ (AMX/CLV)} = 2–8$ μg/mL). Sanfetrinem showed a 4-fold increased activity in the presence of clavulanate ($MIC_{90\ (SFT/CLV)} = 0.5–2$ μg/mL) (Fig. 3). As in the dose response studies with the rifampicin-resistant H37Rv strain (Fig. 2), a detailed analysis revealed that strains with a resistant profile were more likely to benefit from the addition of clavulanate to sanfetrinem therapy (Table 1); kill kinetic assays with a rifampicin resistant strain confirmed this observation (**Figure S1**). In summary, sanfetrinem displayed the best *in vitro* activity of all β-lactams tested against *M. tuberculosis* clinical isolates with strong potential for broad clinical coverage, i.e., active against DS, MDR and XDR strains. Concomitant treatment with clavulanic acid might improve the effectiveness of sanfetrinem.

3.4. Sanfetrinem displays rapid bactericidal in vitro activity against M. tuberculosis

Having identified sanfetrinem in an intracellular single-shot screening assay (Fig. 1), CFU-based intracellular time-kill assay confirmed these data: i.e., β-lactams are active against actively replicating bacteria and highly unstable in the assay media. Thus, sanfetrinem was administered twice (after 1- and 4-days post-infection) to capture the phase of active bacterial replication. A modest bacteriostatic activity could be observed at the highest concentration tested (50 μM) when sanfetrinem was just added once at day 1 post-infection. However, a second dose of sanfetrinem after 4 days of infection (presumably when bacteria have adapted to intracellular growth and are actively replicating) showed a significant effect with bactericidal activity even at a low 1.25 μM concentration (Fig. 4**A**). After validating the intracellular bactericidal activity of sanfetrinem against *M. tuberculosis*, we aimed to further characterize its activity in extracellular conditions. We first performed stability assays of sanfetrinem in the assay media and calculated a half-life ($T_{1/2}$) of ca. 0.6 days, which effectively means that no drug was present in the assay media after 4 days of incubation. Regardless of this degradation, dose response time-kill assays showed a rapid bactericidal activity over the first 24 hours of exposure at concentrations as low as 5 μM. At higher concentrations, this killing was maintained for up to seven days, even reaching the limit of detection with the highest concentration tested. In all conditions, bacterial regrowth was observed after the initial bacterial killing (Fig. 4**B**). Colonies isolated from the regrowth showed MIC values similar to the wild-type, suggesting this was due to the lack of drug in the assay media and not to the acquisition of genetic mutations. The time to regrowth correlated with the initial amount of sanfetrinem, as confirmed by time-to-positivity (TPP) studies (Fig. 4**C**). As such, and considering the $T_{1/2}$ of sanfetrinem, we performed top-up time-kill assays at a fixed 5 μM concentration. Sanfetrinem was replenished in the assay media every day during 3 or 6 consecutive days. As shown in Fig. 4**D**, time to regrowth was dependent on maintained exposure levels of sanfetrinem. Importantly, when drug exposure was maintained, sanfetrinem was also bactericidal against a high inoculum (10^7 cell/mL). Nevertheless, even after one single dose addition of sanfetrinem, there was a lag-phase to regrowth of almost 7 days. This result could suggest a post-antibiotic effect of sanfetrinem (Fig. 4**E**), an *in vitro* feature not observed for other β-lactams (**Figure S2**).

We performed time-lapse microscopy studies to further characterize the activity of sanfetrinem at the single cell level (Fig. 5). Unlike time-kill assays, the microfluidics device allowed us to maintain the concentration of sanfetrinem over the treatment period. A rapid killing profile due to an abrupt cell lysis was observed only 8 hours after drug exposure, from which cells were not able to recover after removing sanfetrinem six days later (Fig. 5**A** and **Supplementary video S1.** Remaining bacteria exposed to sanfetrinem were PI positive (**Supplementary video S2 & S3),** which indicates a compromised cell membrane with a distorted cell morphology (Fig. 5**B**). All together, these studies demonstrated the rapid bactericidal activity of sanfetrinem against *Mycobacterium,* in both extracellular and intracellular conditions, with a long-lasting effect when drug pressure was maintained over the course of the experiment.

3.5. Sanfetrinem displays strong in vitro synergistic interactions with amoxicillin, ethambutol, rifampicin and rifapentine

In vitro synergy assays were performed with sanfetrinem and meropenem in combination with a panel of twenty-six clinically approved (or with known mode of action) antimicrobials to further explore the repurposing potential of sanfetrinem within a TB regimen (Fig. 6). Sanfetrinem displayed strong synergy with amoxicillin and rifampicin with FICI values lower than 0.25 and up to 8-fold reductions in MIC values within the combination, compared to the MIC of the compound alone; synergy was also observed with rifapentine (FICI = 0.38) and ethambutol (FICI = 0.5). This pattern of interaction differed from that of meropenem for which synergy was more prevalent, although strong synergy (FICI < 0.25) was only observed for rifampicin, rifapentine and cephradine, but not for amoxicillin (Fig. 6**A**). A closer look at the sanfetrinem synergistic combinations revealed a shift in the dose response curves when in the presence of sub-MIC concentrations of the companion drugs (Fig. 6**B**), consistent with the observed FICI values. The observation that sanfetrinem and meropenem had different synergy profiles was not uncommon, as it has been observed for other β-lactams (Ramón-García et al., 2016).

Time-kill assays were performed to study the *in vitro* pharmacodynamics of sanfetrinem combinations against *M. tuberculosis.* The activity of sanfetrinem, amoxicillin/clavulanate, and rifampicin was evaluated through the examination of their individual and combined effects with the compounds under investigation, i.e., alone and in pair-wise and triple combinations. Drugs were added at sub-optimal concentrations in order to allow detection of synergistic interactions with an increased killing capacity, demonstrated by higher CFU decline of the combination compared to the drugs alone. As shown in Fig. 7, at the tested concentration sanfetrinem was able to reduce the bacterial burden by 2-logs over the first 24 hours but regrowth occurred after 2 days, similar to previous observations (Fig. 4); this bactericidal effect was stronger than that observed for amoxicillin/clavulanate (with only 1-log reduction over the first 24 hours). The combination of sanfetrinem with amoxicillin/clavulanate exhibited a minor interaction characterized by a slight delay in re-growth. However, the combination of either sanfetrinem or amoxicillin/clavulanate with rifampicin had a strong positive interaction with a post-antibiotic effect between 10 and 15 days preventing the culture from resuming growth. Triple combinations of sanfetrinem and amoxicillin/clavulanate plus either rifampicin had profiles similar or slightly better that the respective pair-wise combos. In both cases, a sanfetrinem-dependent rapid initial killing was observed in addition to maintaining bacteriostasis up to 15 days after treatment (Fig. 7). Our studies thus confirm rifampicin and rifapentine as strong synergistic partners of sanfetrinem and amoxicillin/clavulanate with the potential to be part of a novel combination therapy.

Table 1
Antimicrobial activities of sanfetrinem and other β-lactams against *M. tuberculosis* strains, including drug resistant clinical isolates. Information in this table supports Fig. 3.

				Minimal Inhibitory Concentration (MIC) (μg/mL)																					
				Santrinem			Meropenem			Amoxicillin			Tebipenem			Ertapenem			Doripenem			Faropenem			Cefdinir		
#	Strain	Background	Drug resistant profile	Alone	plus CLV	Ratio CLV	Alone	plus CLV	Ratio CLV	Alone	plus CLV	Ratio CLV	Alone	plus CLV	Ratio CLV	Alone	plus CLV	Ratio CLV	Alone	plus CLV	Ratio CLV	Alone	plus CLV	Ratio CLV	Alone	plus CLV	Ratio CLV
1	H37Rv	H37Rv	DS	1.52	0.76	2	35.00	4.38	8	> 58.46	7.31	8	15.34	1.92	8	> 76.08	19.02	4	33.64	4.21	8	12.29	6.15	2	15.82	3.95	4
2	Erdman	Erdman	DS	0.76	0.76	1	4.38	2.19	2	29.23	3.65	8	1.92	0.48	4	38.04	9.51	4	4.21	2.10	2	6.15	4.61	1	11.86	7.91	2
3	CDC 1551	CDC 1551	DS	0.76	0.76	1	17.50	4.38	4	29.23	5.48	5	3.83	1.92	2	19.02	9.51	2	8.41	4.21	2	12.29	12.29	1	15.82	15.82	1
4	dH37Rv H526D	H37Rv	RIF	1.52	0.76	2	8.75	3.28	3	> 58.46	14.62	4	5.75	0.96	6	> 76.08	9.51	8	4.21	2.10	2	12.29	6.15	2	> 63.27	63.27	1
5	EthA	H37Rv	ETH	1.52	0.48	3	4.38	2.19	2	29.23	3.65	8	1.92	0.48	4	> 76.08	9.51	8	4.21	1.58	3	3.07	3.07	1	23.72	23.72	1
6	Line R	H37Rv	INH, STR	2.27	1.52	2	6.56	4.38	2	29.23	7.31	4	1.92	0.96	2	> 76.08	9.51	8	4.21	2.10	2	12.29	6.15	2	31.63	15.82	2
7	724_R	H37Rv	INH (KasA resistant mutant)	1.90	1.52	1	8.75	2.19	4	29.23	1.83	16	3.83	0.96	4	38.04	9.51	4	8.41	2.10	4	6.15	3.07	2	7.91	3.95	2
8	366_R	H37Rv	MmpL3 resistant mutant	0.38	0.38	1	8.75	2.19	4	29.23	1.83	16	3.83	0.69	6	28.53	4.76	6	4.21	1.58	3	3.07	3.07	1	3.95	3.95	1
9	VdH−15178	Clinical	DS	1.52	0.76	2	26.25	4.38	6	> 58.46	3.65	16	7.67	1.92	4	> 76.08	9.51	8	25.23	1.58	16	12.29	6.15	2	15.82	3.95	4
10	Beijing 1137	Clinical	DS	1.52	1.52	1	70.00	8.75	8	> 58.46	29.60	2	> 61.36	3.83	16	> 76.08	19.02	4	> 67.28	6.31	11	24.58	18.44	1	> 63.27	63.27	1
11	clinical strain S531L	Clinical	RIF	0.58	0.18	3	8.75	1.09	8	29.23	1.37	21	1.92	0.48	4	19.02	3.57	5	4.21	0.79	5	2.30	1.54	2	3.95	2.97	1
12	Clinical Strep R	Clinical	STR, INH	1.52	0.76	2	17.50	2.19	8	> 58.46	10.96	5	15.34	0.96	16	> 76.08	9.51	8	12.62	2.10	6	9.22	6.15	2	31.63	23.72	1
13	Clinical Moxi R 488	Clinical	MOX	1.52	0.76	2	17.50	4.38	4	> 58.46	7.31	8	15.34	0.96	16	> 76.08	9.51	8	8.41	2.10	4	12.29	6.15	2	31.63	19.77	2
14	2 A	Clinical	INH	1.90	1.52	1	8.75	4.38	2	> 58.46	7.31	8	3.83	0.96	4	> 76.08	9.51	8	4.21	2.10	2	12.29	6.15	2	23.72	31.63	1
15	21	Clinical	INH	2.23	nd		2.45	nd																			
16	70	Clinical	INH	2.23	nd		9.82	nd																			
17	223	Clinical	INH	2.23	nd		4.91	nd																			
18	250	Clinical	INH, STR	2.23	nd		4.91	nd																			
19	276	Clinical	INH, STR	2.23	nd		4.91	nd																			
20	280	Clinical	INH, STR	2.23	nd		2.45	nd																			
21	291	Clinical	INH	2.23	nd		2.45	nd																			
22	389 R	Clinical	INH, STR	4.46	nd		2.45	nd																			
23	52 S (car1)	Clinical	DS	2.23	nd		2.45	nd																			
24	215 (car3)	Clinical	DS	2.23	nd		9.82	nd																			
25	275 (car5)	Clinical	DS	2.23	nd		4.91	nd																			
26	2020 S (car6)	Clinical	DS	2.23	nd		2.45	nd																			
27	2166S (car8)	Clinical	DS	2.23	nd		2.45	nd																			
28	CRC−1	Clinical	INH, RIF, EMB, STR, KAN, ETH, PAS, MOX, CAP				1.40	0.10	14																		
29	HN878	HN878	DS	3.79	0.95	4																					
30	K03b00DS 57	Clinical	DS	3.79	0.95	4																					
31	K04b00_rplCT460C	Clinical	LZD	0.95	0.45	2																					
32	K04b00_rrlA2741G	Clinical	LZD	1.90	0.95	2																					
33	K04b00_rrlG2270T	Clinical	LZD	0.95	0.45	2																					
34	K04b00_rrlG2814T	Clinical	LZD	0.32	0.36	1	1.01	0.53	2	1.72	0.57	3															
35	K04b00DS	Clinical	DS	0.95	0.24	4	3.30	0.30	11	> 8	> 8	ND															
36	K07b00DS 41	Clinical	DS	1.90	0.24	8	1.37	0.17	8	6.94	0.04	174															
37	K12b00DS 44	Clinical	DS	3.79	1.90	2																					
38	K13b00DS	Clinical	DS	3.79	0.95	4																					
39	K16b00DS	Clinical	DS	1.90	0.95	2																					
40	K25b00MR	Clinical	INH, RIF, EMB, PZA, RBT, PTH	2.84	0.53	5	10.94	0.53	21	> 18.27	0.57	> 64															
41	K26b00MR	Clinical	INH, RIF, EMB, PZA, RBT, PTH	3.79	0.95	4																					

(*continued on next page*)

Table 1 (*continued*)

				Minimal Inhibitory Concentration (MIC) (μg/mL)																				
				Santrinem			Meropenem			Amoxicillin			Tebipenem			Ertapenem			Doripenem			Faropenem			Cefdinir		
42	K33b00MR	Clinical	INH, RIF, EMB, PZA, STR, KAN, PTH	1.90	0.24	8																				
43	K35b00DS	Clinical	DS	3.79	0.95	4																				
44	K37b00XR	Clinical	INH, RIF, KAN, STR, MOX, LVX	0.95	0.45	2																				
45	Kb005	Clinical	DS	0.45	0.12	4																				
46	Kb019	Clinical	INH, RIF, EMB, PAS, KAN, OFX, PTH	0.95	0.45	2	0.50	0.10	5	> 8	0.10	> 160														
47	NIH_G10R	Clinical	RIF, INH, STR, RBT, EMB	1.90	0.45	4																				
48	NIH_G11R	Clinical	CYC, EMB	1.90	0.45	4																				
49	NIH_G12	Clinical	DS	1.90	0.95	2																				
50	NIH_G15R	Clinical	RIF, INH, RBT, ETH, CYC	3.79	0.45	8																				
51	NIH_G16R	Clinical	STR, CYC	1.90	0.36	5	1.37	0.17	8	9.14	0.07	131														
52	NIH_G17R	Clinical	INH, ETH, CYC	1.90	0.45	4																				
53	NIH_G18R	Clinical	RIF, INH, STR, PAS, ETH	1.90	0.45	4																				
54	NIH_G19R	Clinical	RIF, INH, STR, RBT, CYC, EMB	3.79	0.45	8																				
55	NIH_G1DS	Clinical	DS	1.90	0.36	5	1.37	0.26	5	4.57	0.07	65														
56	NIH_G20R	Clinical	CYC	2.84	1.18	2	2.73	0.53	5	4.57	0.07	65														
57	NIH_G21R	Clinical	RIF, INH, RBT, CYC, EMB	1.90	3.79	1																				
58	NIH_G22R	Clinical	RIF, INH, RBT, EMB	3.79	0.95	4																				
59	NIH_G4XR	Clinical	INH, RIF, EMB, PAS, KAN, OFX, PTH	3.79	0.95	4	4.11	0.68	6	13.52	0.44	31														
60	NIH_G9R	Clinical	MOX, CYC	1.90	0.36	5	1.37	0.26	5	9.14	0.05	183														
61	*M. africanum*	Mtb complex		0.95	0.12	8	1.01	0.53	2	1.72	0.57	3														
62	*M. bovis* 0AF2122	Mtb complex		1.90	0.45	4																				
63	*M. canetti*	Mtb complex		0.76	0.76	1	35.00	4.38	8	> 58.46	2.74	21	15.34	1.92	8	> 76.08	9.51	8	33.64	2.10	16	6.15	4.61	1	7.91	1.98	4

[a]Mycobacterial strains were assayed in liquid 7H9 media containing 0.2 % glycerol, 10 % ADC supplement and 0.05 % Tween 80 or tyloxapol. The MIC was determined as the concentration that inhibited growth compared to control wells. See Material & Methods for more detailed technical information. [b]The concentration of clavulanate used in strains #1 to #14 was 4 μg/mL and in strains #28 to #63 was 25 μM (5.88 μg/mL). DS, drug susceptible; DR: Drug Resistant; MDR: Multidrug resistant; XDR: Extensively drug resistant; CLV, clavulanic acid; CYC, cycloserine; CAP, capreomycin; EMB, ethambutol; ETH, ethionamide; INH, isoniazid; KAN, kanamycin; LVX, levofloxacin; LZD, linezolid; MOX, moxifloxacin; OFX, ofloxacin; PAS, *p*-aminosalicilate; PTH, prothionamide; PZA, pyrazinamide; RBT, rifabutin; RIF, rifampicin; STR, streptomycin.

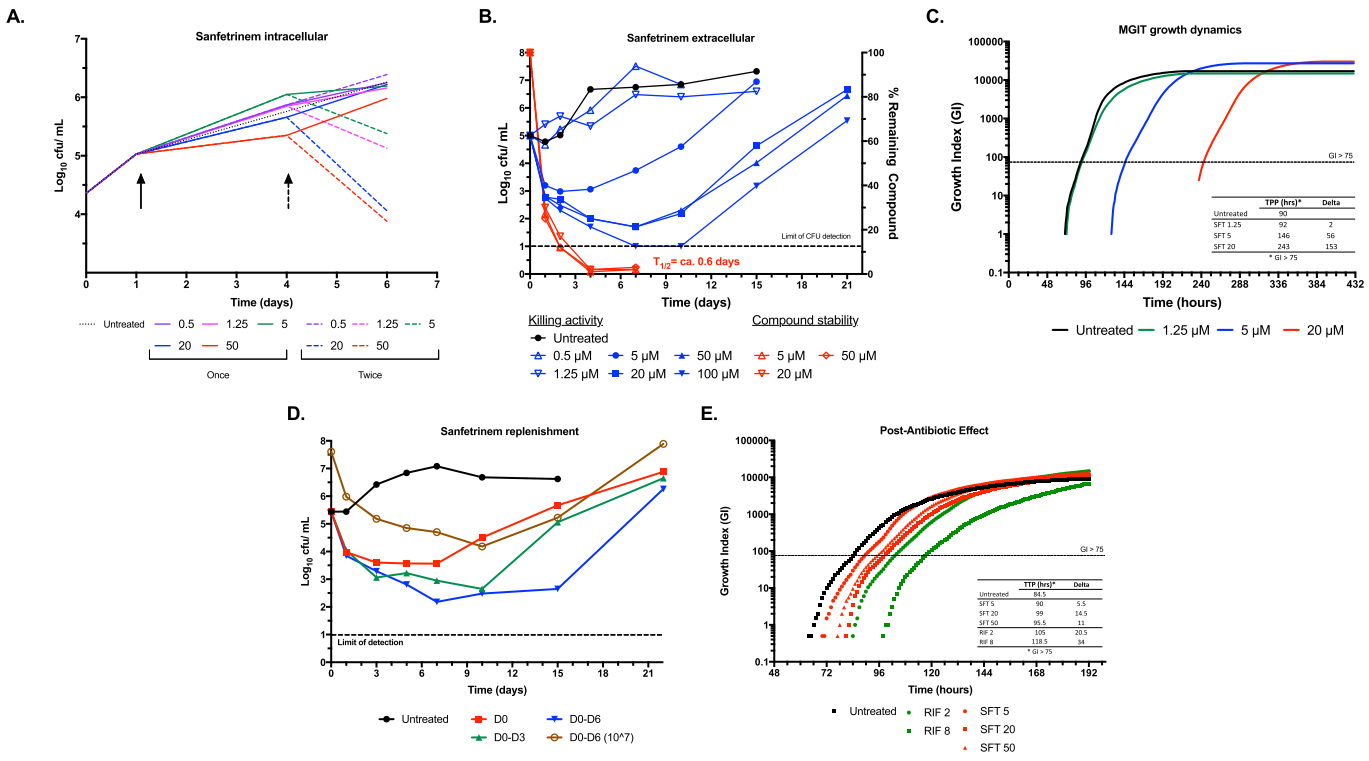


Fig. 4. Intracellular and extracellular time-kill and post-antibiotic effect assays of sanfetrinem against *M. tuberculosis*. **(A)** CFU-based intracellular dose response assays were performed with sanfetrinem (µM) added only on day 1 (solid arrow) or twice on days 1 and 4 (dashed arrow). **(B)** Dose response time-kill assay of sanfetrinem in standard 7H9 medium. Blue lines indicate bacterial density over time after sanfetrinem treatment. Red lines indicate the amount of sanfetrinem present in the assay media over time, degraded due to thermal instability at 37C. **(C)** The activity of sanfetrinem was continuously monitored in the MGIT system and the time-to-positivity (TPP) calculated. Delta indicates the elapsed time to reach positivity (GI > 75) compared to the untreated sample. (D) Sanfetrinem was added at 5 µM only once at day 0, or daily from day 0 to day 3, or from day 0 to day 6 to replenish to the target concentration, accounting for a degradation $T_{1/2}$ of ca. 0.6 days. Two starting inocula were also used: approx. 10^5 and 10^7 cells/mL. Cell density (CFU/mL) was followed over time. (E) The post-antibiotic effect of sanfetrinem was measured against *M. tuberculosis* H37Rv. Cells treated at the corresponding drug concentrations for 2 hours were transferred (10^5 cells) to MGIT tubes and the TTP measured. The 2-hours treatment did not affect CFU counts. Rifampicin was included as positive control. Numbers in legend indicate drug concentration in µg/mL for rifampicin and in µM for sanfetrinem (5 µM equals to ca. 1.7 µg/mL). Delta indicates the increase in TTP compared to untreated. RIF, rifampicin; SFT, sanfetrinem.

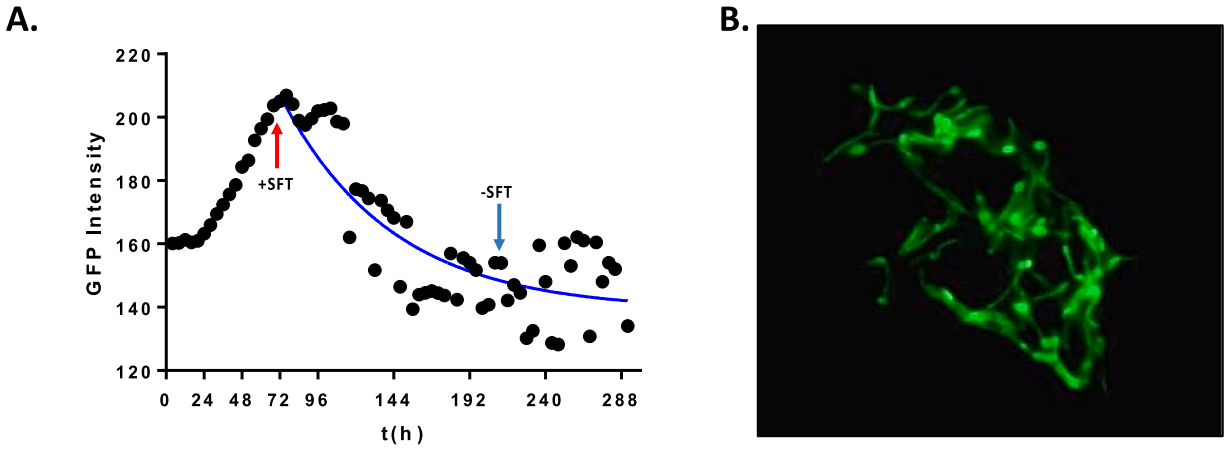
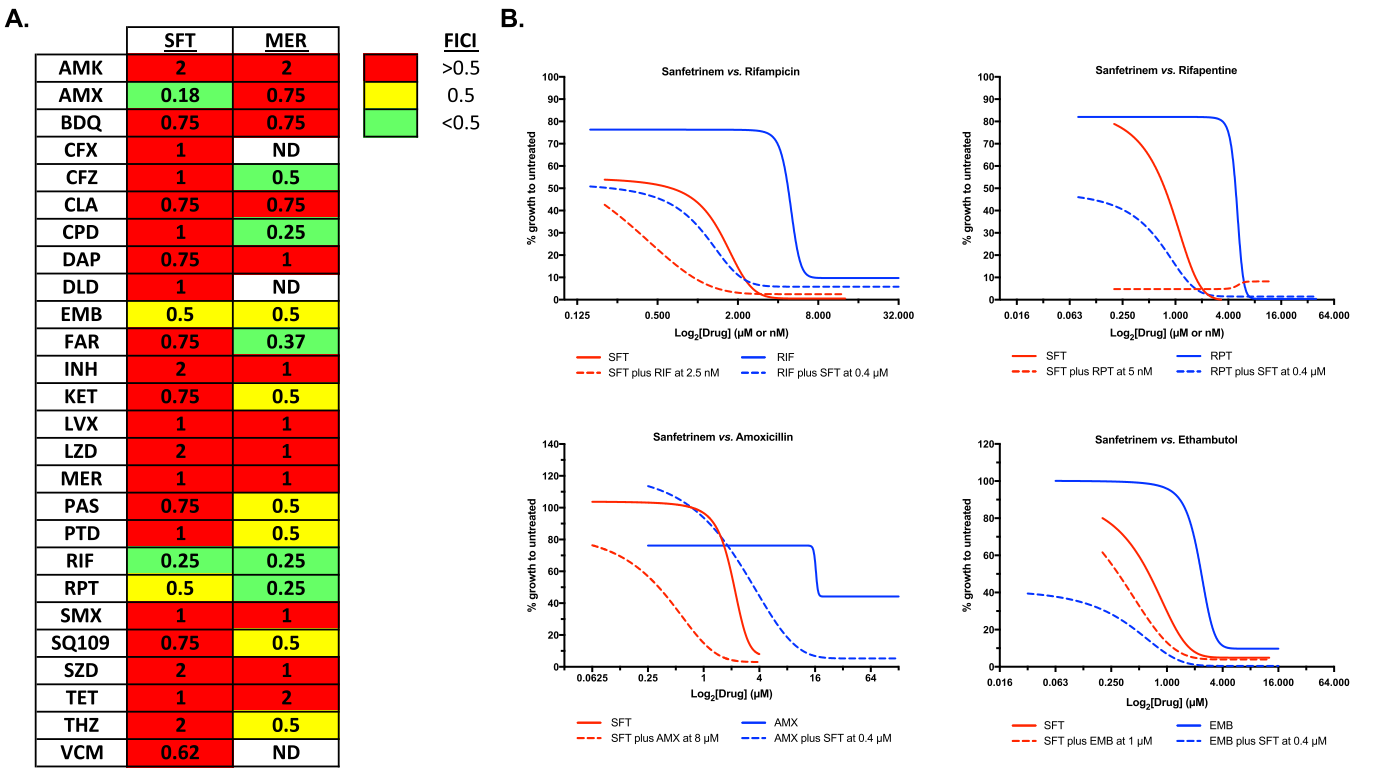


Fig. 5. Time-lapse microscopy of sanfetrinem against *M. bovis* BCG. **(A)** *M. bovis* BCG cells were allowed to adapt for 72 hours until exponential growth was achieved in the microfluidic device. Then, sanfetrinem was added (+SFT) at a fixed concentration of 20 µM for 6 days. Afterwards, sanfetrinem was removed (-SFT) and bacterial cells allowed to recover for 3 days before propidium iodide staining (see also **Supplementary video S1–S3**. **(B)** Bacterial morphology after 6 days exposure to sanfetrinem.

3.6. Sanfetrinem is active against M. tuberculosis in an in vivo model of infection

β-lactams display poor *in vivo* efficacy in traditional murine models of *M. tuberculosis* infection (Veziris et al., 2011); (England et al., 2012); (Solapure et al., 2013). One of the reasons for this lack of efficacy is the increased expression of the murine renal dehydropeptidase (DHP-1) enzyme, several orders of magnitude higher than its human homologue, which limits the exposure of β-lactams in such models. However, developments in the field have demonstrated robust and significant CFU reductions (1–2 log CFU) in the lungs of infected DHP-I-deficient mice, in comparison to the untreated control (Rullas et al., 2015).



	SFT	MER
AMK	2	2
AMX	0.18	0.75
BDQ	0.75	0.75
CFX	1	ND
CFZ	1	0.5
CLA	0.75	0.75
CPD	1	0.25
DAP	0.75	1
DLD	1	ND
EMB	0.5	0.5
FAR	0.75	0.37
INH	2	1
KET	0.75	0.5
LVX	1	1
LZD	2	1
MER	1	1
PAS	0.75	0.5
PTD	1	0.5
RIF	0.25	0.25
RPT	0.5	0.25
SMX	1	1
SQ109	0.75	0.5
SZD	2	1
TET	1	2
THZ	2	0.5
VCM	0.62	ND

Fig. 6. ***In vitro*** **synergy profiling of sanfetrinem and meropenem against** ***M. tuberculosis*** **H37Rv. (A)** Fractional inhibitory concentration indexed (FICI) of sanfetrinem and meropenem in pair-wise combinations of clinically approved or with known mode of action antimicrobials (n = 26). A FICI ≤ 0.5 indicates synergy, while a FICI > 0.5 and ≤ 4 indicates no interaction; **(B)** Dose response interaction curves of sanfetrinem (red) with synergistic partners (blue). Solid lines represent the dose response curve of drugs alone while broken lines represent the dose response curves in the presence of sub-MIC concentrations of the synergistic partner. Concentrations of drugs are expressed in μM, except for rifampicin and rifapentine that are expressed in nM. AMK, amikacin; AMX, amoxicillin; BDQ, bedaquiline; CFZ, clofazimine, CLA, clarithromycin; CPD, cephradine; DAP, dapsone; DLD, delamanid; EMB, ethambutol; FAR, faropenem, INH, isoniazid; KET, ketoconazole; LVX, levofloxacin; LZD, linezolid; MER, meropenem; PAS, p-amino salicilic acid; PTD, pretomanid; RIF, rifampicin; RPT, rifapentine; SFT, sanfetrinem; SMX, sulfamethoxazole; SZD, sutezolid; TET, tetracycline; THZ, thioacetazone; VCM, vancomycin.

In this study, we used DHP-1 knockout mice to evaluate the *in vivo* activity of sanfetrinem in comparison with β-lactams already tested in clinical trials (Fig. 8). A first experiment compared the activity of sanfetrinem cilexetil with a combination of faropenem plus clavulanate. Untreated controls experienced an increase in lung CFU of ca. 1-log over the treatment period (day 9 *vs.* day 15), while treatment groups were able to prevent such growth. In a second experiment, the activities of both forms of sanfetrinem (sodium salt dosed subcutaneously and cilexetil dosed orally) were compared against combinations of meropenem plus either clavulanate or clavulanate and amoxicillin. Similarly, untreated controls displayed an increase in lung CFU over the treatment period (ca. 1.6-log CFU) while this growth was prevented in all treatment groups; also evidenced by a prevention of weight loss in the treated arms (**Figure S3**). When comparing among treatment groups, the combination of meropenem-clavulanate-amoxicillin was the most active, although not significantly different from the sanfetrinem and meropenem-clavulanate groups. The oral sanfetrinem cilexetil arm also resulted in a significant lung CFU reduction, although slightly less than the most active meropenem-clavulanate-amoxicillin group. This could be due to the lower exposure levels obtained with the oral form of sanfetrinem compared to the subcutaneous administration, as observed in mice pharmacokinetic studies (**Figure S4**). However, no significant differences were observed between the activities of the two forms of sanfetrinem. No adverse effects were observed in mice treated with either form of sanfetrinem.

4. Discussion

A seminal Phase 2a EBA clinical study demonstrated the efficacy of meropenem in combination with amoxicillin/clavulanate for the treatment of TB (Diacon et al., 2016). Subsequent studies in treating MDR/XDR patients supported these observations, although recent studies with drug-susceptible TB cast some doubts on the role of meropenem in programmatic TB treatment due to poor tolerability (Jager et al., 2022). In addition, due to the lengthy therapies required for TB treatment, the clinical use of meropenem comes with some limitations, mainly related to the intravenous route of administration and its susceptibility to degradation by intrinsic *Mtb* β-lactamases. The stated goals of this screening of β-lactams, and related ones (Gold et al., 2022) against *Mtb* were to select compounds with: (i) similar or greater potency as compared to the clinically proven meropenem; (ii) inherent stability to BlaC degradation; and (iii) oral bioavailability. Therefore, we were excited and pleased to identify sanfetrinem cilexetil, a tricyclic β-lactam candidate, met such criteria.

Tricyclic carbapenems or "trinems" were originally reported by Glaxo Wellcome in 1966 (Géhanne et al., 1996; Hanessian et al., 1997) with the original aim of inhibiting β-lactamases (Fasoli et al., 2005), (Prezelj et al., 2011). Several *in vitro* and *in vivo* studies demonstrated the efficacy of sanfetrinem against Gram-positive and Gram-negative bacteria (Singh et al., 1996). The oral prodrug sanfetrinem cilexetil was subsequently developed for respiratory tract infections in adults and otitis media in children. Multiple successful Phase 2 trials in these indications demonstrated its ability to achieve therapeutically relevant exposures upon oral dosing. With just a slightly increased tendency toward gastrointestinal tolerability issues in patients taking sanfetrinem cilexetil, these trials and preceding development showed a reasonable safety profile, in line with other β-lactam drugs, including pediatric populations (GSK, unpublished data). Compatibility with pediatric and

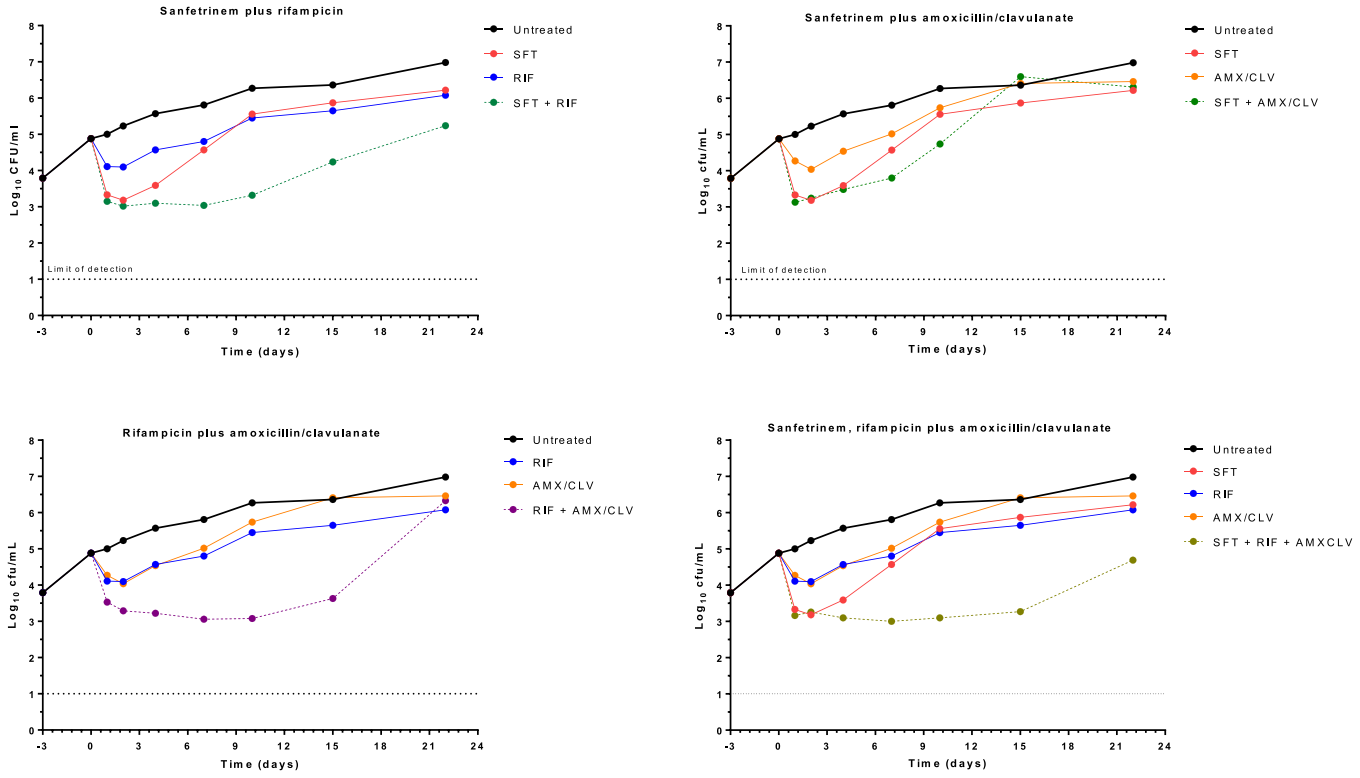


Fig. 7. Combinatorial time-kill assays of sanfetrinem with rifampicin and amoxicillin/clavulanate. Sanfetrinem (SFT), rifampicin (RIF), and amoxicillin/clavulanate (AMX/CLV) were tested alone and in pair-wise and triple combinations against *M. tuberculosis* H37Rv. Cultures were allowed to adapt for three days to exponential growth conditions (Day −3). Then, drugs were added at day 0. Concentrations used were: SFT = 2.5 μM (0.87 μg/mL); RIF = 0.5 μM (0.41 μg/mL); AMX = 2.2 μM (0.8 μg/mL); CLV = 5 μg/mL.

pregnant populations is a major gap in TB drug development, representing another exciting feature for sanfetrinem cilexetil.

β-lactams are rapid bactericidal antibiotics that were largely considered inactive against intracellular pathogens (Kurz and Bonomo, 2012). *Mtb* can be found extracellularly in the caseum of the granulomas but mainly resides in intracellular compartments at the site of infection. Effectively targeting both populations will contribute to TB treatment shortening and control of the disease by means of a rapid decline in extracellular burden and reaching difficult-to-treat intracellular bacteria. We thus designed an intracellular screening program to test a GSK *in-house* library of 1973 unique β-lactams; sanfetrinem cilexetil was identified as one of the most promising hits from this screen with dual intracellular and extracellular activity (Fig. 1). While these findings could be attributed to differences in conditions and readouts between intracellular and extracellular assays, they are also consistent with previous reports demonstrating intracellular activity of sanfetrinem (Cuffini et al., 1998a) and intracellular accumulation of sanfetrinem into human polymorphonuclear granulocytes (Cuffini et al., 1998b). Time-kill and time-lapse microscopy assays further demonstrated the rapid bactericidal capacity of sanfetrinem in both intracellular and extracellular conditions and an extended lag phase despite the poor chemical stability under assay conditions (Fig. 4 and Fig. 5). *Mtb in vitro* assays require several weeks to produce results, and compound stability experiments showed that sanfetrinem, like other carbapenems (Smith et al., 2018), is almost entirely degraded within 1–2 days. Quantifying the actual carbapenemś activity in *Mtb in vitro* assays is non-obvious, with extensive effort and coordination needed due to the biohazard nature of the samples containing a Biosafety Security Level 3 pathogen. The implication of this instability is that traditional MIC determinations (typically used as the main initial proxy for *in vitro* activity) for carbapenems are misleading because their antimicrobial activity is consistently underestimated due to the drug being present only for a fraction of the time assay (Srivastava et al., 2016). Time-kill replenishment studies accounting for the drug degradation in the assay media demonstrated a better proxy to quantify sanfetrinemś activity. By topping up the concentration of sanfetrinem, we were able to show a greater than expected antimicrobial activity when the drug pressure was maintained over time. Time-kill longitudinal data identified a lag-phase upon sanfetrinem treatment prior to bacterial regrowth, which was linked to the post-antibiotic effect of sanfetrinem (Fig. 4**D**), as previously described (Spangler et al., 1997). Further time-lapse microscopy studies confirm the biphasic killing dynamic of sanfetrinem observed in the time-kill assays; i.e., bacterial cytolysis occurred within the first 8 hours of treatment, followed by a slower kill rate, similar to previously observed with faropenem and meropenem (Dhar et al., 2015). Constant drug exposure leaves a surviving weakened sub-population of tolerant bacteria with a club-like morphology, indicative of cell wall damage (Fig. 5), which could facilitate the activity of companion drugs.

The *in vitro* antimicrobial activity of compounds against *Mtb* is typically performed in synthetic 7H9 media supplemented with ADC or OADC, containing BSA and glycerol and dextrose as the main carbon sources. This has proven to be non-representative of *in vivo* activity in some cases (Pethe et al., 2010) and previous *in vitro* studies demonstrated that media composition can affect the activity of some β-lactams (Ramón-García et al., 2016); we thus explored the impact of different media compositions (Fig. 2). The activity of sanfetrinem was affected by the presence of BSA indicating extensive protein binding, which should be taken into consideration when projecting suitable clinical doses. Interestingly, sanfetrinem was more potent in the presence of cholesterol as the sole carbon source, which could be a more clinically relevant *in vitro* model of activity since cholesterol is the main carbon source used by *Mtb* at the site of infection. In MIC determinations against a panel of clinical isolates, sanfetrinem performed favorably compared to several clinically approved β-lactams, including meropenem. The presence of

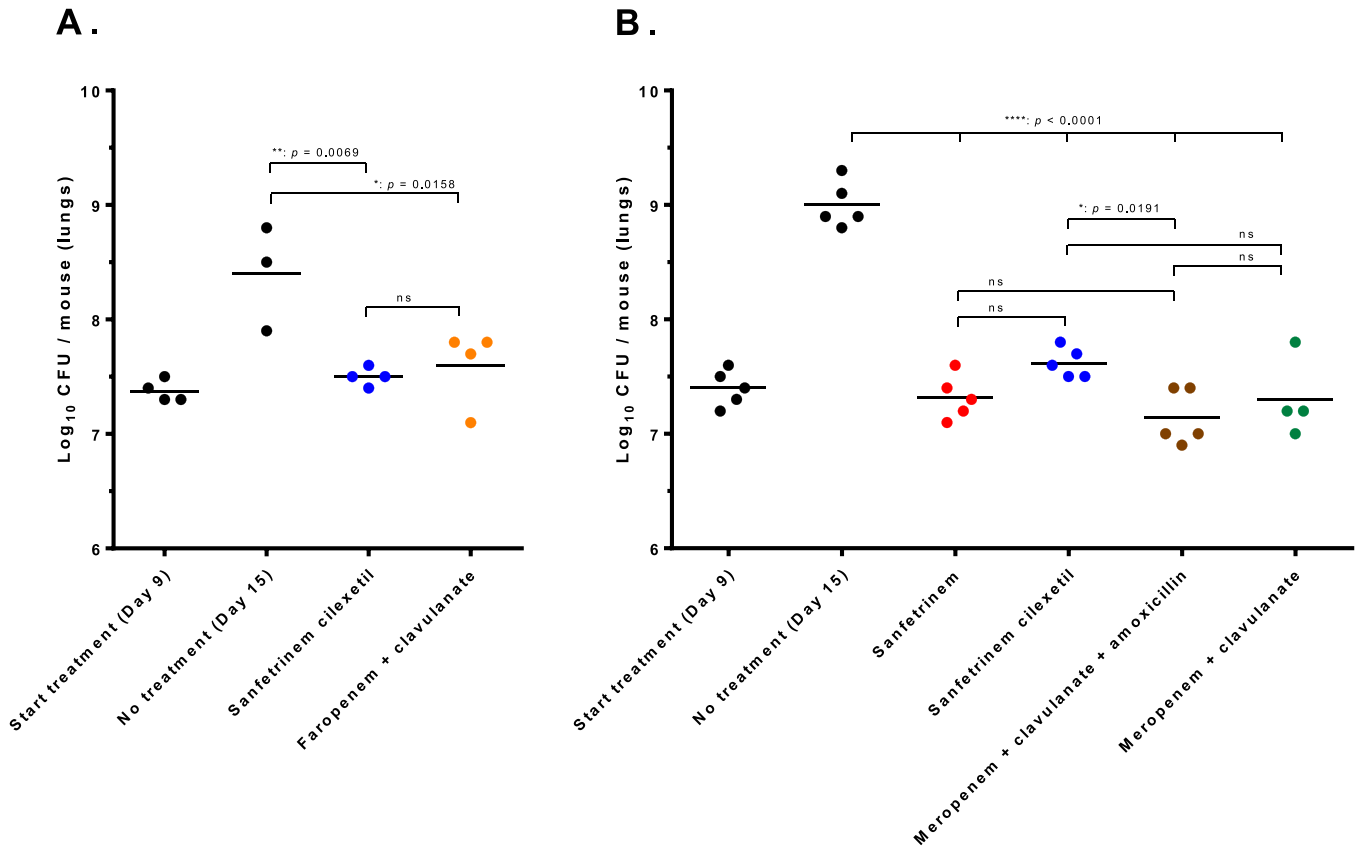


Fig. 8. Anti-tubercular activity of different β-lactams in an acute murine model of *M. tuberculosis* infection. Two independent experiments (**A** and **B**) were performed in which each point represents a data value from a single DHP-1 knockout mouse. Treatment was started nine days after infection and administered twice a day (bid) from day 9 to day 14 after infection. Lungs were harvested on day 15. Meropenem and sanfetrinem were administered subcutaneously and sanfetrinem cilexetil and all other β-lactams by oral gavage at the following doses (mg/kg bw): amoxicillin (200), faropenem (500), meropenem (300), sanfetrinem sodium salt (200), sanfetrinem cilexetil (400) and clavulanate (100). ANOVA analysis and Dunnett´s posttest versus the "No treatment (Day 15)" group indicates significant differences upon treatment for all groups (p-value <0.05). Bonferroni's multiple comparisons tests show significant differences among treatment groups. ns, no significant.

clavulanate improved the overall activity of sanfetrinem against the clinical panel, with a 4-fold increased activity (Fig. 3). This reduced shift in the presence of clavulanic acid, compared to the highly hydrolyzed amoxicillin or meropenem, might suggest that the tricyclic carbapenem backbone is inherently more stable to β-lactamase hydrolysis by BlaC, as previously described (Babini et al., 1998). In our studies, this shift was not observed with the laboratory *Mtb* H37Rv susceptible strain (Fig. 2), but it was clearly noticed against a rifampicin-resistant strain (**Figure S1**). That the effect of clavulanate on the activity of sanfetrinem could be better observed in a rifampicin-resistant strains, it is in agreement with previous reports describing a paradoxical hyper susceptibility of drug resistant *Mtb* to β-lactam antibiotics, which is further enhanced in the presence of clavulanate (Fig. 2, Table 1) (Cohen et al., 2016).

Any new potential drug developed for TB treatment needs to be included in a multi-drug regimen. As such, understanding how to better combine sanfetrinem could help to optimize its inclusion in future combinatorial treatments. In order to initiate the identification of potential companion drugs from an *in vitro* microbiological perspective, we performed synergy assays against a panel of clinically approved antibiotics, including several currently used in TB therapy. The interaction profile of sanfetrinem differed from that of meropenem (Fig. 6**A**). This was not unexpected since previous studies had shown that interaction profiles are β-lactam dependent (Ramón-García et al., 2016). Importantly, sanfetrinem displayed a strong interaction with amoxicillin. Currently, clavulanate is not commercially available and it needs to be added in clinical studies co-formulated with amoxicillin. The inclusion of amoxicillin/clavulanate together with meropenem has proven more effective against MDR/XDR strains than the compounds alone (Gonzalo and Drobniewski, 2013), and this could similarly apply to sanfetrinem. In our study, we observed that the strong interaction between sanfetrinem and amoxicillin against the laboratory strain *Mtb* H37Rv (Fig. 6**B**) was somehow masked in the presence of clavulanate (Fig. 7), which might be competing with the β-lactamase blocking activity of sanfetrinem (Prezelj et al., 2011). Nevertheless, the activity of the triple combination was slightly improved and greatly enhanced in combination with rifampicin. We also showed that sanfetrinem strongly interacts with rifampicin (Fig. 7). The interaction of β-lactams with rifamycins has been described previously *in vitro* (Kaushik et al., 2015) and *in vivo* (Kumar et al., 2017). Specifically, amoxicillin/clavulanate showed prevalent interactions with rifampicin and ethambutol against a panel of drug-susceptible and drug-resistant strains (Pagliotto et al., 2016). As an additional benefit, gastrointestinal side effects associated with the use of amoxicillin/clavulanate in prolonged therapy can be minimized within the synergistic interaction by reducing the dose. This approach is currently under evaluation in ongoing clinical trials aiming to reduce the treatment duration for Buruli ulcer, a disease caused by *Mycobacterium ulcerans* (NCT05169554, PACTR202209521256638), by co-administration of amoxicillin/clavulanate to current rifampicin plus clarithromycin WHO recommended therapy (Johnson et al., 2022).

Before embarking on a clinical study in TB, it is expected that a compound demonstrates *in vivo* efficacy in preclinical models. Unfortunately, β-lactams are notoriously difficult to test in rodents due to the high expression levels of DHP-1 enzymes which, like β-lactamases, hydrolyze the β-lactam core before the drug is able to reach the site of

action (Smith et al., 2018). To quantify the *in vivo* activity of sanfetrinem we employed a DHP-1 knockout model recently developed for the specific purpose of testing β-lactams against TB (Rullas et al., 2015). Impressively, both sanfetrinem administered subcutaneously and sanfetrinem cilexetil dosed orally demonstrated similar efficacy in the reduction of bacterial load in the lungs and in prevention of weight loss (**Figure S3**) compared to meropenem or faropenem co-dosed with clavulanic acid (Fig. 8). Levels of *in vivo* activity were comparable to other β-lactam studies (England et al., 2012; Solapure et al., 2013). Thus, two independent murine experiments confirmed the *in vivo* efficacy of sanfetrinem against *M. tuberculosis*, similar to other β-lactams already validated in clinical trials.

The concept of drug repurposing can be attractive in any therapeutic area, but particularly in the Global Health space with its limited opportunities for commercial return on investment. The opportunity to circumvent the protracted and expensive pre-clinical and early clinical development phases, proceeding directly to patient efficacy studies, is invaluable. Naturally, hurdles remain in any repurposing approach, particularly when the development program has been dormant for decades. In the case of sanfetrinem cilexetil, which showed variable bioavailability in previous clinical studies (GSK, unpublished data), a significant doubt remains as to whether sufficient lung exposure can be achieved and maintained to have an effect in TB treatment. The *in vitro* potencies against *Mtb* are lower than those observed for many of the Gram-negative and Gram-positive organisms that were targeted in previous Phase 2 studies. However, the *Mtb* potencies are likely underestimated due to the instability of sanfetrinem in longer MIC assays for slow growing bacteria. Additionally, the activity of sanfetrinem could be improved if dosed within a synergistic combination. The data here presented, together with the robust pre-clinical and clinical package from the 1990s, were considered sufficient to justify a proof-of-concept clinical study: a Phase 2a EBA study of sanfetrinem cilexetil in South Africa (NCT05388448).

Funding

This work was supported by grants from a People Programme (Marie Skłodowska Curie Actions) of the European Union's Seventh Framework Programme (FP7/2007–2013) under REA Agreement No. 291799 (Tres Cantos Open Lab Foundation - COFUND programme), from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 749058 to SRG, and from the Tres Cantos Open Lab Foundation (Grant No. TC144 and TC256) to SRG. This work was also funded in part by the Intramural Research Program of NIAID (NIH) to HB.

CRediT authorship contribution statement

Ramon Garcia Santiago: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **González del Río Rubén:** Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Arenaz-Callao María Pilar:** Investigation. **Boshoff Helena I.:** Resources, Investigation. **Rullas Joaquín:** Visualization, Methodology, Formal analysis. **Anca Sara:** Investigation. **Cacho Izquierdo Mónica:** Resources. **Porras de Francisco Esther:** Resources. **Pérez Herrán Esther:** Resources. **Santos-Villarejo Angel:** Investigation. **Mendoza-Losana Alfonso:** Writing – review & editing, Conceptualization. **Ferrer-Bazaga Santiago:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Thompson Charles J.:** Writing – review & editing, Supervision, Project administration. **Barros Aguirre David:** Supervision. **Bates Robert H.:** Writing – original draft, Supervision, Conceptualization.

Declaration of Competing Interest

RGdR, MPAC, JR, SA, MCI, EPdF, EPH, ASV, AML, SFB, DBA and RHB are or were employees of GSK, a producer of the generic drug amoxicillin/clavulanate and sanfetrinem. SRG, RGdR, AML, DBA and RHB are inventors of the patent describing the antituberculosis activity of sanfetrinem (WO2018206466A1). CJT declare no conflicts of interest. All authors approved the submission of the document.

Acknowledgements

We would like to acknowledge analytical support by Pablo Gamallo from GSK and Carlos Martín, from the University of Zaragoza, for the kind gift of the *M. tuberculosis* H37Rv and *M. bovis* BCG derived strains. We are also grateful to the many current and former GSK employees who have supported the clinical re-purposing effort.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.drup.2025.101213.

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