Developing synergistic drug combinations to restore antibiotic sensitivity in drug-resistant *Mycobacterium tuberculosis*

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BACKGROUND: *Mycobacterium tuberculosis* is the leading global cause of death owing to an infectious agent, accounting for approximately one in four antimicrobial resistance (AMR) fatalities annually.

OBJECTIVE: We aimed to identify synergistic two-drug combinations with the capacity to restore therapeutic efficacy against drug-resistant mutants of *M. tuberculosis*.

METHODS: Combinations were investigated containing the known translational inhibitors, spectinomycin (SPT) and fusidic acid (FA), or the phenothiazine antibiotic, chlorpromazine (CPZ), which disrupts mycobacterial energy metabolism.

RESULTS: Potentiation was observed between SPT and CPZ. This effect was lost against an *M. tuberculosis* mutant lacking the major facilitator superfamily (MFS) efflux pump, Rv1258c. Notably, the SPT-CPZ combination restored SPT efficacy against an SPT-resistant mutant carrying a g1379t point mutation in *rrs*, encoding the mycobacterial 16S ribosomal RNA. Combinations of SPT with FA, which targets the mycobacterial elongation factor G (EF-G), exhibited potentiating activity against wild-type *M. tuberculosis.* Moreover, this combination produced a small potentiating effect against defined FA-monoresistant and SPT-monoresistant mutants. Finally, combining SPT with the frontline anti-tuberculosis (TB) drugs, rifampicin and isoniazid, resulted in enhanced activity *in vitro* and *ex vivo* against both drug-susceptible *M. tuberculosis* and a RIF-monoresistant *rpoB* S531L mutant.

CONCLUSIONS: These results provide evidence of the utility of novel potentiating drug combinations in restoring susceptibility against *M. tuberculosis* strains carrying genetic resistance to any one of the partner drugs.

KEYWORDS: spectinomycin, spectinamide, chlorpromazine, fusidic acid, potentiation, efflux pump, Rv1258c

Introduction

The increasing prevalence of multidrug-resistant tuberculosis (MDR-TB) – defined as resistance to the frontline anti-TB agents, isoniazid (INH) and rifampicin (RIF) – necessitates the urgent development and implementation of new anti-mycobacterial drugs and therapeutic strategies.[1, 2] A number of anti-TB compounds are currently in the drug discovery pipeline, with several others in advanced preclinical development.[3] However, except for bedaquiline and delamanid, no TB-specific drugs have been introduced in the past 40 years.[4] Therefore, new options need to be explored to address the problem of drug resistance.

Novel combination regimens comprising standard anti-TB agents and repurposed drugs represent an attractive approach, especially where the new partner drug has already been approved for use in other clinical indications.[5-7] Moreover, recent advances in understanding the physicochemical properties which ensure drug distribution into the complex and variable tissue and cellular (micro)environments characteristic of active TB disease,[8, 9] as well as improved methods for rapid selection of multiple potentially synergistic drug partners *in vitro* and *in vivo*,[8, 10, 11,12] suggest the potential for rational development of novel combination approaches. This is important since it might address the long-held concern that developing combinations should be avoided owing to the complexities inherent in ensuring simultaneous and sustained delivery of the chosen partner compounds to the same target site.[11] The impact of pre-existing drug-resistance on the utility of new drug combinations – especially where these combinations comprise current frontline anti-TB agents – presents an additional challenge, particularly if the risks of exposing an individual to effective monotherapy are to be minimized. Consequently, for cases where resistance to individual drugs may exist, intelligent combination approaches for drug

therapies must be designed, and should incorporate multiple attributes beyond simply selecting biologically active individual molecules.[13]

In this study, we employed spectinomycin (SPT) as anchor compound in combination with other experimental antibiotics and existing frontline anti-TB agents. Our interest in SPT stems from the following: an aminocyclitol antibiotic which inhibits protein synthesis by disrupting mRNA interactions with the 30S ribosome[14], SPT has been used extensively in treating *Neisseria gonorrhoeae* infections in patients who cannot tolerate first-line treatments;[10] moreover, unlike other aminocyclitol antibiotics including gentamycin and kanamycin, SPT is not ototoxic.[15] From the perspecective of new regimen design, SPT has been shown in combination screens against *M. tuberculosis* to synergize with several different classes of antimycobacterial compounds, both *in vitro* and in a macrophage model.[10] Unfortunately, a key liability undermining its utility as a single agent is that SPT is subject to active efflux by *M. tuberculosis* – an observation which motivated a very elegant medicinal chemistry solution in the development of the spectinamides as derivative "efflux-resistant" anti-TB antibiotics.[16-18] Like SPT, spectinamides synergize with a variety of antibiotic classes[11]; moreover, the lead molecules are active against MDR *Mtb* strains and synergize with existing and experimental anti-TB drugs *in vivo*.[19] However, developing suitable spectinamide formulations for therapeutic delivery remains the major challenge to the advancement of these compounds as novel anti-TB agents.[20, 21]

We investigated the utility of identifying potentiating combinations which include SPT to circumvent drug resistance and, perhaps, restore (partial) susceptibility where genetically resistant mutants pre-exist to one of the partner compounds. To this end, we applied the checkerboard screening format utilizing (i) chlorpromazine (CPZ), a phenothiazine whose

complex and unresolved mechanism of action involving disruption of the mycobacterial electron transport chain[22] has been implicated in efflux pump inhibition;[23] and (ii) fusidic acid (FA), a translational inhibitor with demonstrated (albeit moderate) activity *in vitro*.[24, 25] FA was selected owing to its potential for repositioning as anti-TB agent[26] and because it possesses a unique mechanism of action: specifically, inhibition of bacterial protein synthesis by binding to elongation factor G (EF-G).[27] The antimicrobial-potentiating effect of FA with other antibiotics including the frontline anti-TB drug, ethambutol, as well as its lack of cross-resistance to other antimicrobial classes, provided additional motivation for our choice of FA.[12, 28] By testing the chosen combinations against both drug-susceptible *M. tuberculosis* H37Rv and selected drug-resistant mutants, we not only explore new potentiating combinations but also establish the utility of developing potent combinations against bacilli carrying pre-existing genetic resistance to either of the partner drugs. Notably, this work also reveals that the addition of SPT as third agent to the existing first-line anti-TB drug combination of rifampicin (RIF) and (INH) restores activity against defined pre-MDR mutants of *M. tuberculosis.*

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and solvents were purchased from Sigma-Aldrich. Working solutions of all antimicrobial agents were prepared in DMSO.

Bacterial strains and culture conditions

Freezer stocks of *M. tuberculosis* H37Rv and derivative mutants were inoculated in standard Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (Difco) at 37°C for approximately 3 days, sub-cultured, and grown further to attain an OD_{600} ~0.5. A reporter mutant, *M. tuberculosis* H37Rv::(pSMYC::mCherry)[29] that constitutively

expresses mCherry, was grown in media containing 50 mg/L hygromycin. Cell suspensions were then adjusted to give a final concentration of 10^5 cells/ml at the time of inoculation into the microplate for MIC assays.

Checkerboard assays

2D Checkerboard

Standard "two-dimensional" (2D) drug-drug interactions were determined by checkerboard titration in a 96-well plate **(Figure S1a)**. The 2D microdilution was carried out as described,[30] with slight modification. Briefly, the first serially diluted drug was dispensed (2 µl) along the x-axis (columns 3 to 11) at a starting concentration 100 times higher than the final concentration in the well and the second serially diluted (2 µl) drug in the y-axis (row B to H) at a starting concentration 100 times higher than the final concentration in the 96-well microtitre plate. The first column (column 1) and last column (column 12) contained drug-free controls and a control drug concentration giving maximum inhibition, respectively. The second column from B2-H2, and first row from A3-A11, contained individual drugs, thus providing the MIC for each drug alone in each assay. The plates were placed in zip-lock bags and incubated at 37 °C for 7 days. Resazurin dye was then added and the plates incubated for a further 24 h. Fluorescence readings were obtained using BMG Labtech POLARstar Omega plate reader (BMG Labtech, Offenburg, Germany, excitation: 544 nm; emission: 590 nm). Drug interactions were interpreted based on their inhibitory effects and the results plotted as % inhibition as a function of drug concentration.

3D Checkerboard

In the three-drug ("three-dimensional", 3D) combinations **(Figure S1b)**, microdilutions for the first two drugs were initially set up principally following the standard 2D checkerboard assay protocol described above. The third drug (2 µl) was then added at a starting concentration 100

times higher than the final concentration in the well as an overlay at sub-inhibitory concentrations ranging from 1/32 to 1/2 of the single-drug MIC. Well A2 on all plates contained the third drug only, providing the single-drug MIC for the third drug in each assay. After inoculation with a log-phase culture of *M. tuberculosis* (50 µl) to each well, the plates were placed in zip-lock bags and incubated for 7 days at 37°C before the results were read in the BMG Labtech plate reader (excitation 544 nm; emission 590 nm). The mean fluorescence value for the "maximum inhibition" wells (column 12) was subtracted from all other wells to control for background fluorescence. Percent inhibition was defined as 1-(test well fluorescence units/mean fluorescence units of maximum inhibition wells) × 100 on day 8 after incubation. The lowest drug concentration effecting inhibition of 90% was considered the MIC_{90}. Alamar Blue dye was used to visualize the 96-well microtitre plates.

Macrophage assays

Cell culture and maintenance

Human promonocytic THP-1 cells were maintained in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at an initial density of 8×10^5 cells/ml at 37°C in a humidified, 5% CO_2 atmosphere. Prior to plating of the cells, viability was assessed by trypan blue exclusion method[31]. Maturation of THP-1 cells into macrophages was induced by adding 200 nM PMA (phorbol 12-myristate 13-acetate; Sigma) in cell culture medium for 24 h. Differentiated macrophages were then washed with pre-warmed phosphate buffer saline (PBS) three times to remove the PMA and replenished with cell culture medium.

Infection of macrophages and drug treatment

To check the efficacy of drugs in macrophages, 5×10^4 THP-1 cells/well/100µl in 96-well flat-bottomed tissue culture plates were differentiated into macrophages. To infect macrophages, an

exponentially growing *M. tuberculosis* H37Rv::(pSMYC::mCherry) culture was harvested by centrifugation and washed twice with PBS. The pellet was resuspended in PBS and passed through a 5 µm filter to generate a single bacterial suspension. The bacterial density was quantified by measuring OD of bacterial suspension at 600 nm, correlating an OD_{600} ~0.5 to 1×10^8 CFU/ml. To make infection medium, the number of bacteria required to achieve a multiplicity of infection (MOI) of 5:1 (5 bacilli to one macrophage cell)[32] was added to the cell culture medium. The cells were overlaid with infection medium and incubated at 37°C in 5% CO_2 for the phagocytic period of 3 h. Thereafter, cells were washed gently and thoroughly with prewarmed PBS to remove extracellular bacteria. The cells from three wells were lysed in triton X-100 (0.05% in PBS) and plated onto 7H10 to score colony forming units (CFUs) for day zero. The cells used as un-treated control were refreshed with cell culture medium. The cells in remaining wells were treated with the indicated antibiotic either alone or in combination at 1× MIC_{90} or 5× MIC_{90}, as determined in liquid culture. Hygromycin was added into the cell culture medium used for both untreated or treated cells to maintain the plasmid expressing mCherry.

Fluorescence measurement

The fluorescence of the mCherry reporter was measured at different time points on a BMG Labtech plate reader (excitation 544 nm; emission 590 nm).

CFU enumeration

To count live bacteria after drug treatment, untreated and drug-treated *M. tuberculosis*-infected cells were lysed in triton X-100 (0.05% in PBS) on days 2, 4 and 6, and serial dilutions of the cell lysate were plated onto 7H10 agar. Colonies were counted after 3-4 weeks of incubation.

RESULTS

CPZ potentiates SPT activity by inhibiting Rv1258c-mediated efflux. The combination of SPT and CPZ was previously reported as synergistic against *M. smegmatis*.[33] When tested against wild-type *M. tuberculosis* H37Rv **(Fig 1a),** the same combination yielded a sum fractional inhibitory concentration (FIC) value of 0.09 **(Table 1)**, suggesting strong synergy.[34] We next investigated whether this effect resulted from CPZ-mediated disruption of the activity of the Major Facilitator Superfamily (MFS) efflux pump, Rv1258c, which has been implicated in innate resistance to SPT.[17] To investigate this possibility, we performed checkerboard assays using the efflux-defective Δ*Rv1258c* knockout mutant which was exploited in the development of the spectinamides (SPD),[17] as well as its complemented derivative, Δ*Rv1258c* pCRS4. Both mutants exhibited the same MIC_{90} for CPZ (22 mg/L; **Fig. 1** & **Table 1**), whereas the Δ*Rv1258c* knockout mutant was hypersusceptible to SPT, returning an MIC_{90} of 3.9 mg/L (**Fig. 1b** & **Table 1**), as observed previously.[35] Importantly, the synergy detected on exposing wild-type *M. tuberculosis* to a combination of CPZ and SPT (**Fig. 1a**) was eliminated in the Δ*Rv1258c* mutant (**Fig. 1b**) — yielding an FIC value of 0.76 **(Table 1)**, — but restored in the complemented Δ*Rv1258c* pCRS4 strain (**Fig. 1c**), FIC 0.12 **(Table 1)**. This observation suggested that CPZ abrogated efflux-mediated intrinsic resistance to SPT in wild-type *M. tuberculosis* in a manner dependent on Rv1258c, perhaps as a result of CPZ-mediated inhibition of energy metabolism.[22] Previous studies have reported no significant alteration in *Rv1258c* transcription in response to CPZ treatment,[36] supporting the conclusion that the inferred CPZ-SPT synergy is likely to derive from disrupted Rv1258c function in cells exposed to the phenothiazine.

A spontaneous SPT-resistant mutant, SPT^R, carrying a g1379t point mutation in the mycobacterial 16S ribosomal RNA, *rrs*, was associated with >64-fold increase in the SPT MIC_{90}. In contrast, CPZ retained activity against this strain – confirming a mechanism of action

independent of *rrs* inhibition.[37] Moreover, while SPT^R was resistant to SPT at concentrations >248 mg/L in the absence of CPZ, combinations utilizing CPZ at sub-MIC concentrations ([CPZ] ≤ 11.1 mg/L) restored SPT sensitivity, at least partially (**Fig. 1d**). These results suggested the capacity for synergistic combinations to restore drug activity against mutant strains genetically resistant to either of the partner compounds.

Antibiotics acting on the mycobacterial translational machinery exhibit synergy.

The systematic application of drug combinations can reveal interactions that result in synergy against bacteria. One form of synergy occurs when drug(s) which perturb normal cell physiology trigger (compensatory) cellular responses that can in turn affect (potentiate) the activities of other drugs.[38] For example, Nichols *et al*. demonstrated that the synergistic interaction between sulfamethoxazole and trimethoprim was a result of the two drugs targeting tetrahydrofolate biosynthesis.[39] With this in mind, a combination comprising SPT and FA – translation inhibitors which act at discrete steps of the elongation process – was tested against wild-type *M. tuberculosis* and two resistant strains, FA^R and SPT^R. As noted above, the SPT^R mutant carries a g1379t mutation in *rrs*.[33] Isolation of spontaneous FA^R mutants yielded a strain carrying a c1384t (H462Y) substitution in *fusA1* (*Rv0684*), encoding the essential mycobacterial elongation factor G (EF-G)[40]. The histidine residue is highly conserved across multiple bacterial species and, using the *T. thermophilus* structure as template,[41] it has been inferred that *M. tuberculosis* H462 corresponds to *T. thermophilus* H458,[42] mutations of which are likely to alter the FA-binding pocket.[43] In MIC assays, the H462Y mutant consistently yielded an MIC90 ≥25mg/L, confirming heritable FA^R.

With these strains in hand, we evaluated the interaction between SPT and FA and, furthermore, examined the potential to use potent combinations to counter pre-existing drug

resistance. The combination of SPT and FA returned a sum FIC value = 0.49 **(Table 2)** against wild-type H37Rv; upon addition of sub-MIC concentrations of FA ([FA] = 0.8 and 1.6 mg/L), the MIC_{90} of SPT exhibited a 4-fold decrease from 248 mg/L to 62 mg/L (**Fig. 2a**). Notably, FA restored the activity of SPT against a SPT^R mutant (**Fig 2b**). Although not manifest as a "synergistic" sum FIC **(Table 2),** the effect was marked and reproducible, with sub-MIC concentrations of FA (1.6 mg/L and 3.3 mg/L) achieving more than 90 % inhibition in combination with SPT. The same combination did not return any synergy against the FA^R mutant (**Fig 2c)**, identifying FA as the major contributor to the SPT-FA combination. **Table 3** summarizes the inhibitory effects demonstrated by the CPZ-SPT and FA-SPT combinations, respectively.

The three-drug combination of SPT with RIF and INH enhances activity against both susceptible and resistant *M. tuberculosis* strains.

The premise that synergistic combinations might be usefully applied to overcome existing drug resistance was further explored using SPT in combination with the frontline anti-TB agents, RIF and INH. In a 2D pairwise screening assay, two-drug permutations were performed utilizing RIF, INH and/or SPT. The RIF-INH combination showed no interaction (**Fig S2a)** while sub-MIC concentrations of RIF (0.0025 mg/L) and INH (0.63 mg/L) halved the effective SPT concentration from 125 mg/L to 62 mg/L (**Fig S2b & S2c**). To leverage the potential effect of SPT, a 3D combination assay was performed in which RIF and INH were titrated against decreasing sub-MIC_{90} concentrations of SPT (*i.e.*, 1/2×, 1/4×, 1/8×, 1/16× MIC_{90}), as illustrated in **Fig. S1b**. When tested against drug-susceptible *M. tuberculosis*, RIF at sub-MIC ([RIF] = 0.0006 mg/L) plus 1/2x MIC SPT resulted in a 4-fold decrease in the effective concentration of INH; from 0.125 mg/L to 0.03 mg/L (**Fig S3a**). A kill kinetics assay showed that the addition of

SPT to the RIF-INH combination elicited a 1 $\log_{10}$ unit reduction in the viable bacillary population following 8-day exposure to the drugs (**Fig. S3b**).

Since *M. tuberculosis* survives and replicates in macrophages,[44] the synergy of the RIF-INH plus SPT combination was evaluated against intracellular bacilli in *M. tuberculosis*-infected THP-1 cells. The three-drug combination showed inhibitory activity at 1× MIC_{90} of the combined drugs. (**Fig. S4**). In contrast, the inhibitory effect was reduced when similar concentrations of each drug were applied individually, or when the standard RIF-INH combination was used. The intracellular activity of this triple combination was further confirmed by CFU enumeration (**Fig. S5**) which revealed a 2-$\log_{10}$ decrease in CFU/ml when using 1× MIC_{90} RIF-INH-SPT compared to the untreated control. Finally, to evaluate the efficacy of RIF-INH-SPT against known drug-resistant strains, the combination was tested against two pre-MDR strains: a RIF-monoresistant mutant carrying the common *rpoB* S531L allele, and an INH-monoresistant strain harbouring an *inhA* promoter -C15T mutation that confers low-level INH resistance. Duplicate checkerboard experiments showed that, for the rpoBS531L mutant, addition of 1/2x MIC SPT to the RIF-INH plate resulted in a two-fold decrease in the effective concentrations of both RIF (0.005 mg/L) and INH (0.05 mg/L) (**Fig. 3**) – suggesting partial restoration of drug susceptibility in this strain. A similar effect was not observed for the INH^R mutant.

DISCUSSION

The capacity for evolution through mutagenesis means that it is difficult, even conceivably impossible, to overcome antibiotic resistance sustainably.[45] However, different approaches can be used to circumvent resistance transiently, ensuring efficacy despite the pre-existence of resistant organisms in an infecting population. Combination therapies represent one such

approach. In a previous study, Chen *et al*. demonstrated a synergistic interaction between SQ109 and RIF when tested against RIF^R isolates: at 0.5x MIC, SQ109 was able to increase RIF's activity against *de facto* resistant organisms in a dose-dependent manner.[30] Recently, Yang *et al* reported the enhanced efficacy of the imipenem-colistin combination against multiple drug-resistant *Enterobacter cloacae* using both *in vitro* activity and a *Galleria mellonela* model.[46] Very few studies, though, have been undertaken to illustrate the association between potentiating drug interactions and the ability of the particular drug combination to overcome pre-existing genetic resistance. In a clinical study, Ankomah *et al*. suggested that drugs acting synergistically can prevent treatment failure even when bacteria resistant to one of the drugs are present at the beginning of therapy.[47] Our interaction studies between SPT and CPZ **(Fig. 1)** reaffirmed the susceptibility of SPT to Rv1258c-mediated efflux, an observation which suggests that efforts to modify SPT – including through novel chemical modifications that engineer resistance to efflux[17] – should be pursued.

This study reveals interaction between FA and SPT against drug-susceptible bacteria via a mechanism independent of the efflux inhibition seen with SPT-CPZ. Notably, the same FA-SPT combination exerted a greater inhibitory effect against a genotypically confirmed SPT^R mutant compared to a FA^R mutant. Although there is no definitive explanation for this finding, we postulate that the relative potency of FA (~3.3-6.5 mg/L) against the drug-susceptible H37Rv parent compared to that of SPT (~50mg/L) could impact the FA-SPT combination against the SPT^R mutant, restoring susceptibility. A similar effect was not evident in a SPT-FA combination against FA^R mutant owing to the diminished activity of FA and high MIC_{90} of SPT. Of interest is the impact of individual active drugs in driving synergy.

Other explanations for potentiation include the sustained drug pressure emanating from the drug interactions which leads to an increased effective dose of the drug combination. Moreover, some studies have demonstrated that the drug susceptibility of pathogens can be significantly enhanced as a result of a reduced efflux pump efficiency either by genetic manipulation[48] or addition of efflux pump inhibitors.[49, 50] The clinical relevance of this finding is that, despite the existence of bacterial resistance against a combination partner, it would still be possible to achieve optimal therapeutic outcomes with the use of appropriate potent drug combinations.

Previous studies have demonstrated the potential of having three-drug combinations when compared to individual or two-drug regimens.[51, 52] Recently, Tekin *et al*. reported that combinations of three different antibiotics can often overcome bacteria resistance to antibiotics, even when none of the three antibiotics on their own — or even two of the three together — is effective.[53] In addition, based on drug interaction studies, Ramon-Garcia *et al*. hypothesized that the synergistic activity of the triplet combination might have multiplicative effects.[10] Here, SPT was deployed as part of a three-drug regimen which also included RIF and INH, the two drugs that form the cornerstone of TB treatment. Previous studies have shown the interaction between RIF and INH against *M. tuberculosis* to have no interaction or to be mildly antagonistic.[8, 54] The inclusion of SPT in this drug regimen was underpinned by studies elsewhere, which reported that 24 out of 70 random combinations tested were synergistically active in *M. smegmatis*.[10] This suggests a large unexplored pool of synergistic combinations. Notably, SPT exhibited synergy with several compounds both *in vitro* and *ex vivo*[10] even though the compound has high MIC_{90} against *M. tuberculosis* when administered on its own.[17]

In the three-drug combination assay, synergy resulted when sub-inhibitory concentrations (1/2x and 1/4x MICs) of SPT were titrated into media containing RIF and INH **(Fig. S3a)**. This finding correlated well with the results of time-kill kinetics **(Fig. S3b)**. The time-kill assay suggested, however, that the inhibitory effect of this three-drug interaction was bacteriostatic (0≥ $\log_{10}$ CFU <3 reduction) and not bactericidal. This observation reveals that the combination of RIF and INH — two bactericidal drugs that are most potent with actively dividing cells — shows bacteriostatic effects. Furthermore, the inhibition of growth induced by a bacteriostatic drug, SPT, results in an overall static effect.when the drug is used in combination with a bactericidal drug. Other studies have shown that in similar interactions, the resulting effect achieves a more efficient clearance at lower concentrations.[35]

In attempting to exploit synergy for potential optimal treatment outcomes, an investigation of the RIF-INH plus SPT interaction was performed in *rpoB* and *inhA* mutants. The RIF *rpoB* resistant strain had an MIC value >50 times the MIC_{90} for drug susceptible *M. tuberculosis* **(Table S1)**. **Fig. 3a** shows that the addition of 1/2 and 1/4× MICs of SPT also facilitated inhibition of the drug resistant *M. tuberculosis* strain. As with the drug susceptible H37Rv strain, a mechanistic explanation for the synergy between RIF-INH plus SPT combination against the *rpoB* mutant is presently lacking. INH targets mycobacterial cell wall synthesis, possibly enhancing permeation of SPT into the bacilli. However, access alone may not necessarily contribute to the synergistic interaction. Chen *et al*. reported synergy between SQ109, a presumed cell wall synthesis inhibitor, and RIF.[30] On the other hand EMB, which also affects mycobacterial cell wall synthesis, did not exhibit synergy with RIF.[55]

Studies have shown RIF to be an efficient inducer of cytochrome P450 (CYP 450), a superfamily of haem-containing enzymes involved in the biosynthesis of compounds such as

sterols, steroids, and fatty acids as well as detoxification of xenobiotics and chemicals.[56] RIF has been linked with the induction of CYP both in humans and in *M. tuberculosis*.[57] The elevated levels of CYP have been associated with drug resistance due to the enhanced rate of elimination of the drugs by metabolism and detoxification pathways. INH, on the other hand, inhibits CYP in *M. tuberculosis*.[57] This ability of INH to inhibit CYP may contribute to synergy in the RIF-INH plus SPT combination when the active form of INH is not rapidly eliminated inside *M. tuberculosis* and when SPT acts by further reducing the activiy of CYPs.

There are prospects to combine SPT with RIF-INH. SPT is given by intramuscular injection to achieve therapeutic concentrations in serum of about 100 mg/L 1 h after a single 2-g dose; therefore, a ≥ 4-fold increase in its effectiveness within a triple SPT-RIF-INH combination would potentially allow for oral formulation. This is critical, especially in treating TB out-patients. In summary, these *in vitro* and *ex vivo* results suggests that the RIF-INH plus SPT triple-combination may be an effective therapeutic option for the treatment of both the drug susceptible and resistant *M. tuberculosis* infections. They also reinforce a growing body of evidence supporting the utility of drug potentiation strategies in improving treatment outcomes.

Acknowledgements

This work was supported by the University of Cape Town, the Strategic Health Innovations Partnership (SHIP) unit of the South African Medical Research Council (SAMRC) (to KC and DFW), the SAMRC (to KC and VM), the South African Research Chairs Initiative (to KC) and Department of Science and Technology administered through the National Research Foundation, and the Schlumberger Foundation Faculty for the Future scheme (to EK and AW). We thank

Prof. J.A. Aínsa for providing the *M. tuberculosis* Δ*Rv1258c* knockout mutant and its complemented derivative, and Dr. Krupa Naran for advice with checkerboard assays.

Funding

The University of Cape Town, Novartis Research Foundation, South African Medical Research Council, and South African Research Chairs Initiative of the Department of Science and Technology, administered through the South African National Research Foundation are gratefully acknowledged for support (K.C). and Carnegie Mellon Scholarship Awards.

Transparency declarations

None to declare.

REFERENCES

1. Sullivan T, Ben Amor Y. Global Introduction of New Multidrug-Resistant Tuberculosis Drugs-Balancing Regulation with Urgent Patient Needs. Emerg Infect Dis 2016; 22.
2. Hoagland DT, Liu J, Lee RB et al. New agents for the treatment of drug-resistant Mycobacterium tuberculosis. Adv Drug Deliv Rev 2016; 102: 55-72.
3. Evans JC, Mizrahi V. Priming the tuberculosis drug pipeline: new antimycobacterial targets and agents. Curr Opin Microbiol 2018; 45: 39-46.
4. Zumla A, Nahid P, Cole ST. Advances in the development of new tuberculosis drugs and treatment regimens. Nat Rev Drug Discov 2013; 12: 388-404.
5. Nzila A, Ma Z, Chibale K. Drug repositioning in the treatment of malaria and TB. Future Med Chem 2011; 3: 1413-26.
6. Oprea TI, Bauman JE, Bologa CG et al. Drug Repurposing from an Academic Perspective. Drug Discov Today Ther Strateg 2011; 8: 61-9.
7. Zumla AI, Schito M, Maeurer M. Advancing the portfolio of tuberculosis diagnostics, drugs, biomarkers, and vaccines. Lancet Infect Dis 2014; 14: 267-9.
8. Cokol M, Kuru N, Bicak E et al. Efficient measurement and factorization of high-order drug interactions in Mycobacterium tuberculosis. Sci Adv 2017; 3: e1701881.
9. Greenwood DJ, Dos Santos MS, Huang S et al. Subcellular antibiotic visualization reveals a dynamic drug reservoir in infected macrophages. Science 2019; 364: 1279-82.
10. Ramon-Garcia S, Ng C, Anderson H et al. Synergistic drug combinations for tuberculosis therapy identified by a novel high-throughput screen. Antimicrob Agents Chemother 2011; 55: 3861-9.
11. Bruhn DF, Scherman MS, Liu J et al. In vitro and in vivo Evaluation of Synergism between Anti-Tubercular Spectinamides and Non-Classical Tuberculosis Antibiotics. Sci Rep 2015; 5: 13985.
12. Yilancioglu K, Cokol M. Design of high-order antibiotic combinations against M. tuberculosis by ranking and exclusion. Sci Rep 2019; 9: 11876.
13. Brooks BD, Brooks AE. Therapeutic strategies to combat antibiotic resistance. Adv Drug Deliv Rev 2014; 78: 14-27.
14. Hoeksema H, Knight JC. The production of dihydrospectinomycin by Streptomyces spectabilis. J Antibiot (Tokyo) 1975; 28: 240-1.
15. Novak E, Gray JE, Pfeifer RT. Animal and human tolerance of high-dose intramuscular therapy with spectinomycin. J Infect Dis 1974; 130: 50-5.
16. Cully M. Antibacterial drugs: Redesigned antibiotic combats drug-resistant tuberculosis. Nat Rev Drug Discov 2014; 13: 256.
17. Lee RE, Hurdle JG, Liu J et al. Spectinamides: a new class of semisynthetic antituberculosis agents that overcome native drug efflux. Nat Med 2014; 20: 152-8.
18. Liu J, Bruhn DF, Lee RB et al. Structure-Activity Relationships of Spectinamide Antituberculosis Agents: A Dissection of Ribosomal Inhibition and Native Efflux Avoidance Contributions. ACS Infect Dis 2017; 3: 72-88.
19. Robertson GT, Scherman MS, Bruhn DF et al. Spectinamides are effective partner agents for the treatment of tuberculosis in multiple mouse infection models. J Antimicrob Chemother 2017; 72: 770-7.
20. Rathi C, Lukka PB, Wagh S et al. Comparative pharmacokinetics of spectinamide 1599 after subcutaneous and intrapulmonary aerosol administration in mice. Tuberculosis (Edinb) 2019; 114: 119-22.
21. Stewart IE, Lukka PB, Liu J et al. Development and Characterization of a Dry Powder Formulation for Anti-Tuberculosis Drug Spectinamide 1599. Pharm Res 2019; 36: 136.
22. Weinstein EA, Yano T, Li LS et al. Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs. Proc Natl Acad Sci U S A 2005; 102: 4548-53.
23. Jaiswal I, Jain A, Verma SK et al. Effect of efflux pump inhibitors on the susceptibility of Mycobacterium tuberculosis to isoniazid. Lung India : official organ of Indian Chest Society 2017; 34: 499-505.

24. Castanheira M, Watters AA, Bell JM et al. Fusidic acid resistance rates and prevalence of resistance mechanisms among Staphylococcus spp. isolated in North America and Australia, 2007-2008. Antimicrob Agents Chemother 2010; 54: 3614-7.
25. Collignon P, Turnidge J. Fusidic acid in vitro activity. Int J Antimicrob Agents 1999; 12 Suppl 2: S45-58.
26. Tsang TF, Qiu Y, Lin L et al. Simple Method for Studying in Vitro Protein-Protein Interactions Based on Protein Complementation and Its Application in Drug Screening Targeting Bacterial Transcription. ACS Infect Dis 2019; 5: 521-7.
27. Turnidge J. Fusidic acid pharmacology, pharmacokinetics and pharmacodynamics. Int J Antimicrob Agents 1999; 12 Suppl 2: S23-34.
28. Fuursted K, Askgaard D, Faber V. Susceptibility of strains of the Mycobacterium tuberculosis complex to fusidic acid. APMIS 1992; 100: 663-7.
29. Carroll P, Schreuder LJ, Muwanguzi-Karugaba J et al. Sensitive detection of gene expression in mycobacteria under replicating and non-replicating conditions using optimized far-red reporters. PLoS One 2010; 5: e9823.
30. Chen P, Gearhart J, Protopopova M et al. Synergistic interactions of SQ109, a new ethylene diamine, with front-line antitubercular drugs in vitro. J Antimicrob Chemother 2006; 58: 332-7.
31. Strober W. Trypan blue exclusion test of cell viability. Current protocols in immunology 2001; Appendix 3: Appendix 3B.
32. Stokes RW, Doxsee D. The receptor-mediated uptake, survival, replication, and drug sensitivity of Mycobacterium tuberculosis within the macrophage-like cell line THP-1: a comparison with human monocyte-derived macrophages. Cell Immunol 1999; 197: 1-9.
33. Kigondu EM, Wasuna A, Warner DF et al. Pharmacologically active metabolites, combination screening and target identification-driven drug repositioning in antituberculosis drug discovery. Bioorg Med Chem 2014; 22: 4453-61.
34. Odds FC. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother 2003; 52: 1.
35. Lee A, Mao W, Warren MS et al. Interplay between efflux pumps may provide either additive or multiplicative effects on drug resistance. J Bacteriol 2000; 182: 3142-50.
36. Li P, Gu Y, Li J et al. Mycobacterium tuberculosis Major Facilitator Superfamily Transporters. J Membr Biol 2017; 250: 573-85.
37. Rubin H. The respiratory chain of M. tuberculosis. The FASEB Journal 2007; 21: A207-A.
38. Bollenbach T. Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution. Curr Opin Microbiol 2015; 27: 1-9.
39. Nichols RJ, Sen S, Choo YJ et al. Phenotypic landscape of a bacterial cell. Cell 2011; 144: 143-56.
40. DeJesus MA, Gerrick ER, Xu W et al. Comprehensive Essentiality Analysis of the Mycobacterium tuberculosis Genome via Saturating Transposon Mutagenesis. mBio 2017; 8: e02133-16.
41. Gao YG, Selmer M, Dunham CM et al. The structure of the ribosome with elongation factor G trapped in the posttranslocational state. Science 2009; 326: 694-9.
42. Ramakrishnan G, Chandra NR, Srinivasan N. Recognizing drug targets using evolutionary information: implications for repurposing FDA-approved drugs against Mycobacterium tuberculosis H37Rv. Mol Biosyst 2015; 11: 3316-31.
43. Besier S, Ludwig A, Brade V et al. Molecular analysis of fusidic acid resistance in Staphylococcus aureus. Mol Microbiol 2003; 47: 463-9.
44. Takaki K, Davis JM, Winglee K et al. Evaluation of the pathogenesis and treatment of Mycobacterium marinum infection in zebrafish. Nature Protocols 2013; 8: 1114.
45. Wright GD. Antibiotic Adjuvants: Rescuing Antibiotics from Resistance: (Trends in Microbiology 24, 862-871; October 17, 2016). Trends Microbiol 2016; 24: 928.

46. Yang H, Chen G, Hu L et al. Enhanced efficacy of imipenem-colistin combination therapy against multiple-drug-resistant Enterobacter cloacae: in vitro activity and a Galleria mellonella model. J Microbiol Immunol Infect 2016.
47. Ankomah P, Johnson PJ, Levin BR. The pharmaco -, population and evolutionary dynamics of multi-drug therapy: experiments with S. aureus and E. coli and computer simulations. PLoS Pathog 2013; 9: e1003300.
48. Lomovskaya O, Lee A, Hoshino K et al. Use of a genetic approach to evaluate the consequences of inhibition of efflux pumps in Pseudomonas aeruginosa. Antimicrob Agents Chemother 1999; 43: 1340-6.
49. Markham PN, Neyfakh AA. Inhibition of the multidrug transporter NorA prevents emergence of norfloxacin resistance in Staphylococcus aureus. Antimicrob Agents Chemother 1996; 40: 2673-4.
50. Markham PN. Inhibition of the emergence of ciprofloxacin resistance in Streptococcus pneumoniae by the multidrug efflux inhibitor reserpine. Antimicrob Agents Chemother 1999; 43: 988-9.
51. Cokol-Cakmak M, Bakan F, Cetiner S et al. Diagonal Method to Measure Synergy Among Any Number of Drugs. J Vis Exp 2018.
52. Cokol M, Weinstein ZB, Yilancioglu K et al. Large-scale identification and analysis of suppressive drug interactions. Chem Biol 2014; 21: 541-51.
53. Tekin E, Beppler C, White C et al. Enhanced identification of synergistic and antagonistic emergent interactions among three or more drugs. J R Soc Interface 2016; 13.
54. Bhusal Y, Shiohira CM, Yamane N. Determination of in vitro synergy when three antimicrobial agents are combined against Mycobacterium tuberculosis. Int J Antimicrob Agents 2005; 26: 292-7.
55. Torella JP, Chait R, Kishony R. Optimal drug synergy in antimicrobial treatments. PLoS Comput Biol 2010; 6: e1000796.
56. Sarathy JP, Zuccotto F, Hsinpin H et al. Prediction of Drug Penetration in Tuberculosis Lesions. ACS Infect Dis 2016; 2: 552-63.
57. Zhang L, Yan K, Zhang Y et al. High-throughput synergy screening identifies microbial metabolites as combination agents for the treatment of fungal infections. Proc Natl Acad Sci U S A 2007; 104: 4606-11.

Figures and Table

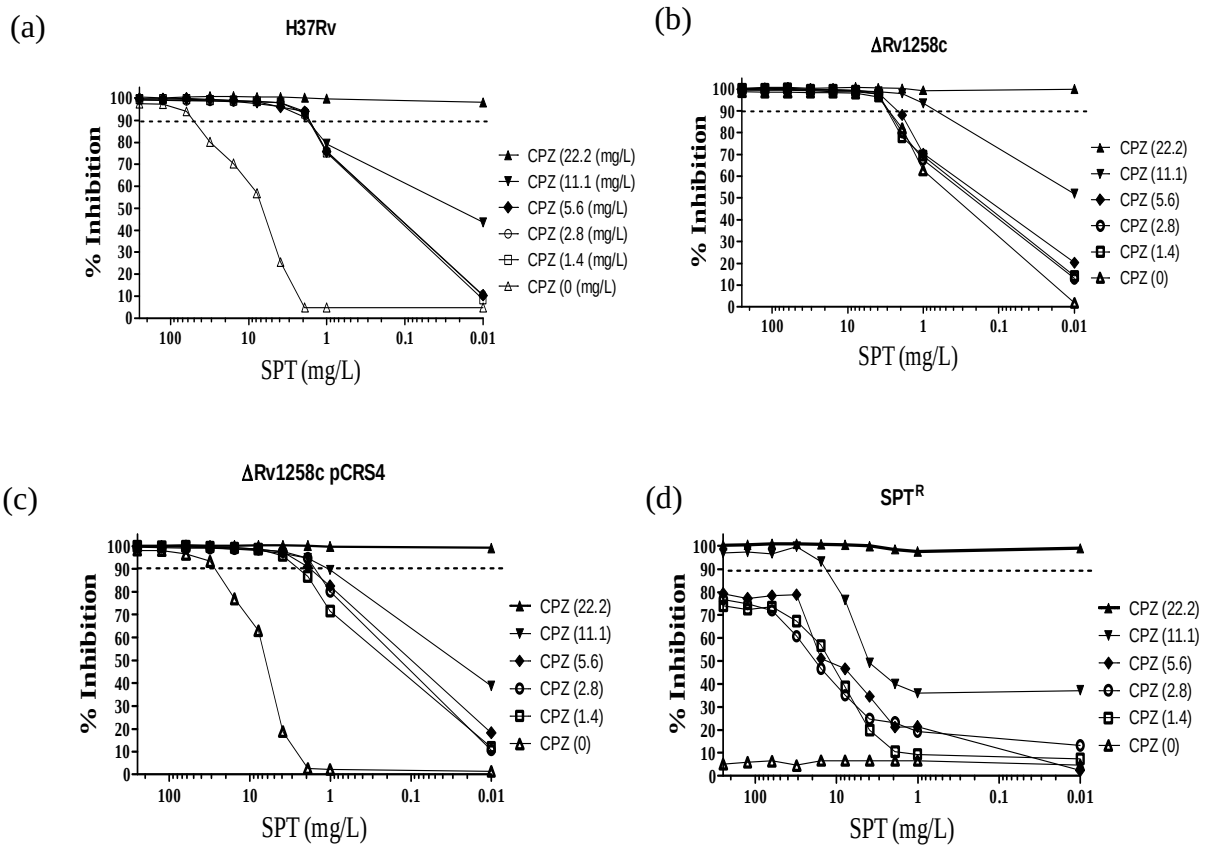

Figure 1. Inhibition of Rv1258c-mediated efflux by the synergistic interaction between SPT and CPZ**.** Combinations comprising CPZ and SPT were applied in checkerboard assays against (*a*) wild-type H37Rv, (*b*) Δ*Rv1258c*, (*c*) Δ*Rv1258c* pCRS4, and (*d*) SPT^R mutant. Bacterial viability was assessed in two independent experiments by fluorescence-based resazurin assay. The numbers in parentheses denote the concentration of CPZ (mg/L). The dashed horizontal line indicates 90% inhibition.

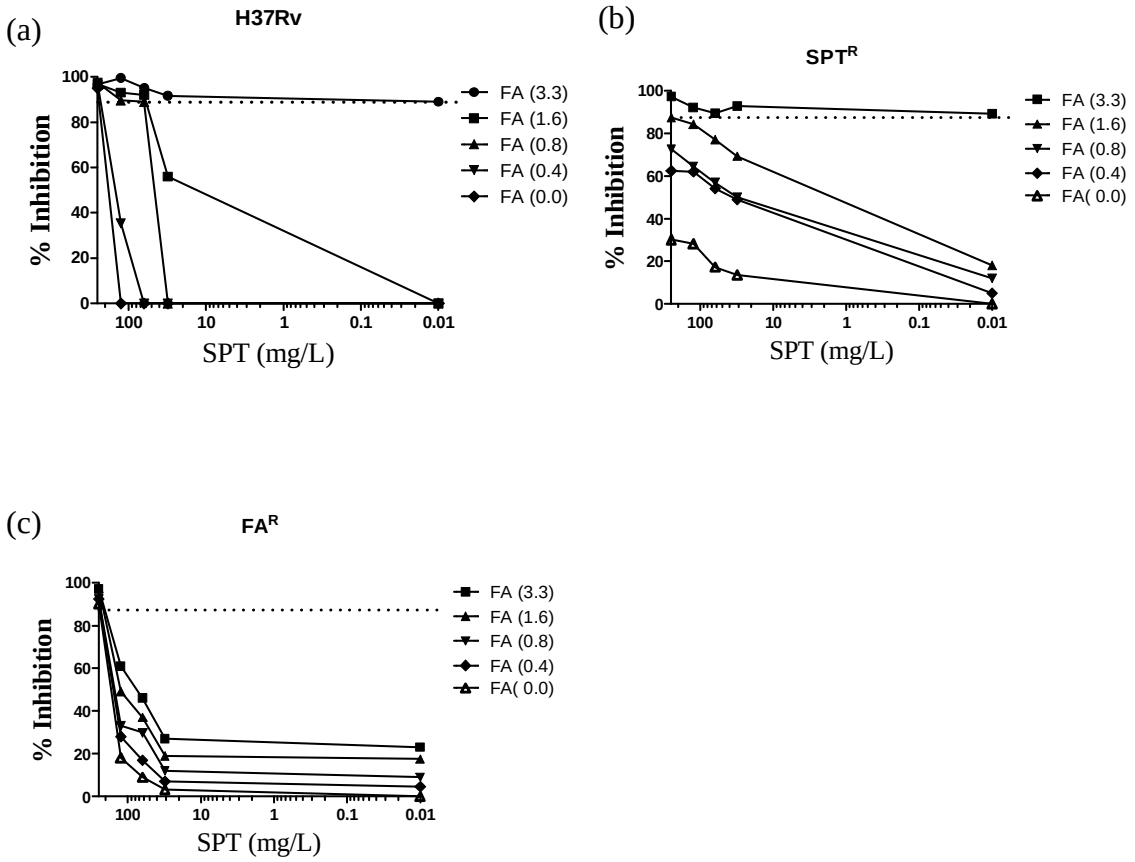

Figure 2. *In vitro* interaction between SPT and FA**.** Combinations comprising SPT and FA were applied in checkerboard assays against (*a*) wild-type H37Rv and (*b*) the SPT resistant mutant, SPT^R. Bacterial viability was assessed in two independent experiments by fluorescence-based resazurin assay. The numbers in parentheses denote the concentration of FA (mg/L). The dashed horizontal line indicates 90% inhibition.

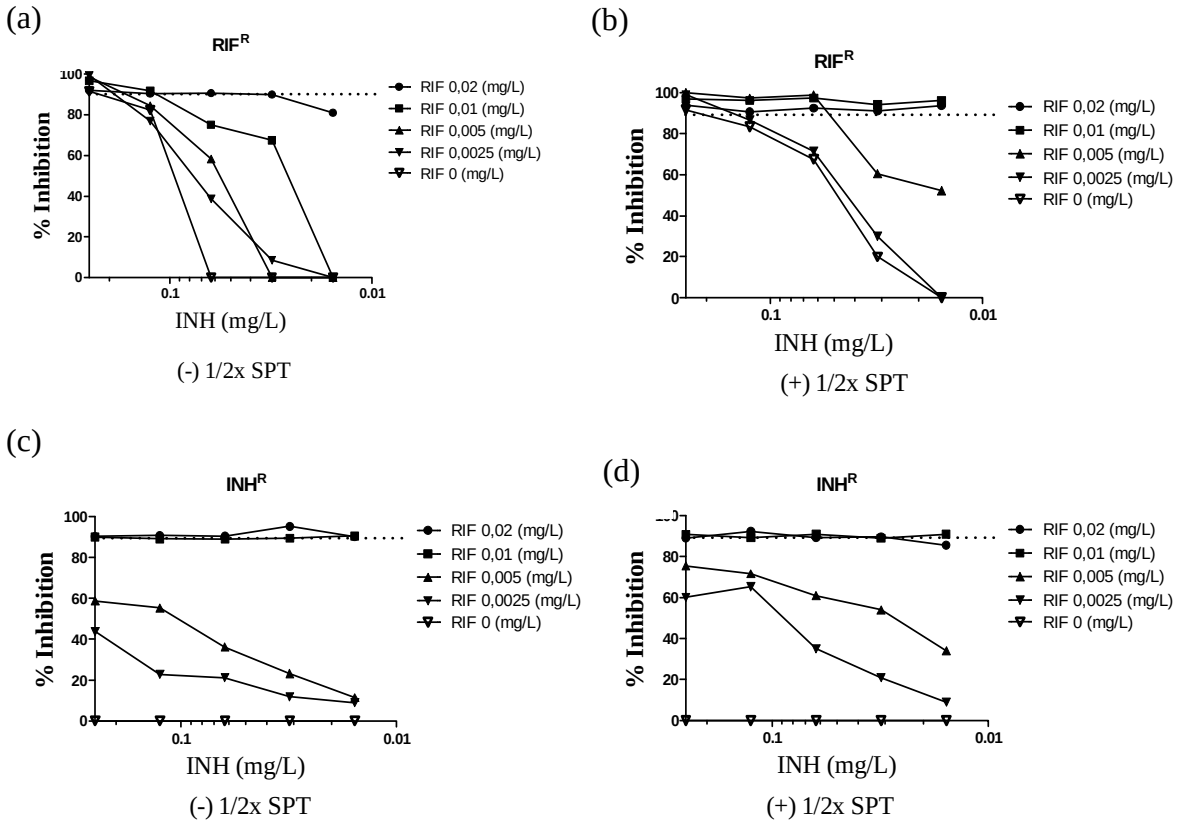

Figure 3. Activity against pre-MDR *M. tuberculosis. In vitro* activity of RIF-INH against RIF^{R} *M. tuberculosis rpoB*^{S531L} mutant in the absence (a) or presence (b) of 1/2x MIC SPT, and *M. tuberculosis* INH^{R} *inhA* mutant in the absence (c) or presence (d) with 1/2x MIC SPT. Bacterial viability was assessed in two independent experiments by fluorescence-based resazurin assay. The numbers in parentheses denote the concentration of RIF (mg/L). The dashed horizontal line indicates 90% inhibition

Table 1 Checkerboard titration of SPT and CPZ

Mycobacterium mutant	**Drug combination**	**MIC (mg/L)**		**ΣFIC**
		Alone	**Combination**	
H37Rv	SPT	62	1.9	0.09
	CPZ	22.2	1.4	
Δ*Rv1258c*	SPT	3.9	1.0	0.76
	CPZ	22.2	11.1	
Δ*Rv1258c* pCRS4	SPT	31.0	1.9	0.12
	CPZ	22.2	1.4	
SPT^R	SPT	>248	15.5	0.56
	CPZ	22.2	11.1	

Drug interactions were determined by checkerboard titration of SPT and CPZ by a 96-well microdilution assay described previously using *M. tuberculosis* H37Rv. The FIC of each drug was calculated as (MIC of drug in combination)/(MIC of individual drug). The ΣFIC is the sum of the FICs of the two drugs. ΣFIC ≤0.5 is synergistic; ≥4.0 is antagonistic and in between is additive or no interaction.

Table 2 Checkerboard titration of FA and SPT.

Mycobacterium mutant	**Drug combination**	**MIC (mg/L)**		**ΣFIC**
		Alone	**Combination**	
H37Rv	SPT	62	15.5	0.49
	FA	3.3	0.8	
SPT^R	SPT	≥248	124	≤1.0
	FA	6.5	3.3	
FA^R	SPT	62	31.5	≥1.5
	FA	≥25	≥25	

Drug interactions were determined by checkerboard titration of FA by a 96-well microdilution assay described previously using *M. tuberculosis* H37Rv.

Table 2 In vitro activities of drugs/combinations against M. tuberculosis, wild-type and mutant strains.

Drugs/Combination			
Strains	**CPZ**	**SPT**	**CPZ-SPT**
WT	✓	✗	⊙
Rv1258c	✓	✓	⊗
Rv1258c::WT	✓	✗	⊙
SPT^R	✓	✗	⊙

Drugs/Combination			
Strains	**FA**	**SPT**	**FA-SPT**
WT	✓	✗	⊙
FA^R	✗	✗	⊗
SPT^R	✓	✗	⊙

Legend:

✓	$MIC_{90} \leq 10mg/L$
✗	$MIC_{90} > 10mg/L$
⊙	Synergy
⊗	No interaction