Synergistic interactions of SQ109, a new ethylene diamine, with front-line antitubercular drugs in vitro

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Objectives: The purpose of this study was to determine interactions of SQ109, a new asymmetric diamine tuberculosis (TB) drug candidate, with existing antitubercular drugs in vitro and assess its potential to improve combination drug activities against Mycobacterium tuberculosis.

Methods: Two-drug combinations at various concentrations below their MICs were tested for growth inhibition of M. tuberculosis using the BACTEC 460 system in vitro. Drug interactions were evaluated based on the quotient values that were derived numerically from the growth indices of cultures treated with a single antibiotic or combination treatment with two antibiotics.

Results: SQ109 at 0.5 of its MIC demonstrated strong Synergistic activity with 0.5MIC isoniazid and as low as 0.1 MIC rifampicin in inhibition of M. tuberculosis growth. Additive effects were observed between SQ109 and streptomycin, but neither synergy nor additive effects were observed with the combination of SQ109 with ethambutol or pyrazinamide. The synergy between SQ109 and rifampicin was also demonstrated using rifampicin-resistant (RIFR) M. tuberculosis strains; SQ109 lowered the MIC of rifampicin for these drug-resistant strains.

Conclusions: SQ109 interacts Synergistically with isoniazid and rifampicin, two of the most important front-line TB drugs. This finding supports efforts to further evaluate new combination therapies containing SQ109 in experimental animal models of TB that emulate future clinical trial studies in humans.

Keywords: antibiotics, SQ109, rifampicin, isoniazid, tuberculosis, Mycobacterium tuberculosis, in vitro, combination treatment, synergy

Introduction

The current WHO1 and CDC2 recommended treatment of active pulmonary TB patients infected with drug-susceptible Mycobacterium tuberculosis requires four drugs to be delivered concurrently for at least 6 months in a staged therapeutic regimen: isoniazid, rifampicin, ethambutol and pyrazinamide are given daily for 2 months (intensive phase), and isoniazid and rifampicin are continued, usually daily, for an additional 4–7 months (continuation phase). Failure to provide adequate drugs to patients, or failure of patients to complete the long-term therapy with all four drugs, results in the emergence of drug-resistant M. tuberculosis. In order to control the current TB epidemic and finally eradicate the disease, new and more potent drug combinations that can shorten treatment of active disease and eliminate latent M. tuberculosis from asymptomatic patients are desperately needed.

Using combinatorial chemistry, a 67 238 compound library was generated based on a [1,2]-ethylenediamine pharmacophore of ethambutol, one of the four drugs recommended in the current therapeutic regimen for treatment of active pulmonary TB. The library was screened for compounds that (i) activate a genetic sensor in live recombinant M. tuberculosis to signal cell wall disruption and (ii) inhibit M. tuberculosis growth in culture. SQ109 was selected as best in class of this library, the lead drug candidate, with high potency against live M. tuberculosis (MIC range 0.16–0.64 mg/L in more than 30 drug-susceptible and drug-resistant laboratory and clinical isolates, P. Chen and F. Barbosa, unpublished data), low cytotoxicity in cultured mammalian cells, efficacy in killing M. tuberculosis within infected macrophages and in experimental animals as a single agent, and drug-like pharmacokinetic and pharmacodynamic profiles. More importantly, SQ109 differed in activity against M. tuberculosis from the original active pharmacophore and differed in...
the genes that were up- or down-regulated in drug-treated *M. tuberculosis*. Thus, SQ109 is not a better ethambutol: it is a totally different and very potent new antimicrobial in the broader class of diamine antibiotics.

Any new proposed anti-TB drug will be used in combination with other drugs in order to prevent the emergence of drug-resistant *M. tuberculosis*. How SQ109, a new drug compound with unique attributes, interacts with existing first-line anti-TB agents is important to understand, as this will enable us to design the most efficacious therapeutic combination and to avoid antagonistic side effects in a new combination therapy. In the present study, we investigated the interaction of SQ109 in combination with other anti-TB drugs in vitro.

**Materials and methods**

**Antimicrobial drugs**

Isoniazid, rifampicin, streptomycin and ethambutol were purchased from Sigma-Aldrich (St Louis, MO, USA). Stock solutions of isoniazid, streptomycin and ethambutol were prepared in distilled and deionized water at 10 mg/mL, sterilized by filtration and stored frozen at −80°C. Stock solutions of rifampicin, at 1 or 10 mg/mL, were prepared in methanol and stored at −80°C. Pyrazinamide was purchased as the drug reconstituting kit from Becton Dickinson (Cockeysville, MD, USA) and a stock solution was prepared following instructions by the manufacturer. Stock solutions of SQ109 were prepared in methanol at 1 mg/mL and stored at −80°C.

*M. tuberculosis* strains

*M. tuberculosis* strain H37Rv was the same strain used in previous studies documenting activity of chemical compounds in our library. The mono rifampicin-resistant (RIF) *M. tuberculosis* clinical isolates used in the study were obtained from the Department of Health and Mental Hygiene, Central Laboratory, State of Maryland. The drug susceptibility profiles of strains 3185 and 2482 were previously determined at the state laboratory and later confirmed at Sequella. Both strains were susceptible to 0.1 mg/L isoniazid, 2.5 mg/L ethambutol or 2 mg/L streptomycin. Rifampicin MIC was 24 mg/L for strain 3185 and >100 mg/L for strain 2482. The nature of *spoT* mutations associated with rifampicin resistance was not determined.

**Media**

Middlebrook 7H11 agar (Difco, Becton Dickinson) supplemented with 10% OADC (Difco) was used to grow *M. tuberculosis* for BACTEC inocula. BACTEC 7H12B medium (Becton Dickinson) was used for all drug combination experiments except for pyrazinamide + SQ109: this combination was evaluated in PZA Test Medium (Becton Dickinson).

**Assessment of drug effects on *M. tuberculosis* growth**

BACTEC 460 system (Becton Dickinson) was used to determine MIC for individual drug and to study the combined effects of drugs on *M. tuberculosis growth in vitro*. *M. tuberculosis* H37Rv or RIF *M. tuberculosis* isolates were grown on 7H11 agar plates. Bacterial inocula were prepared from 4- to 6-week-old plates by transferring loopfuls of bacteria into capped glass tubes containing dilution fluid and glass beads and vortexing to break the clumps. Suspensions of *M. tuberculosis* at turbidities equivalent to that of a 1 McFarland standard, free of clumps, were inoculated into Middlebrook 7H12 medium vials containing various combinations of test drugs (SQ109, isoniazid, rifampicin, ethambutol, streptomycin). The growth of the bacilli, expressed as the growth index or GI, was monitored daily by measuring the release of 14CO2 after the bacteria consumed 14C-labelled palmitic acid in the medium. To determine the combined effects of pyrazinamide and SQ109, we used the special pyrazinamide test medium from Becton Dickinson. No-drug controls, including the undiluted and 1:100 diluted bacterial inocula, were included in each experiment to monitor bacterial growth. When the GI value of the 1:100 inoculum vial reached 30 or greater, ΔGI, the difference in GI values between the latest GI and the previous reading for all testing vials was derived. The MIC of a given drug was defined as the lowest concentration at which the ΔGI of the drug vial was less than the ΔGI of the 1:100 control. All the experiments were completed within 5–8 days.

**Data analysis**

The effects of drugs in combination were evaluated based on GI values using the technique that was described previously. Synergy was defined as $x/y < 1/2$, where $x$ is the GI of the test vial with the combination of drugs, $y$ is the lowest GI of the single drugs of the combination and $z$ is the number of drugs combined. For a two-drug combination, a quotient of <0.5 was indicative of a Synergistic effect, a quotient of 1 indicated no interaction, a quotient of >2 showed an antagonistic effect and a quotient of <0.75 but >0.5 indicated an additive effect.

**Results**

**Antimicrobial activity of SQ109 in vitro**

We previously determined that SQ109 MIC is 0.2 µM (0.11 mg/L) by micro-broth dilution or 0.63 µM (0.35 mg/L) by BACTEC for the laboratory strain H37Rv *M. tuberculosis*. Recently completed determination of SQ109 MIC on more than 30 *M. tuberculosis* clinical isolates (drug susceptible and drug resistant, including ethambutol-resistant strains) found the susceptibility of these clinical strains to be indistinguishable from H37Rv (MIC range 0.16–0.64 mg/L). These *in vitro* activities of SQ109 suggest that it is equally active against drug-sensitive and drug-resistant *M. tuberculosis*, including strains resistant to the parent pharmacophore ethambutol.

**Interaction of SQ109 with isoniazid, streptomycin, ethambutol and pyrazinamide**

Using a chequerboard titration, in which series of dilutions of two antibiotics are studied for effects on bacterial growth inhibition at all possible concentrations, both alone and in combination, the nature of the interaction between the two antibiotics can be determined algebraically. The interaction between two antibiotics in combination can be described as Synergistic, additive, no effect or antagonistic. In order to translate the experimental data into the types of drug–drug interactions, the quotient $x/y$ (where $x$ is the data obtained when two antibiotics are in combination and $y$ is the data with the lower value of the two agents when tested separately at the same concentration) can be used. If the $x/y$ value equals 1, it is interpreted that one of the drugs in the combination is inactive. If $x/y$ is less than 0.5 for a two-drug combination, it implies that the two drugs when used together are more effective than when they are used separately, suggesting Synergistic effects. If $x/y$ values fall between 0.5 and 0.75, an additive effect...
may exist between the two drugs, suggesting a weak enhancement between them. If \( xy \) values are greater than 2, it suggests antagonistic interactions between the two drugs. The window between \( xy \) greater than 1 and less than 2 is the transition area from no effect to antagonistic. By applying simple algebra as published and validated elsewhere, we can evaluate the potential outcome of two drugs in combination in an in vitro assay system.

To determine the optimal drug combination(s) that might include SQ109 in future human efficacy trials, we analysed the interaction of SQ109 with antibiotics that are currently used to treat TB patients. The MIC of each drug is the lowest concentration that inhibits the growth of 99% of the bacterial inoculum, thus it is clear that Synergistic, additive or antagonistic effects of combination drugs must be evaluated at individual drug concentrations that are below the level of the MIC for effects to be observed. Table 1 lists the MIC of each individual drug, as well as quotient values (see definition in the Data analysis section) for combinations of SQ109 with isoniazid, streptomycin, ethambutol and pyrazinamide at drug concentrations below their MIC values. The concentration of each of the drugs used in combination was expressed as the fraction of the MIC value. This table includes only the combinations at which synergy or additive effects were observed. For the drug combinations with no observed effect, only the quotients obtained from the combination with the highest suboptimal dose tested in the experiments were listed. SQ109 showed synergy with isoniazid when both drugs were used at 0.5 MIC and showed an additive effect when combined with streptomycin. SQ109 did not show any positive interaction (additive or Synergistic) with either ethambutol or pyrazinamide, even though the combination with ethambutol was at the borderline for additivity. No antagonism was observed with any two-drug combination tested in the present study.

**Interaction of SQ109 with rifampicin**

When SQ109 was used in combination with rifampicin, we observed marked synergy between the two drugs, as indicated by the average quotient values (Table 2). This synergy worked both ways: SQ109 Synergistically enhanced rifampicin activity, and rifampicin Synergistically enhanced SQ109 activity. SQ109 at 0.5 MIC showed synergy with rifampicin at concentrations as low as 0.1 MIC. Synergy was also observed when 0.2 MIC SQ109 was combined with 0.5 MIC rifampicin. Interestingly, the combination with both drug concentrations below 0.5 MIC (0.2 MIC SQ109 + 0.25 MIC rifampicin) showed an additive interaction.

**SQ109 and rifampicin interaction in RIF\(^R\) M. tuberculosis**

To examine whether the Synergistic interaction between SQ109 and rifampicin also facilitated inhibition of drug-resistant *M. tuberculosis* strains, we evaluated drug interactions with RIF\(^R\) *M. tuberculosis* clinical isolates. The rifampicin MICs for RIF\(^R\) *M. tuberculosis* isolates 3185 and 2482 were 24 and >100 mg/L, respectively, much higher than the average MIC for drug-susceptible *M. tuberculosis* H37Rv. Figure 1 shows the GI profiles of strain 3185 when its susceptibility to rifampicin was tested in the presence of 0.5 MIC of SQ109 (a) or 0.5 MIC ethambutol (b). The RIF\(^R\) bacilli grew well in the presence of 0.6 MIC rifampicin (16 mg/L, over 90-fold higher than the average MIC determined for *M. tuberculosis* H37Rv). Addition of 0.5 MIC SQ109 (0.16 mg/L) to rifampicin-treated RIF\(^R\) bacteria inhibited >99% growth. Rifampicin dose-dependent inhibition of RIF\(^R\) *M. tuberculosis* growth in the presence of SQ109 decreased with decreasing rifampicin concentration in the test vials. The \( xy \) quotients for rifampicin at 16, 12 and 8 mg/L were 0.35, 0.40 and 0.48, respectively, indicating Synergistic interaction between SQ109 and rifampicin. An additive interaction was observed at 6 and 4 mg/L rifampicin (\( xy = 0.55 \) and 0.64, respectively). This synergy was not observed when 0.5 MIC ethambutol, a different diamine antibiotic, was used in combination with rifampicin (Figure 1b).

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**Table 1.** Synergy quotients for SQ109 tested in two-drug combinations with isoniazid (INH), streptomycin (STR), ethambutol (EMB) or pyrazinamide (PZA)

<table>
<thead>
<tr>
<th>Drug</th>
<th>INH*</th>
<th>STR*</th>
<th>EMB**</th>
<th>PZA**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>0.5 MIC</td>
<td>0.5 MIC</td>
<td>0.4 MIC</td>
<td>0.5 MIC</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.45(^b)</td>
<td>0.57(^c)</td>
<td>0.75(^d)</td>
<td>1.03(^d)</td>
</tr>
</tbody>
</table>

MICs: INH, 0.05 mg/L; STR, 0.25 mg/L; EMB, 1.25 mg/L; PZA, 100 mg/L; SQ109, 0.32 mg/L or 0.64 mg/L.

\(^a\)Synergy was defined as \( xy < 1/z \), where \( x \) is the growth index (GI) of the test vial with the combination of drugs, \( y \) is the lowest GI of the single drugs of the combination and \( z \) is the number of drugs combined. For a two-drug combination, a quotient of <0.5 was indicative of a synergistic effect, a quotient of 1 indicated no interaction, a quotient of >2 showed an antagonistic effect and a quotient of <0.75 but >0.5 indicated an additive effect.

\(^b\)Synergistic.

\(^c\)Additive.

\(^d\)No difference.

**Table 2.** Synergy quotients for SQ109 tested in combination with rifampicin (RIF)

<table>
<thead>
<tr>
<th>MIC fraction SQ109(^a) + RIF(^b)</th>
<th>Quotients (mean ( x/y ) ± SD)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50 0.50</td>
<td>0.11 ± 0.06</td>
</tr>
<tr>
<td>0.25(^d) 0.10</td>
<td>0.16 ± 0.08</td>
</tr>
<tr>
<td>0.20 0.05</td>
<td>0.38 ± 0.21</td>
</tr>
<tr>
<td>0.05 0.43</td>
<td>0.81 ± 0.18</td>
</tr>
<tr>
<td>0.25(^d) 0.69 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>0.10 0.87</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)MIC of SQ109 = 0.32 mg/L.

\(^b\)The average MIC of RIF from three experiments (0.1, 0.2 and 0.2 mg/L) was 0.17 ± 0.06 mg/L. The MIC fractions in the table represent the concentrations used in combinations in respect to the MIC of that particular experiment.

\(^c\)Data obtained from three separate experiments. Please refer to the Table 1 footnotes for \( x/y \) quotient definition.

\(^d\)In one experiment, the concentration of RIF in this group was 0.2 MIC.
Experiments with RIFR strain 3185 were repeated with a different RIFR M. tuberculosis, strain 2482, whose rifampicin resistance was even more profound: MIC >100 mg/L. As shown in Figure 2, the bacteria grew modestly in the presence of 100 mg/L rifampicin. Adding 0.5 MIC SQ109 to this culture inhibited growth more than 99%. The x/y quotients for rifampicin at 100, 50 and 25 mg/L were 0.33, 0.39 and 0.497, respectively, indicating Synergistic interaction between SQ109 and rifampicin. Additive interactions were observed at 12.5 and 6.25 mg/L rifampicin (x/y = 0.62 and 0.71, respectively). In addition, an additive effect was also observed at 0.25 MIC SQ109 (0.08 mg/L) and 100 mg/L rifampicin (x/y = 0.66). Again, 0.5 MIC
isoniazid. These in vivo tuberculosis results described in this paper were consistent with the recent results obtained from the study of SQ109, a new diamine antitubercular drug candidate, with commonly used anti-TB drugs in vitro. The possible interactions that we could measure in these studies using growth inhibition included antagonistic, Synergistic, additive and no effect at all. We found no antagonistic interactions between SQ109 and any of the five front-line TB drugs tested in this study. The combinations of SQ109 + ethambutol or SQ109 + pyrazinamide showed no interactions, positive or negative, and the effects on growth of M. tuberculosis of these combinations were indistinguishable from those obtained with single drug treatment. An additive interaction was observed between SQ109 and streptomycin at certain concentrations, but no synergy was observed. In contrast, SQ109 showed synergy with two different front-line drugs: isoniazid and rifampicin. The Synergistic interactions between SQ109 and rifampicin were particularly interesting and quite potent: >99% inhibition of M. tuberculosis growth was achieved at very low concentrations of the individual drugs, 0.1 MIC rifampicin + 0.5 MIC SQ109. The in vitro synergy results described in this paper were consistent with in vivo studies (B. Nikonenko and R. Samala, unpublished data) that demonstrated drug synergy in experimental animals infected with M. tuberculosis when SQ109 was combined with rifampicin and isoniazid. These in vivo results showed enhanced bacterial activity and faster elimination of M. tuberculosis by SQ109-containing drug combinations compared with similar combinations containing ethambutol. Together, the in vitro and in vivo data presented in these two studies can guide us in achieving the most efficacious drug combination design in future SQ109 clinical trials for treatment of active TB disease.

Interestingly, data in the present study also showed that SQ109 is fully active against RIFR M. tuberculosis clinical isolates. This is consistent with the recent results obtained from the study of over 30 drug-susceptible and drug-resistant M. tuberculosis, where the strains had the same SQ109 MIC (range 0.16–0.64 mg/L), suggesting that there is no pre-existing resistance to this compound. Synergy of SQ109 with rifampicin was also observed when tested against RIFR isolates. At 0.5 MIC, SQ109 was able to increase rifampicin activity against the resistant organisms in a dose-dependent manner. This activity was not observed with 0.5 MIC ethambutol, suggesting that a specific interaction between SQ109 and rifampicin is likely to be responsible for the observation. Although this observation may not have direct clinical relevance, it points out additional differences between SQ109 and its parent pharmacophore, ethambutol, and could be an interesting experimental model to tease out SQ109 actions on M. tuberculosis.

A definitive explanation for the profound synergy between rifampicin and SQ109 against M. tuberculosis is not yet available. Hypothetically, the Synergistic interaction could result from the differences in drug action. Although the precise target of SQ109 is unknown, it does affect mycobacterial cell wall synthesis.3,5 Perhaps the effect of SQ109 on the mycobacterial cell wall results in increasing permeability, allowing more rifampicin to enter the bacteria. Subinhibitory concentrations of cell wall inhibitors such as ethambutol were shown to increase bactericidal activity of clarithromycin for M. tuberculosis, presumably by decreasing the permeability barrier for drug entry.10 However, rifampicin, a cell wall inhibitor, did not show any synergy when used in combination with rifampicin in our experiments with RIFR H37Rv or the RIFR M. tuberculosis clinical isolates (Figure 1) or in other drug-susceptible strains,11 suggesting that just any effect on the cell wall structure of mycobacteria does not necessarily contribute to the Synergistic interaction between rifampicin and other drugs. In addition, rifampicin is already known to rapidly penetrate the hydrophobic cell wall of mycobacteria due to its lipophilic nature. As a result, the effect(s) on cell wall permeability by SQ109 is not likely to be the sole contributor to the observed synergy between rifampicin and SQ109 in vitro.

It is also possible that the expression level of the currently unknown primary target of SQ109 is tightly regulated at the transcriptional level. Rifampicin is an inhibitor of DNA transcription. Since transcripts of mRNA of various genes differ in half-life, inhibition of transcription could have profound effects on those transcripts with shorter half-life than those with longer half-life. Thus, rifampicin treatment, even at suboptimal concentrations, could exert a noticeable effect on the level of a short-lived mRNA target for SQ109 activity. However, this hypothesis is inconsistent with the observation that synergy between the two drugs was not affected by the RIFR phenotype of the M. tuberculosis strains. This suggests that other effects of rifampicin, rather than only its primary action as an antibiotic, contributed to its Synergistic interaction with SQ109. That rifampicin antibiotic activity might certainly be a contributing factor, but perhaps not the only factor that determined the interesting interaction with SQ109, was suggested by data on the MIC for RIFR strains. As the MIC of rifampicin becomes greater in RIFR as compared with RIFR strains, the concentrations of rifampicin showing synergy with the same amount of SQ109 are also greater, even though the combinations expressed in terms of fractions of MIC were similar between the strains studied. To fully evaluate the effect(s) of rifampicin transcription inhibition on SQ109 activity requires the identification of the SQ109 target(s), which is ongoing in our laboratory.

Another possible effect of rifampicin on the drug synergy interaction between rifampicin and SQ109 is the rifampicin action as an efficient inducer of cytochrome P450 (CYP).12 The CYP are a superfamily of haem-containing enzymes involved in a wide array of NADPH/NADH-dependent reactions, and they play pivotal roles in biosynthesis of compounds such as steroids, steroids and fatty acids, as well as in detoxification of xenobiotics and chemicals.13 Rifampicin is a potent inducer of a variety of CYP in human hepatocytes, as well as in peripheral blood lymphocytes.1,4

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The complete genome of *M. tuberculosis* reveals at least 20 CYP, but precise functions for these genes remain to be elucidated. Like their mammalian counterparts, the mycobacterial CYP were induced by rifampicin. Ramachandran and Gurumurthy determined CYP activity present in bacterial membrane fractions extracted from rifampicin-treated *Mycobacterium smegmatis* and *M. tuberculosis*. In both cases, the CYP activities in the treated fractions were elevated as compared with the untreated controls, and the increase in CYP activity was statistically significant. In parallel, they found that isoniazid did not induce CYP activity in its treated fractions. The ability of rifampicin to induce CYP in *M. tuberculosis* may contribute to its synergy with SQ109. In this case, instead of inactivating the active compounds, CYP may in fact activate SQ109 by producing oxidized metabolites of SQ109 within *M. tuberculosis*.

Recently obtained data on SQ109 metabolism showed that SQ109 was metabolized rapidly after incubation with human liver microsomes: only 8% SQ109 remained after 40 min incubation. Analysis of SQ109 metabolites produced by the action of the microsomes revealed four chemical groups based on molecular mass. Two of the groups contained oxidized metabolites. Furthermore, the study found that SQ109 was metabolized by human CYP2D6 and CYP2C19 to generate these metabolites. Based on the finding that SQ109 was metabolized rapidly by CYP, it is possible that its antimycobacterial activity may come from one of its metabolites. It is conceivable that SQ109 is a prodrug and requires activation by mycobacterial CYP. We speculate that when SQ109 is used in combination with rifampicin, the latter induces certain CYP activity within the mycobacteria. Elevated CYP activity activates the SQ109 prodrug more efficiently, resulting in an apparent Synergistic activity of the two drugs. In fact, the enhanced activity seen with the combination of SQ109 + rifampicin may be the more efficient and effective activity of an SQ109 active metabolite, rather than enhanced activity of rifampicin itself.

SQ109 has a very narrow spectrum: it is active against *M. tuberculosis* and *Mycobacterium bovis* BCG, but much less active against *M. smegmatis* and *Mycobacterium avium* (P. chen and C. Hanrahan, unpublished data). By comparing the putative CYP open reading frames present in various mycobacterial genomes, Kelly et al. showed there were several CYP that are unique in *M. tuberculosis* and *M. bovis*. These CYP are strong candidates for actors that might be responsible for converting SQ109 to an active metabolite(s) within *M. tuberculosis*, and they are the subjects of ongoing investigations.

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Transparency declarations

None to declare.