Sulfamethoxazole enhances the antimycobacterial activity of rifampicin

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Objectives: To investigate the effect of trimethoprim/sulfamethoxazole on the survival of Mycobacterium tuberculosis and trimethoprim and sulfamethoxazole individually and combined with the first-line tuberculosis drugs (isoniazid, rifampicin and ethambutol).

Methods: M. tuberculosis strains were exposed to either trimethoprim/sulfamethoxazole combination or sulfamethoxazole and trimethoprim alone at various concentrations. The strains were also exposed to sulfamethoxazole in combination with existing antibiotics to assess the combined effect on the growth of M. tuberculosis in the BACTEC 460TB system. The effect of the drugs was compared with vehicle-treated controls. Drug interactions were interpreted using quotient values obtained from the growth index of cultures treated with a single drug or the combination.

Results: Trimethoprim showed a negligible effect on the growth of M. tuberculosis while sulfamethoxazole inhibited 80% of the growth of M. tuberculosis at 4.75 mg/L. There was no synergistic activity between sulfamethoxazole and trimethoprim, although an additive effect was observed. A statistically significant synergistic effect was observed between sulfamethoxazole and rifampicin. Sulfamethoxazole also had an additive effect with ethambutol, but there was no interaction with isoniazid.

Conclusions: Sulfamethoxazole is the main active compound against M. tuberculosis in the combination trimethoprim/sulfamethoxazole and has a synergistic effect with rifampicin. These findings suggest that sulfamethoxazole has potential in the multidrug regimen against M. tuberculosis.

Keywords: trimethoprim, isoniazid, ethambutol, synergy

Introduction

The emergence of extensively drug-resistant Mycobacterium tuberculosis strains (although quite rare in some parts of the world) is a medical and public health concern as the inexpensive and easily administered first-line drugs lose efficacy. Therefore, there is a need for new drugs and drug combinations. While new drugs are being sought, it is important to re-examine available, registered and inexpensive compounds for their potential role as part of antituberculosis regimens.

Trimethoprim/sulfamethoxazole is an old drug combination used in the last few decades for treating various bacterial infections, such as urinary tract infection.¹ More recently, however, trimethoprim/sulfamethoxazole has been used extensively in HIV-infected patients for the prevention and treatment of Pneumocystis jirovecii and Toxoplasma gondii infections.² Trimethoprim inhibits dihydrofolate reductase, thereby blocking the reduction of dihydrofolate to tetrahydrofolate. Sulfamethoxazole is the structural analogue of para-aminobenzoic acid (PABA) and inhibits dihydropterotate synthetase, a key enzyme in folate biosynthesis, encoded by folPI.³ A recent study reported that a patient, initially thought to have nocardiosis, improved on trimethoprim/sulfamethoxazole, but later was found to have had tuberculosis without nocardiosis or any other infection. This study suggested that M. tuberculosis was susceptible to the combination of sulfamethoxazole and trimethoprim.⁴

Sulfonamides were used for the treatment of tuberculosis in the 1940s, but toxicity from early sulfonamides and the fact...
that the newer antimycobacterial compounds were much safer and more effective resulted in sulfonamides being abandoned for the treatment of tuberculosis.\(^4\) With the new knowledge about trimethoprim/sulfamethoxazole and the fact that this combination is well tolerated, this study presents the investigation of the antimycobacterial activity of trimethoprim/sulfamethoxazole. The combined effect of sulfamethoxazole with trimethoprim or with the first-line antituberculosis drugs isoniazid, rifampicin and ethambutol against \textit{M. tuberculosis} was also studied.

**Materials and methods**

Ethics approval for this study was obtained from the Health Research Ethics Committee of Stellenbosch University (reference no. N11/07/230).

**Reagents and antibiotics**

Drugs were purchased from Sigma-Aldrich (St Louis, MO, USA). Stock solutions of 76000 mg/L sulfamethoxazole, 40 mg/L trimethoprim and 332 mg/L rifampicin were prepared in 100% sterile DMSO. Stock solutions of 332 mg/L isoniazid and 200 mg/L ethambutol were prepared in deionized water and filter sterilized with a 0.2 μm Supor\(^\text{m}\) membrane Acrodisc\(^\text{m}\) syringe filter (Pall Corporation, USA). All stock solutions were prepared under sterile conditions and stored at \(-20\)°C.

**\textit{M. tuberculosis} strains**

The reference strain of \textit{M. tuberculosis} H37Rv (ATCC 27294) was used for drug evaluation. The H37Rv strain was cultured at 37°C in 7H9 Middlebrook medium supplemented with 10% (v/v) oleic acid/albumin/dextrose/catalase (OADC) (Becton Dickinson, Sparks, MD, USA) and 0.025% (v/v) Tween 80 to an optical density (OD\(_{600}\)) of 0.7. Ziehl–Neelsen staining and blood agar cultures were performed to control for contamination of the bacterial stocks. Bacterial stocks were stored at \(-80\)°C until further use.

**Inoculum preparation**

A volume of 0.1 mL of each \textit{M. tuberculosis} frozen stock was inoculated in BACTEC 12B medium (Becton Dickinson). The cultures were incubated at 37°C and the growth monitored daily in a BACTEC 460TB system (Becton Dickinson) to establish a working culture. The growth was monitored until the culture reached a maximum growth index (GI) of 999. A volume of 0.1 mL of the working culture was subsequently inoculated into a new vial containing BACTEC 12B medium and grown to a GI of 500 (GI\(_{500}\)). This culture was used for susceptibility testing and synergistic effect determinations.

**Drug susceptibility testing**

The MICs of trimethoprim/sulfamethoxazole, sulfamethoxazole, trimethoprim, isoniazid, rifampicin and ethambutol for \textit{M. tuberculosis} H37Rv were determined using the BACTEC 460TB system as described by Tortoli et al.\(^5\) Briefly, 0.1 mL from a culture at GI\(_{500}\) was inoculated into BACTEC vials containing a drug at the required concentration. The control vials contained a drug solvent with undiluted bacterial inoculum and a 1:100 diluted control culture. The cultures were monitored daily until the GI of the 1:100 control culture was \(>30.\)\(^6\)

\textit{M. tuberculosis} strains were categorized as susceptible to a specific drug when the GI of the drug-exposed culture at a specific concentration on the final day of the experiment (GI 1:100 \(>30\)) was below or equal to the GI of the preceding day, indicating a reduction of metabolic activity in the test vial. The GI of the drug-exposed culture on the final day was also compared with the GI of the drug-free vial containing undiluted inoculum on the same day and MIC was defined as the minimum concentration of a drug that inhibited 99\% of bacteria.

**Sulfamethoxazole in combination with trimethoprim and the first-line antituberculosis drugs isoniazid, rifampicin and ethambutol**

The drug–drug interaction of sulfamethoxazole in combination with isoniazid, rifampicin and ethambutol was assessed by evaluating drug combinations that were two to four times less than the MICs of the individual drugs and for sulfamethoxazole and trimethoprim in a 19:1 ratio. Growth was monitored daily in the BACTEC 460TB system, as described earlier.

**Synergism**

The effect of the drug combinations was interpreted using a formula in which synergism is defined as \(x/y < 1/2\), where \(x\) is the GI of the vial with two drugs on the final day (day 5), \(y\) is the lowest GI of the single drug in the combination and \(z\) is the number of drugs in the combination. In this instance, two drugs were used in a combination, hence \(z = 2\).

Therefore, \(x/y < 0.5 \Rightarrow \text{synergy}\), \(x/y \geq 0.5\) and \(< 0.75 \Rightarrow \text{additive}\), \(x/y \geq 0.75\) and \(< 2 \Rightarrow \text{no interaction}\), and \(x/y \geq 2 \Rightarrow \text{antagonism}\).\(^7\)

**Results**

Sulfamethoxazole and trimethoprim using a fixed ratio of 19 sulfamethoxazole to 1 trimethoprim\(^8\) displayed antimycobacterial activity. In order to determine which of the two drugs was responsible for the observed antimycobacterial effect, the individual drugs were tested for growth inhibition of \textit{M. tuberculosis}. The antimycobacterial activities of these compounds on \textit{M. tuberculosis} strain H37Rv are shown in Figure 1. Trimethoprim (Figure 1a) had negligible activity against \textit{M. tuberculosis}, showing only 22% growth inhibition at 76 mg/L, doubling to 44% at 152 mg/L. In contrast, sulfamethoxazole (Figure 1b) showed 93% growth inhibition at 76 mg/L and 95% growth inhibition at 152 mg/L. At 9.5 mg/L, sulfamethoxazole still showed 90% growth inhibition, which was determined as the MIC of sulfamethoxazole for \textit{M. tuberculosis} (Figure 1).

**Combined effect of sulfamethoxazole and trimethoprim on H37Rv**

Table 1 lists the quotient values for the combination of sulfamethoxazole and trimethoprim. The drug–drug interaction was assessed at concentrations that were below the cytotoxic level in a 19:1 ratio.\(^9\) There was no synergistic killing between the two compounds. However, an additive effect was observed between 9.5 mg/L sulfamethoxazole (MIC) and 0.5 mg/L trimethoprim.

**Combined effect of sulfamethoxazole with first-line drugs on H37Rv**

The MICs of the compounds for the laboratory strain H37Rv were determined using the BACTEC 460TB system and Table 2 lists the MIC of each drug. A concentration below the MIC of sulfamethoxazole (2 mg/L, approximately five times less than the MIC) was tested in combination with the first-line
were calculated using Excel. TMP, trimethoprim; SMX, sulfamethoxazole.

Activity of trimethoprim. (b) Activity of sulfamethoxazole. Results were obtained from three separate experiments and standard deviations after the first day until the 30th day. BACTEC vials were incubated at 37°C and GI readings were obtained after the first day until the ΔGI of the 1:100 culture was >30. (a) Activity of trimethoprim. (b) Activity of sulfamethoxazole. Results were obtained from three separate experiments and standard deviations were calculated using Excel. TMP, trimethoprim; SMX, sulfamethoxazole.

Discussion

We have shown that sulfamethoxazole possesses antimycobacterial activity that could be explored further for clinical use. Trimethoprim/sulfamethoxazole is readily available and is an inexpensive combination. A single dose of trimethoprim/sulfamethoxazole (160/800 mg) yields a sulfamethoxazole Cmax of 45 mg/L at ~2 h and a sulfamethoxazole Cmin of 13 mg/L 24 h after ingestion. Both are in excess of the MIC for 90% inhibition for M. tuberculosis strain H37Rv in this study. An early randomized study in HIV-infected adults with active tuberculosis in Côte d’Ivoire comparing trimethoprim/sulfamethoxazole with placebo showed efficacy in reducing mortality and morbidity, largely due to reduced septicaemia and enteritis.11

The table shows the interaction between sulfamethoxazole (SMX) and trimethoprim (TMP), rifampicin (RIF), ethambutol (EMB) and isoniazid (INH). The data were obtained at day 5 when the GI of the 1:100 culture was ≥30 or when the GI of the control treated with DMSO (solvent) reached 999. Synergy was defined as x/y < 1/z, where x is the GI value of the combination, y is the lowest GI of the single drug in the combination and z is the number of drugs combined (which was two in our case). Synergy, x/y < 0.5; additive, x/y ≥ 0.5 and < 0.75; no interaction, x/y ≥ 0.75 and < 2; antagonism, x/y ≥ 2. All results were obtained from three separate determinations and standard deviations were calculated using Excel.

Table 1. Interaction between sulfamethoxazole and trimethoprim, rifampicin, ethambutol and isoniazid

<table>
<thead>
<tr>
<th>SMX (mg/L)</th>
<th>TMP (mg/L)</th>
<th>Quotients (mean x/y ± SD)</th>
</tr>
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<tbody>
<tr>
<td>9.5</td>
<td>0.5</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td>4.75</td>
<td>0.25</td>
<td>1.06 ± 0.02</td>
</tr>
<tr>
<td>2.4</td>
<td>0.125</td>
<td>1.18 ± 0.26</td>
</tr>
<tr>
<td>SMX (mg/L)</td>
<td>RIF (mg/L)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>0.16 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.19 ± 0.16</td>
</tr>
<tr>
<td>SMX (mg/L)</td>
<td>EMB (mg/L)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>SMX (mg/L)</td>
<td>INH (mg/L)</td>
<td></td>
</tr>
<tr>
<td>0.025</td>
<td></td>
<td>1.03 ± 0.05</td>
</tr>
</tbody>
</table>

The table shows the interaction between sulfamethoxazole (SMX) and trimethoprim (TMP), rifampicin (RIF), ethambutol (EMB) and isoniazid (INH). The data were obtained at day 5 when the GI of the 1:100 culture was ≥30 or when the GI of the control treated with DMSO (solvent) reached 999. Synergy was defined as x/y < 1/z, where x is the GI value of the combination, y is the lowest GI of the single drug in the combination and z is the number of drugs combined (which was two in our case). Synergy, x/y < 0.5; additive, x/y ≥ 0.5 and < 0.75; no interaction, x/y ≥ 0.75 and < 2; antagonism, x/y ≥ 2. All results were obtained from three separate determinations and standard deviations were calculated using Excel.

Table 2. MICs of the first-line drugs and sulfamethoxazole for M. tuberculosis strain H37Rv

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td>0.05</td>
</tr>
<tr>
<td>EMB</td>
<td>1.6</td>
</tr>
<tr>
<td>RIF</td>
<td>0.8</td>
</tr>
<tr>
<td>SMX</td>
<td>9.5</td>
</tr>
</tbody>
</table>

INH, isoniazid; EMB, ethambutol; RIF, rifampicin; SMX, sulfamethoxazole. MICs were determined using the BACTEC 460TB system following the manufacturer’s recommendations.

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tuberculosis, they then showed an antituberculosis effect in clinical isolates, including multidrug-resistant tuberculosis.\textsuperscript{4}

Our data support the findings of Ong and colleagues,\textsuperscript{12} who reported that in the combination of trimethoprim and sulfamethoxazole, it is only sulfamethoxazole that has an activity against \textit{M. tuberculosis}. We evaluated the interaction of sulfamethoxazole with trimethoprim and the first-line drugs isoniazid, rifampicin and ethambutol and there was no synergistic killing between sulfamethoxazole and trimethoprim. Sulfamethoxazole showed a strong synergistic effect with rifampicin, one of the two key drugs in the tuberculosis regimen.\textsuperscript{13} This synergy was achieved at very low concentrations of both drugs, where sulfamethoxazole at four times less than its MIC of 9.5 mg/L in combination with rifampicin between two and three times its MIC of 0.8 mg/L gave the same killing effect as rifampicin at its MIC. The combination of sulfamethoxazole and ethambutol showed an additive effect, with no positive or negative interaction between sulfamethoxazole and isoniazid. There was no antagonism in all combinations involving sulfamethoxazole. The synergy between sulfamethoxazole and rifampicin may be the result of their respective targets; sulfamethoxazole indirectly inhibits RNA synthesis by inhibiting DNA-dependent RNA polymerase,\textsuperscript{3,14} rifampicin directly inhibits the RNA synthesis by inhibiting DNA-directed RNA synthesis by rifamycin.\textsuperscript{14}

Our findings support reports that sulfamethoxazole is the active compound in the trimethoprim/sulfamethoxazole combination. Sulfamethoxazole has synergistic activity with rifampicin and an additive effect with ethambutol. Trimethoprim/sulfamethoxazole is a registered drug combination for other indications, is inexpensive and is widely available. Mouse studies will be undertaken before proceeding to clinical trials to clarify the potential of sulfamethoxazole and sulfamethoxazole/rifampicin in drug-susceptible tuberculosis. Also, we plan to evaluate clinical isolates.

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Transparency declarations

None to declare.

References