Original Article

Dihydrofusarubin and rifampicin combination prevents pulmonary tuberculosis in murine models of tuberculosis

Zhijian Bao, Yanfei Cui, Yunfeng Sheng, Min Zhu

Department of Tuberculosis, Hangzhou Red Cross Hospital, Hangzhou 310000, China

Received March 13, 2017; Accepted May 5, 2017; Epub August 15, 2017; Published August 30, 2017

Abstract: Tuberculosis is the leading cause of death among the infectious diseases owing to the failure of the present to control the epidemics of the disease. Search for new chemical entities remains the desperate need to control this global scourge. Recently dihydrofusarubin has been reported to exhibit antimicrobial potential. The present study was designed to evaluate the anti-mycobacterial activity of dihydrofusarubin and to examine the efficacy of dihydrofusarubin chemotherapy in murine models of pulmonary tuberculosis (TB). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by broth microdilution method and viable count method respectively. In vitro drug interaction by checker board assay and dynamics of killing by kill curve method. The two-drug combination was also evaluated for in vivo efficacy in murine models of tuberculosis The MIC of dihydrofusarubin was found to be 8 µg/ml against M. tuberculosis and was found to be bactericidal at 4 × MIC. It demonstrated synergistic interactions with rifampicin and additive interactions with isoniazid and ethambutol under in vitro. Dynamics of the compound was found to be both concentration as well as time dependent. Acute single dose toxicity studies in BALB/c mice demonstrated it to be safe for in vivo studies. In vivo studies revealed its better efficacy both alone as well as in combination with rifampicin. Dihydrofusarubin interacts synergistically with rifampicin both under in vitro and in vivo conditions, thus supporting the view that dihydrofusarubin based drug scaffolds could be promising drug candidates for the treatment of pulmonary tuberculosis.

Keywords: Chemotherapy, dihydrofusarubin, M. tuberculosis, pulmonary tuberculosis, synergism

Introduction

Tuberculosis (TB) is the leading cause of death among infectious diseases originating from single infectious agent, Mycobacterium tuberculosis. It is leads to a death of approximately 1.5 million people each year globally [1]. Although, there are continuous efforts for development of effective treatments, TB continues to be a major public health burden. In developing world in particular, where the treatment and diagnostics are not adequate, the control measures for TB quite often results in failure. TB causes leads to approximately one-third of HIV-associated deaths [2]. The major bottle necks in TB are the issue of cost, poor health assets, lengthy treatment and adverse effects associated with the anti-TB medication that has to be given a due attention so as to avoid relapse, or the emergence of drug resistant isolates of M. tuberculosis. Additionally, new resistant isolates increase owing to poor treatment adherence and by spontaneous innate mutations, making treatment even more complicated [3]. The World Health Organization (WHO) estimates that about 2 billion humans might be infected by this M. tuberculosis, and about 9 million new and active cases of the disease emerge annually. M. tuberculosis isolates resistant to two (rifampicin and isoniazid) of four (isoniazid, rifampicin, pyrazinamide and ethambutol) first line anti-TB drugs known as Multidrug resistant (MDR) isolates also emerge. Furthermore, some strains of M. tuberculosis are MDR plus they also exhibit resistance to one of the flouroquinolone (moxifloxacin/levofloxacin) and to one of the injectable agent (amikacin, kanamycin/capreomycin) have also been isolated. The morbidity and mortality due to TB remains unabated because of its synergy with
the HIV/AIDS pandemic and the evolution of drug resistant strains of *Mycobacterium tuberculosis* [4-6]. Despite some successes in the recent past, such as the discovery of the anti-TB drug such as bedaquiline [7] and the high drug attrition rate, great effort is required to find better drugs in order to meet these challenges. Therefore, the need for new and effective compounds that comprise a simple, shorter and effective treatment is vital need.

**Materials and methods**

**Antibiotics**

Isoniazid (INH), rifampicin (RIF), and ethambutol (EMB) were purchased from Sigma-Aldrich (USA). Stock solutions of INH and EMB were made in sterile double distilled water at 10 mg/mL. Stock solutions of RIF (10 mg/mL) and test compound were made in 99% dimethyl sulfoxide (DMSO) at 15 mg/mL. Stock of test compound was prepared in DMSO at 50 mg/ml. All antimicrobial stock solutions were stored at -80°C till use. For *in vivo* study the test compound preparations were made in 0.5% carboxymethyl cellulose (CMC).

**Determination of minimum inhibitory concentration**

The Minimum inhibitory concentration (MICs) of the dihydrofusarubin and standard anti-TB drugs (INH and RIF) against *M. tuberculosis* H37Rv were determined by the slight modification of the procedure as described previously [8]. Briefly, a range of concentrations of the test compound (0.5-256 µg/ml) and the reference antibiotics INH/RIF (0.019-10 µg/ml) were made in 99% dimethyl sulfoxide (DMSO) at 15 mg/mL. Stock of test compound was prepared in DMSO at 50 mg/ml. All antimicrobial stock solutions were stored at -80°C till use. For *in vivo* study the test compound preparations were made in 0.5% carboxymethyl cellulose (CMC).

**Determination of minimum bactericidal concentration (MBC)**

Minimum bactericidal concentration (MBC) of the test compound was determined by diluting the concentrations above MIC to sub-MIC levels and platting them onto Middle brook 7H11 agar plates for determinations of the viability counts. The agar plates were incubated at 37°C in an incubator and bacterial counts were read after 3 weeks of incubation. MBC was defined as the lowest concentration of antimicrobial agent which kills 99% of the bacterial population of the initial inoculum.

**In vitro kill curve studies**

50 ml tubes containing 10 ml of middle brook 7H9 broth with antimicrobial agents at concentrations at 1 ×, 4 × and 8 × MICs were inoculated with *M. tuberculosis* H37Rv at 10⁵ CFU/mL and incubated at 37°C in an orbital shaker. Tubes without an antimicrobial agent were also included to serve as growth control. Aliquots from each concentration were removed at predetermined time points (0, 4, 8, 16 and 20 days) post-inoculation and serially diluted with PBS and plated onto Middlebrook 7H11 agar plates for viability counts after proper incubation at 37°C for 3 weeks.

**In vitro drug-drug interaction studies**

**In vitro** drug interaction studies of the test compound with the commonly used anti-TB drugs were evaluated by checker board titration assay using the 96 well plates. Briefly, one drug was diluted along the rows of the 96 well plates and the second drug was isolated on the same plate along the columns. Growth control wells were also included in the assay. Plates were incubated at 37°C and monitored after 12 days for MIC determination of the drugs alone as well as in combination.

Fractional inhibitory concentrations (FICs) were calculated by use of the following formula:

\[
FIC (a + b) = \frac{MIC of compound a in combination with b}{MIC of a alone}.
\]

The fractional inhibitory index (ΣFIC) was calculated as FIC of compound X + FIC of compound Y to evaluate interaction profiles. FICs of > 0.5 shows synergistic activity, FICs of > 4.0 indicate antagonism, and values in between correspond to additive interaction.

**In vivo study**

**Experimental animals:** Seven week old BALB/C mice weighing about 25 ± 2 g were used in the
Dihydrofusarubin against tuberculosis

study. The rats were given free access to pellet diet and water. Animals were kept in well ventilated rooms with controlled setting of light/dark cycle and temperature of 24 ± 2. The animal protocols for the study were approved by animal ethical committee of the institute.

Acute toxicity study

The acute oral toxic study was performed using test guidelines on acute oral toxicity test 423 according to OCDE 2001 [9]. Fifteen mice fasted O/N, but allowed free access to purified water was randomly divided into the three groups with each group consisted of six mice of either sex in 1:1 sex ratio. Control group received distilled water and the remaining two groups received the test compound extract at the dosage of 500 and 1000 mg/kg of body weight. The animals were fasted for 3 h post administration. The animals were observed for 1 week after test drug administration for signs of toxicity and/or mortality. Afterwards, body weight was recorded for consecutive 14 days.

Induction of experimental pulmonary tuberculosis

Acute pulmonary tuberculosis was induced by single dose administration of M. tuberculosis H37Rv to mice via aerosol route as described previously [10].

Treatment groups

After infection mice were randomly divided into 5 groups with 5-6 animals/group. Group 1 consisted of 6 mice that were administered vehicle only (CMC: Carboxy methyl cellulose). The remaining four were treatment groups. Group 2 were treated with RIF at the dosage of 10 mg/kg body weight, group 4 animals were treated with the test compound at the dosage of 150 mg/kg body weight and group 5 animals were treated with rifampicin and test drug combination. Treatment was started on day 3 after infection and study continued for 10 days. A wash out period of 2 day was also given in which animals were given free access to pellet diet and water but without any medication. Three animals in the control group were sacrificed at the start of experiment to determine the baseline CFU values in the lungs of mice or at the end of the study.

Statistical analysis

Statistical analysis was analysed by 2-tailed Student’s t test. In all statistical analysis, \( P < 0.05 \) were considered to be statistically significant.

Results

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of the test compound and the standard anti-TB drugs against M. tuberculosis H37RV were determined by broth microdilution method (Table 1). The MIC of the test compound was observed to be 8 µg/ml. The MICs of the commonly used first line TB drugs such as RIF, INH and EMB were observed to be 2, 1 and 2.5 µg/ml respectively.

Minimum bactericidal concentration (MBC)

MBC of the test compound was determined by viable count method. In this assay INH was used a positive control. The study demonstrated that the MBC of the test compound is 16 µg/ml as at this concentration, the test molecule reduced the bacterial load of the inoculum by 3.5 log\(_{10}\). Isoniazid proved to be strongly bactericidal as it reduced 3.2 logs of the initial inoculum (5 log\(_{10}\) ) at its MIC. Thus demonstrating that MIC and MBC of the INH is same i.e 1 µg/ml.

Kill curve studies

Checkerboard assay: To interaction of the test drug with conventional drugs checkerboard assays were performed and the fractional inhibitory index (ΣFIC) was calculated for each drug combination. The ΣFIC data are summarized in Table 2. The test compound when combined with INH resulted in two fold improve-

Table 1. Minimum inhibitory concentration (MIC) of test compound and commonly used first line anti-tuberculosis drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrofusarubin</td>
<td>8</td>
</tr>
<tr>
<td>INH</td>
<td>2</td>
</tr>
<tr>
<td>RIF</td>
<td>1</td>
</tr>
<tr>
<td>EMB</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Where, INH is isoniazid, RIF is rifampicin, EMB is ethambutol.
Table 2. Interaction of dihydrofusarubin with first line anti-tuberculosis drugs against M. tuberculosis H37RV. All experiments were carried out in triplicates

<table>
<thead>
<tr>
<th>Drug/drug combination</th>
<th>MIC (µg/ml)</th>
<th>FIC</th>
<th>ΣFIC</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Compound alone</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>Additive</td>
</tr>
<tr>
<td>RIF</td>
<td>2</td>
<td>0.25</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>Test Compound + RIF</td>
<td>8</td>
<td>1</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>Test compound</td>
<td>8</td>
<td>8</td>
<td>1</td>
<td>Additive</td>
</tr>
<tr>
<td>EMB</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Where, INH is isoniazid, RIF is rifampicin, EMB is ethambutol.

Bacterial load decreases with time showing that the compound demonstrated time dependent activity. At these concentrations the bacterial load was reduced by 3 log₁₀ on day 16. At 8 × MIC, a large reduction in bacterial load was observed proving it to be the most effective concentration among the tested concentrations.

**Acute toxicity study:** A single dose of 500 or 1000 mg/kg body weight did not led to any in change animal behaviour. No death was observed over the course of the study. On the 14th day, animals were sacrificed by cervical dislocation macroscopic pathology examination, revealed no visible lesions in any animal’s liver. The orally administered LD₅₀ value of PC extract must be > 1000 mg/kg body weight.

**Efficacy in mice models of tuberculosis**

Test compound demonstrated good efficacy in mouse models of tuberculosis. Test compound orally administered at the dosage of 150 mg/kg body weight prevented the increase in lung CFU compared to vehicle control in which there was a sharp rise in bacterial load in the lungs of the mice. Test compound also reflected better efficacy in combination with RIF resulting in about 2.5 log₁₀ reduction in CFUs.

**Discussion**

TB treatment requires the use of better and effective drugs to control the present TB epidemics thus emphasizing the need for evaluating the anti-TB potential of new chemical entities (NCEs) towards the same purpose. In the present study we have evaluated the potential of dihydrofusarubin for TB. Our study was based on the previous studies which have reported the antimicrobial potential of this compound against both gram positive and gram negative bacteria [11] MIC of the dihydrofusarubin against standard laboratory H37Rv strain of M. tuberculosis was observed to be 8 µg/ml. We have also used standard anti-TB drugs (INH, RIF and EMB) in the MIC determination assay to validate the assay. The MIC values of these anti-TB drugs fall well within the literary reports [12] MBC of the compound reflected that the compound is bactericidal against M. tuberculosis at 2 × MIC. Any compound that has MIC
equal to less than 4 fold of its MIC is considered bactericidal [13]. The chemotherapy for TB involves the use of combination of drugs for at least 6 months; it is therefore necessary to evaluate the \textit{in vitro} activities of the new drug candidates in combination with the common or experimental TB drugs under preclinical stages. Some reports have already questioned whether the conventional anti-TB drugs display the optimal interaction \textit{in vivo}. Pharmacokinetic antagonism has been observed in murine models during the initial phase of chemotherapy between INH and RIF-Pyrazinamide [14]. Thus a rational for the development of new drug regimens is to evaluate the interactions of a drug candidate with other drugs in preclinical trials, in order to guess which drug combinations would have the best efficacy in the clinic. In the current study, we determined how our experimental compound interacts with other existing anti-TB compounds \textit{in vitro}. Our observations demonstrated that no antagonistic interaction of the test compound was observed with INH, RIF or EMB. Interestingly, test compound demonstrated synergistic interaction with RIF. The interactions with INH and EMB proved to be additive. Kill curve studies of the test compound revealed that its activity is concentration as well as time dependent (Figure 1) as it its activity increases with increase in concentration and rate of killing becomes prominent with duration of the drug exposure. In earlier studies, similar activity has been also reported for RIF with clear concentration and time dependent activity [5]. In the same study, INH showed the concentration-dependent killing activity, which was more rapid compared with RIF. moderate killing activity have reported for EMB, that was more or less independent of its concentration, and not time dependent even when tested at the highest concentrations. From the above discussion, it is clear that our experimental compound has demonstrated excellent \textit{in vitro} potential therefore it was subjected to \textit{in vivo} studies in acute mice models of TB (Figure 2). Prior to \textit{in vivo} study, we evaluated the acute toxicity of the test compound and we have indicated that LD\textsubscript{50} value of dihydrofusarubin must be greater than 1000 mg/kg and no sign of toxicity was observed after sub-chronic administration at tested doses. For \textit{in vivo} study, the test compound was administered orally at the dosage of 150 mg alone and in combination with RIF. Test compound when administered alone prevented growth of bacilli in the lungs and the number of bacilli in the lungs was similar to that of control mice. When the test compound was given along with RIF, there was a significant reduction in the number of bacilli in the lung of mice.

Conclusion

In conclusion the dihydrofusarubin exhibits potent anti-TB potential and is active both under \textit{in vitro} and in murine models of tuberculosis thus offers a hope for further validation of dihydrofusarubin based scaffolds for tuberculosis drug development.

Acknowledgements

The present study was supported by Zhejiang province health development planning (project number: 2015KYB312).

Disclosure of conflict of interest

None.

Address correspondence to: Min Zhu, Department of Tuberculosis, Hangzhou Red Cross Hospital, Hangzhou 310000, China. Tel: 0086-0571-5610-9823; Fax: 0086-0571-56109823; E-mail: minzhu987@hotmail.com

References

Dihydrofusarubin against tuberculosis


