Antitubercular Activity of Disulfiram, an Antialcoholism Drug, against Multidrug- and Extensively Drug-Resistant *Mycobacterium tuberculosis* Isolates

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The antimycobacterial activities of disulfiram (DSF) and diethyldithiocarbamate (DDC) against multidrug- and extensively drug-resistant tuberculosis (MDR/XDR-TB) clinical isolates were evaluated in vitro. Both DSF and DDC exhibited potent antitubercular activities against 42 clinical isolates of *M. tuberculosis*, including MDR/XDR-TB strains. Moreover, DSF showed remarkable bactericidal activity *ex vivo* and *in vivo*. Therefore, DSF might be a drug repurposed for the treatment of MDR/XDR-TB.

According to the updated guidelines of the World Health Organization, the medications effective against multidrug- and extensively drug-resistant tuberculosis (MDR/XDR-TB) are confined because only a limited selection of drugs is available; therefore, the development of novel or repurposed drugs with activity against MDR/XDR-TB is strongly desired (9). Disulfiram (DSF; tetraethyl thiuram disulfide) has been used orally in the clinical treatment of alcoholism since 1949 and has been proven to exert an inhibitory effect on aldehyde dehydrogenase *in vivo* with 80% bioavailability and established safety profiles (13, 33). Both DSF and its first metabolite, diethyldithiocarbamate (DDC), were reported to exhibit growth-inhibitory activity against bacteria, fungi, protozoa, and viruses (2, 18, 23, 26, 27). In the mid-1950s, the tuberculostatic effects of DSF and DDC were demonstrated in *vivo* using guinea pigs (17). Subsequently, it was reported that DDC enhances monocyte-induced antitubercular activity in both *in vivo* and *ex vivo* and the bactericidal activity of DSF was demonstrated (4). In addition, we reported the unique antimycobacterial activities of dithiocarbamates and also the potent antitubercular activities of compounds containing dithiocarbamate groups, such as dimethyldithiocarbamate (DMDC), DDC, and PDTC (14, 15). More recently, the mode of action of dithiocarbamates against *M. tuberculosis* has been reported to be through β-class carbonic anhydrases (β-CAs), which are considered possible drug targets (19). However, the mechanism of action of DSF remains unknown.

In the present study, we evaluated the antimycobacterial activities of DSF and its metabolites against *M. tuberculosis*, including MDR/XDR-TB clinical isolates, in more detail. Furthermore, the intracellular bactericidal activities of these compounds against a virulent strain, *M. tuberculosis* H$_{37}$Rv, within macrophages were examined *ex vivo*, and the bactericidal activity of DSF *in vivo* was determined using the mouse model of chronic TB. Finally, the mechanisms of action of these compounds were investigated by means of gene-overexpressing strains *in vitro*.

**MATERIALS AND METHODS**

**Bacterial strains.** *M. tuberculosis* H$_{37}$Rv ATCC 25618, *M. tuberculosis* H$_{37}$Ra ATCC 25177, *Mycobacterium avium* ATCC 25291, and *M. avium* ATCC 35718 were purchased from the American Type Culture Collection (ATCC). *Mycobacterium smegmatis* JATA 64-01 was provided by M. Takahashi (Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo, Japan). Mycobacterial strains were cultured in Middlebrook 7H9 broth (Difco) supplemented with 10% ADC (5% bovine serum albumin [fraction V], 2% dextrose, and 0.005% bovine liver catalase) including 0.05% Tween 80 or on Middlebrook 7H11 agar (Difco) supplemented with 10% OADC (5% bovine serum albumin [fraction V], 2% dextrose, 0.005% bovine liver catalase, and 0.05% alkaline oleic acid) at pH 6.6. *Staphylococcus aureus* 209PJC-1, *S. aureus* RN4220, *S. aureus* MF490, *Enterococcus faecalis* ATCC 19433, *Enterococcus faecium* ATCC 19434, *Escherichia coli* JM109, *Klebsiella pneumoniae* ATCC BAA-1705, and *Pseudomonas aeruginosa* PA01 were grown on Mueller–Hinton agar (Becton, Dickinson).

**Drug and reagent preparation for *in vitro* and *in vivo* studies.** Iso-niazid (INH), rifampin (RIF), streptomycin (STR), ethambutol (EMB),...
TABLE 1 Antimycobacterial activities of DSF and its metabolites

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>DSF</td>
</tr>
<tr>
<td><strong>M. tuberculosis H₃Rv</strong></td>
<td>1.56</td>
</tr>
<tr>
<td><strong>M. tuberculosis H₃Ra</strong></td>
<td>3.13</td>
</tr>
<tr>
<td><strong>M. ivisi BCG strain Tokyo 172</strong></td>
<td>25</td>
</tr>
<tr>
<td><strong>M. avium subsp. avium ATCC 25291</strong></td>
<td>25</td>
</tr>
<tr>
<td><strong>M. avium subsp. avium ATCC 35718</strong></td>
<td>25</td>
</tr>
<tr>
<td><strong>M. avium subsp. hominissuis 104</strong></td>
<td>25</td>
</tr>
<tr>
<td><strong>M. smegmatis IATA 64-01</strong></td>
<td>25</td>
</tr>
</tbody>
</table>

a The antimycobacterial activities of each agent were determined by broth dilution test using Middlebrook 7H9 broth containing albumin, dextrose, catalase, and Tween 80 at pH 6.6. Experiments were performed in duplicate. These results are representative of three separate experiments. DSF, disulfiram; DDC, diethyldithiocarbamate; S-Me-DDC, S-methyl N,N-diethyldithiocarbamate; S-Me-DTC, S-methyl N,N-diethyldithiocarbamate.

RESULTS AND DISCUSSION

In accordance with previous reports, DCF exhibited antitubercular activity, with MICs of 1.56 to 3.13 µg/ml. Despite the use of conditions similar to those in a previous study (4), DCF exhibited potent antitubercular activity, with an MIC of 1.56 µg/ml at pH 6.6 using Middlebrook 7H9 broth (Table 1). Importantly, the MIC of the bulk powder of DCF provided by Mitsubishi Tanabe Pharma, which is generally used in the clinical treatment of alcoholism in Japan, was comparable to that of chemical reagent DCF (data not shown). Additionally, the MIC of DCF against BCG Tokyo using 7H11 agar plates was also equivalent to the MICs of anti-TB drugs, i.e., EMB and KAN (MICs = 1 to 4 µg/ml) (see Table S1 in the supplemental material). Among mycobacterial species, although the antibacterial spectrum of DCF is broad, the antitubercular activity of DCF was highly specific to slow-
grown strains, such as M. tuberculosis and BCG Tokyo, indicating that the antibacterial spectrum of DSF is quite distinct from that of DDC (Table 1). Meanwhile, the antibacterial spectrum of DSF against other Gram-positive bacteria, such as S. aureus (MIC = 16 μg/ml), E. faecalis (MIC = 32 μg/ml), and E. faecium (MIC = 32 μg/ml), was similar to that of DDC (see Table S2 in the supplemental material). As previously reported, unlike DSF, DDC exhibited antibacterial activity, with MICs of 128 μg/ml against Gram-negative bacteria, i.e., E. coli, K. pneumoniae, and P. aeruginosa (see Table S2 in the supplemental material) (28).

DSF is well-known to be a prodrug and is enzymatically metabolized to DDC in blood, followed by activation owing to certain reactions, such as S-methylation, oxidation, and sulfonation (1). The antimycobacterial activities of the metabolites of DSF, i.e., S-Me-DDC, S-Me-DTC, S-Me-DTC sulfoxide, and S-Me-DTC sulfone, were determined by BDT in vitro. The results revealed that S-methylation of the sulfhydryl group of DDC led to deactivation (MIC > 100 μg/ml), in spite of its oxidation, and the metabolites obtained by sulfonation had partially restored antimycobacterial activities (MICs = 12.5 to 25 μg/ml) (Table 1). Therefore, the results suggest that not only DSF but also its metabolites, namely, DDC, S-Me-DTC sulfoxide, and S-Me-DTC sulfone, are biologically active in the human body, which complicated the assessment of the antitubercular activity of DSF after oral administration.

We next further determined the antitubercular activities of DSF and DDC against clinical isolates of M. tuberculosis in vitro. As expected, these compounds exhibited potent antitubercular activities against more than 40 clinical isolates of M. tuberculosis, including MDR/XDR-TB strains (Table 2). The MIC₅₀ₐₜ of DSF and DDC against clinical isolates were 1.56 μg/ml and 3.13 μg/ml, respectively (Table 2). Importantly, there was no cross-resistance of DSF or DDC to the currently available anti-TB drugs, including fluoroquinolones such as levofloxacin, sparfloxacin, and CIP (see Table S3 in the supplemental material). Thereby, DSF and DDC may be implemented in future pharmacological regimens against MDR/XDR-TB.

It has been reported that DDC enhances monocyte-induced antitubercular activity ex vivo (16). Therefore, in order to confirm whether DSF and its metabolites are effective within macrophages, we determined the bactericidal activities of these compounds against intracellular M. tuberculosis in differentiated THP-1 cells. As shown in Fig. 1, these compounds exhibited potent bactericidal activities at 6 to 30 μg/ml and 10 to 30 μg/ml, respectively, in a dose-dependent manner, unlike STR and the bacteriostatic drug EMB. Likewise, S-Me-DTC sulfone, the active metabolite of DSF, exhibited intracellular bactericidal activity at 30 μg/ml, but S-Me-DTC did not, in agreement with the results presented in Table 1 (Fig. 1). These data indicate that DSF, DDC, and S-oxidized metabolites, especially S-Me-DTC sulfone, are effective against intracellular M. tuberculosis.

Subsequently, we examined whether DSF and DDC exhibited bactericidal activity in vivo by means of a serum bactericidal test. The serum bactericidal activities of INH and RIF were significant at a titer of 1:32 compared to the activity of the vehicle control. As expected, the serum sample collected at 2 h after DSF administration orally exhibited bactericidal activity at a titer of 1:2, which was comparable to that of DDC administered intravenously, whereas the serum sample collected at 0.083 h after administration exhibited less activity (Table 3). Likewise, the serum sample collected at 2 h after DDC administration orally exhibited bactericidal activity, and the serum sample collected at 0.083 h exhibited less activity at a titer of 1:8; the serum sample from mice treated orally with DSF, but not DDC, exhibited bactericidal activity. Therefore, DSF has more potent bactericidal activity than DDC (Table 3). These results also indicated that DDC exhibits bactericidal activity in vivo.

Previously, the tuberculostatic effects of dithiocarbamates and thiomuram disulfides have been examined in experimental tuberculosis of guinea pigs (n = 10) (17). Whereas this report is considerably important for the development of drugs with activity against TB, only evidence based on pathological findings without bacteriological examination of the lungs had been shown. This prompted us to evaluate the bactericidal activity of DSF in vivo in the mouse model of chronic TB. In the group administered DSF, significant reductions in the numbers of CFU were observed in both lungs and spleen at 80 to 160 mg/kg (P < 0.01) compared to the numbers of CFU for the vehicle control group (Fig. 2). The reduction in the numbers of pulmonary CFU by DSF at 80 mg/kg was similar to that by RIF at 10 mg/kg (Fig. 2). Whereas it has been

<table>
<thead>
<tr>
<th>Clinical isolate</th>
<th>No. of strains</th>
<th>Agent</th>
<th>MIC (μg/ml)</th>
<th>Range</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS-TB</td>
<td>20</td>
<td>DSF</td>
<td>0.78–1.56</td>
<td>0.78</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DDC</td>
<td>1.56–3.13</td>
<td>1.56</td>
<td>3.13</td>
<td>3.13</td>
</tr>
<tr>
<td>DR-TB</td>
<td>22</td>
<td>DSF</td>
<td>0.78–1.56</td>
<td>0.78</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DDC</td>
<td>1.56–6.25</td>
<td>3.13</td>
<td>3.13</td>
<td>3.13</td>
</tr>
</tbody>
</table>

a Experiments were performed in duplicate. These results are representative of two separate experiments. DS-TB, drug-susceptible TB; DR-TB, drug-resistant TB; DSF, disulfiram; DDC, diethyldithiocarbamate.

b DR-TB includes multidrug-resistant TB (n = 13) and extensively drug-resistant TB (n = 5). Drug resistance profiles are depicted in Table S3 in the supplemental material.

FIG 1 Intracellular antitubercular activities of each agent in differentiated THP-1 cells. The bactericidal activities of DSF, DDC, S-Me-DTC, and S-Me-DTC sulfone against intracellular M. tuberculosis H₃₇Rv were assessed by the amount of CFU reduction compared to the amount achieved with antibacterial drugs, i.e., INH, RIF, STR, and EMB. Error bars represent means ± SDs (n = 3). Experiments were performed in triplicate and were carried out more than three times, and representative data are shown.
reported that DSF possessed a tuberculostatic effect at 20 mg/kg in the experimental model of tuberculosis in guinea pigs, there was no significant bactericidal activity in either the lungs or spleens of mice treated with DSF at 40 mg/kg (Fig. 2) (17). Taken together, these results suggest that DSF would exhibit a therapeutic effect against M. tuberculosis infection. According to previous reports, DSF could be administered at 6 g without considerable harm to humans, and intriguingly, it has been demonstrated that DSF and its metabolites preferentially transfer to lungs rather than to plasma, brain, or liver, suggesting that the use of DSF is feasible for the treatment of TB, especially for MDR/XDR-TB (8, 29).

The currently available anti-TB drugs, i.e., isoniazid, streptomycin, ethambutol, and p-aminosalicylic acid, have hitherto been known to be metal chelators (10, 11, 12). Intriguingly, for example, it has been reported that PAS may exert a synergistic effect with copper ion on antibacterial activity (11a). β-CAs, reported to be the drug target of dithiocarbamates, belong to the metalloenzyme family and include Zn ion at the active site when the enzymatic reaction is catalyzed (19). On the other hand, dithiocarbamates, e.g., DDC and PDTC, have been known to be metal chelators and have been reported to possess synergistic effects with copper ion on killing activity against protozoa, i.e., Plasmodium falciparum and Toxoplasma gondii (5, 21). Thus, to assess whether metal ions, namely, Zn\(^{2+}\), Cu\(^{2+}\), Co\(^{2+}\), or Al\(^{3+}\), affect the antitubercular activities of DSF and DDC, we prepared metal ion-containing Sauton broth medium and determined the MICs of these compounds. Increased antitubercular activities of these compounds were observed in CuSO\(_4\)-containing broth in a dose-dependent manner, unlike the findings for broth containing ZnSO\(_4\), CoCl\(_2\), or Al\(_2\)(SO\(_4\))\(_3\), indicating that the antitubercular activities of these compounds are dependent on small amounts of copper ion (see Table S4 in the supplemental material). Consequently, these results indicate that DSF and DDC exert synergistic effects with copper ion (>1 nM), unlike Zn\(^{2+}\), Co\(^{2+}\), or Al\(^{3+}\). For this reason, the antitubercular activities of these compounds were determined using the copper ion chelator BCPS. BCPS was reported to inhibit the activation of signal transduction and the cell growth activated by DSF in melanoma via chelating copper ion (3). As expected, the antitubercular activities of these compounds were suppressed by BCPS in 7H9 broth, normally supplemented with 4 \(\mu\)M Cu\(^{2+}\) ion, and similar results were obtained for INH and ETH (Table 4). These results suggest that these compounds possess synergistic effects with the small amount of copper ion existing in the human body (30). Based on our knowledge, β-CAs have been reported to be the drug targets of sulfonamides and sulfamates (25). Thereby, we considered that dithiocarbamates required sulfonation via intracellular enzymes, e.g., monoxygenase (EthA), for activation (1, 7). Meanwhile, DDC is catalyzed by the monoxygenase CYP2E1 in the human body. Hence, we hypothesized that DDC may possess a mechanism of activation that relies on the monoxygenase EthA and studied whether ethA expression was required for the

### TABLE 3 Serum bactericidal activities of each agent compared to vehicle control

<table>
<thead>
<tr>
<th>Titer</th>
<th>DSF, p.o.</th>
<th>DDC, p.o.</th>
<th>INH, p.o., 1 h</th>
<th>RIF, p.o., 1 h</th>
<th>DDC, i.v., 0.083 h</th>
<th>INH, i.v., 0.083 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.083 h</td>
<td>2 h</td>
<td>0.083 h</td>
<td>2 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:2</td>
<td>1.84 ± 0.03</td>
<td>2.66 ± 0.01</td>
<td>1.93 ± 0.05</td>
<td>2.46 ± 0.01</td>
<td>4.60 ± 0.0</td>
<td>ND</td>
</tr>
<tr>
<td>1:4</td>
<td>1.55 ± 0.003</td>
<td>1.79 ± 0.11</td>
<td>1.57 ± 0.04</td>
<td>1.58 ± 0.05</td>
<td>4.22 ± 0.12</td>
<td>4.600 ± 1.0</td>
</tr>
<tr>
<td>1:8</td>
<td>1.41 ± 0.01</td>
<td>1.49 ± 0.1</td>
<td></td>
<td>3.67 ± 0.06</td>
<td>3.85 ± 0.21</td>
<td>1.36 ± 0.07</td>
</tr>
<tr>
<td>1:16</td>
<td>—</td>
<td>—</td>
<td>3.83 ± 0.08</td>
<td>3.58 ± 0.03</td>
<td>3.32 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>1:32</td>
<td>—</td>
<td>—</td>
<td>2.32 ± 0.02</td>
<td>2.68 ± 0.03</td>
<td>2.70 ± 0.12</td>
<td></td>
</tr>
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</table>

*Each value (sample-vehicle control) represents the mean ± SD (n = 3). ND, not detected (>5.0 log\(_{10}\) CFU/ml); —, <1.0 log\(_{10}\) CFU/ml; DSF, disulfiram; DDC, diethyldithiocarbamate; INH, isoniazid; RIF, rifampin; p.o., per os; i.v., intravenous injection.*
antimycobacterial activities of DSF and DDC. As control results, the ethR-overexpressing strain exhibited high levels of resistance to ETH, whereas the ethA-overexpressing strain was hypersusceptible to ETH (see Table S5 in the supplemental material). However, the MICs of both DSF and DDC against either the ethR- or ethA-overexpressing strain were equivalent to those against the wild-type strain (BCG pMV261), indicating that the antitubercular activities of these compounds do not depend on ethR activity (see Table S5 in the supplemental material). Albeit the mechanisms of sulfonation of these compounds are currently unknown, this mechanism via monoxygenase must be necessary to exert the antitubercular activities.

In conclusion, DSF is effective against MDR/XDR-TB, exhibits bacterial activity within macrophages, and kills M. tuberculosis in mice, indicating that DSF might be a drug that may be repurposed for the treatment of MDR/XDR-TB. Further investigation on the mechanisms of action is now required to identify the potent drug targets, which will be important for the design of novel anti-tubercular drugs.

ACKNOWLEDGMENTS

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REFERENCES


