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.

A cording 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 healthy volunteers and human immunodeficiency virus-infected patients ex vivo (16). Recently, the antitubercular activities of DDC and the nitric oxide synthase inhibitor pyrrolidine dithiocarbamate (PDTC) against nonreplicating Mycobacterium tuberculosis have been 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, Japan). Mycobacterium bovis BCG strain Tokyo 172 (BCG Tokyo) was purchased from BCG Japan, Co. Ltd. M. avium 104 was provided by Deborah Geiman (John Hopkins University). Clinical isolates of M. tuberculosis were isolated at the Higashi Nagoya National Hospital (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% ADC (5% bovine serum albumin [fraction V], 2% dextrose, 0.005% bovine liver catalase, and 0.05% alkalineized oleic acid) at pH 6.6. Staphylococcus aureus ATCC 209PJC-1, S. aureus RNN4220, S. aureus M4990, 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>
<th>DSF</th>
<th>DDC</th>
<th>S-Me-DDC</th>
<th>S-Me-DTC</th>
<th>S-Me-DTC sulfoxide</th>
<th>S-Me-DTC sulfone</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis H$_3$Rv</td>
<td>1.56</td>
<td>3.13</td>
<td>&gt;100</td>
<td>&lt;100</td>
<td>25</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>M. tuberculosis H$_3$Ra</td>
<td>1.56</td>
<td>3.13</td>
<td>&gt;100</td>
<td>&lt;100</td>
<td>25</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>M. leprae BCG strain Tokyo 172</td>
<td>3.13</td>
<td>6.25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>50</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>M. avium subspar. avium ATCC 25291</td>
<td>25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>M. avium subspar. avium ATCC 35718</td>
<td>25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>M. avium subspar. hominisuis 104</td>
<td>25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>M. smegmatis JTA 64-01</td>
<td>25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*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, diethylthiocarbamates; S-Me–DDC, S-methyl N,N-diethylthiocarbamate; S-Me–DTC, S-methyl N,N-diethylthiocarbamate.

etionamide (ETH), p-aminosalicylic acid (PAS), ciprofloxacin (CIP), and bathocuproinedisulfonic acid disodium salt (BCPS) were purchased from Sigma-Aldrich Co. Thriurum, DSF, DMDC, DDC, PDTC, and amikacin disulfate salt (AMK) were purchased from Wako Pure Chemical Industries, Ltd., Japan. Kanamycin (KAN) was purchased from Meiji Seika Kaisha, Ltd., Japan. The bulk powder of disulfiram was provided by Mitsubishi Tanabe Pharma Corporation, Japan. Methyl N,N-diethylthiocarbamate (S-Me–DDC), S-methyl N,N-diethylthiocarbamate (S-Me–DTC), S-Me–DTC sulfoxide, and S-Me–DTC sulfone were purchased from Toronto Research Chemicals Inc., Canada. Hydrophilic or hydrophobic agents were dissolved in distilled deionized water (DDW) and dimethyl sulfoxide, respectively. Before examination, the stock solutions were diluted with assay broth, i.e., 7H9 broth. For *in vivo* use, RIF and DSF were dissolved or suspended in 5% gum arabic solution.

**BDT and agar dilution method.** The broth dilution test (BDT) for the determination of MICs was performed as previously described (31). The starting drug concentration was 100 µg/ml. In the cases of INH and RFP, the concentrations were 10 and 1 µg/ml, respectively. The MICs of test compounds against clinical isolates were defined as the antimicrobial concentrations that showed 90% growth inhibition of the strains. The agar dilution method for the determination of MICs using 7H11 agar at pH 6.6 was performed according to the *Manual of Clinical Microbiology* (22). The starting concentration of the drugs INH, RIF, and CIP was 3.2 µg/ml, and that of DSF, DDC, STR, EMB, KAN, and PAS was 0.125 µg/ml. The drug susceptibility test for clinical isolates was performed according to the method previously described (32).

**Intracellular antimycobacterial activities of DSF and DDC in differentiated THP-1 cells.** Cells of the human acute monocye leukemia cell line THP-1 (ATCC TIB-202) were purchased from ATCC. THP-1 cells were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, including 100 units/ml penicillin G (Meiji Seika Kaisha, Ltd., Japan) and 100 µg/ml streptomycin (Meiji Seika Kaisha, Ltd., Japan) in a humidified 5% CO2 atmosphere at 37°C. The intracellular antimycobacterial assay was performed according to the method previously described (20).

**Therapeutic efficacy in an experimental mouse model of chronic TB.** In order to examine the therapeutic efficacy of DSF and to determine the therapeutic dose range, an experimental mouse model of chronic TB was used as previously described (20). Mice obtained from the Institute of Cancer Research (ICR) (*n* = 5 per group) were inoculated intravenously with 1 × 106 CFU/ml of *M. tuberculosis* H$_3$Rv through the caudal vein, and the infection was allowed to develop for 28 days. The test compounds were then administered orally once daily for 28 days (RIF, 5 to 20 mg/kg; DSF, 40 to 160 mg/kg [2-fold dilutions]). The extracted lungs and spleen were homogenized with 1 ml DDW. The bacterial burden (CFU/organ) in either organ was counted using 7H11 agar plates to determine the therapeutic efficacy. Statistical analysis was conducted using Microsoft Office Excel 2007 software. Statistical significance was set at *P* values of <0.05, <0.01, and <0.001 compared to the vehicle control group.

**Sulfonylation mechanisms of DSF and DDC.** *ethA*- or *ethR*-overexpressing strains were prepared as described previously and were used for MIC determination (1, 7).

**RESULTS AND DISCUSSION**

In accordance with previous reports, DSF 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), DSF 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 DSF provided by Mitsubishi Tanabe Pharma, which is generally used in the clinical treatment of alcoholism in Japan, was comparable to that of chemical reagent DSF (data not shown). Additionally, the MIC of DSF 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 DSF is broad, the antimycobacterial activity of DDC was highly specific to slow-
The MIC90s of DSF and DDC exhibit potent antitubercular activities against more than 40 clinical isolates of *M. tuberculosis*. Therefore, the results suggest that not only DSF but also its metabolites obtained by sulfonation had partially restored antimycobacterial activities (MICs of DSF and DDC were determined by BDT in *vivo*). 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 sulfone, 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 *vivo*. 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 MIC90s 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 S2 in the supplemental material). 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 thiuram 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 growing 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 antitubercular activities of the metabolites of DSF, i.e., S-Me-DDC, S-Me-DTC, S-Me-DTC sulfoxide, and S-Me-DTC sulfoxide, were determined by BDT in *vivo*. 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 sulfone, 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 *vivo*. 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 MIC90s 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*.

**TABLE 2 Antitubercular activities of DSF and DDC against drug-susceptible and -resistant clinical isolates of *M. tuberculosis***

<table>
<thead>
<tr>
<th>Clinical isolate</th>
<th>No. of strains</th>
<th>Agent</th>
<th>MIC (μg/ml)</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>
</tr>
<tr>
<td></td>
<td></td>
<td>DDC</td>
<td>1.56–3.13</td>
<td>1.56</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>
</tr>
<tr>
<td></td>
<td></td>
<td>DDC</td>
<td>1.56–6.25</td>
<td>3.13</td>
<td>3.13</td>
</tr>
</tbody>
</table>

* 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.

* 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 antibiotic 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.
TABLE 3 Serum bactericidal activities of each agent compared to vehicle control

<table>
<thead>
<tr>
<th>Titer</th>
<th>Log reduction (Δlog_{10} CFU/ml)</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>1:2</td>
<td>0.083 h</td>
<td>2 h</td>
<td>0.083 h</td>
<td>2 h</td>
<td>INH, p.o., 1 h</td>
<td>RIF, p.o., 1 h</td>
<td>DDC, i.v., 0.083 h</td>
</tr>
<tr>
<td></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>
<td>2.63 ± 0.03</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</td>
<td>1.90 ± 0.08</td>
</tr>
<tr>
<td>1:8</td>
<td>1.41 ± 0.01</td>
<td>1.49 ± 0.1</td>
<td>—</td>
<td>—</td>
<td>3.57 ± 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>—</td>
<td>—</td>
<td>2.83 ± 0.09</td>
<td>3.58 ± 0.03</td>
<td>—</td>
</tr>
<tr>
<td>1:32</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>2.32 ± 0.02</td>
<td>2.68 ± 0.03</td>
<td>—</td>
</tr>
</tbody>
</table>

*Each value (sample-vehicle control) represents the mean ± SD (n = 3). ND, not detected (Δlog_{10} CFU/ml); —, <1.0 Δlog_{10} CFU/ml; DSF, disulfiram; DDC, diethyldithiocarbamate; INH, isoniazid; RIF, rifampin; p.o., per os; i.v., intravenous injection.

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 therapeutically synergistic 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).

Intriguingly, for example, it has been reported that PAS may exert a synergistic effect against the antitubercular activities of DSF and DDC, we prepared metal ion-concentrations from 40 to 160 mg/kg, and vehicle (5% arabic gum) alone was administered as a control. Error bars represent means ± SDs (n = 5). The Student t test was used to compare different treatment groups. Statistical significance was set at P values of <0.05 (*), <0.01 (**), and <0.001 (***).
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 antimycobacterial activities of these compounds do not depend on ethA expression (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 antimycobacterial activities.

In conclusion, DSF is effective against MDR/XDR-TB, exhibits bactericidal 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**

We thank Mitsubishi Tanabe Pharma Corporation, Japan, for supplying the bulk powder of disulfiram, an antialcoholism drug. We also thank Taku Chiba (Kinjo Gakuin University, Japan) for his valuable comments on this work.

This work was supported in part by a Grant-in-Aid for Scientific Research on category (C) from the Japan Society for the Promotion of Sciences, a grant for Research on Publicly Essential Drugs and Medical Devices (KHC1016) from the Japan Health Sciences Foundation, and a Grant-in-Aid for Scientific Research from the U.S.-Japan Cooperative Medical Sciences Program, Ministry of Health, Labor and Welfare, Japan, and the Fugaku Foundation.

**REFERENCES**


