In Vitro Antimicrobial Activity of Diethyldithiocarbamate and Dimethyldithiocarbamate Against Methicillin-resistant Staphylococcus

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ABSTRACT

Staphylococcus aureus has appeared which is highly resistant to both methicillin and aminoglycosides. Current therapy involves long-term intravenous therapy of vancomycin. Since vancomycin is currently the only drug used to treat these patients, there is a need to develop additional antimicrobial therapy. The in vitro antimicrobial effect of the metal chelator, diethyldithiocarbamate (DDTC) and its structural analog dimethyldithiocarbamate (DMTC) were investigated. Both DDTC and DMTC were effective against S. aureus including methicillin-resistant S. aureus (MRSA). By agar diffusion, DDTC at 10 |xg per disk produced zone sizes of 12 to 21 mm and at 100 |xg per disk produced zone sizes of 26 to 34 mm against MRSA. The DMTC produced slightly greater zone sizes against MRSA of 16 to 24 mm and 24 to 37 mm for 10 µg per disk and 100 µg per disk, respectively. The minimum inhibitory concentration (MIC) for DMTC against MRSA was 6 µg per ml. Both DDTC and DMTC were also effective against enterococci, Proteus mirabilis, Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia coli, Enterobacter cloacae, Enterobacter aerogenes, Salmonella species, Serratia marcescens and Citrobacter freundii at 100 µg per disk. The MICs of DMTC for Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia coli, Salmonella and Citrobacter freundii were approximately 128 µg per ml while the MICs for Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa and Serratia marcescens was ≥256 µg per ml. In addition, DMTC was synergistic with gentamicin against MRSA and coagulase-negative staphylococcus species, Enterobacter cloacae, Klebsiella pneumoniae and Pseudomonas aeruginosa. Additive and synergistic effects of DMTC were displayed with gen-

* Supported in part by a grant from the University of Arkansas for Medical Sciences Research Foundation.
tamicin against *S. aureus* including methicillin-resistant *S. aureus*. Since DDTC, a current orphan drug, is a metabolite of disulfiram, a drug currently clinically in use in the treatment of alcoholism, there may exist a potential for *in vivo* antimicrobial therapy with these drugs.

**Introduction**

*Staphylococcus aureus* is frequently responsible for post-operative sepsis. Recently, highly resistant strains of *S. aureus* have appeared\(^6,15,20\) which are resistant to both methicillin and gentamicin. *In vitro* resistance of methicillin-resistant *S. aureus* (MRSA) has been demonstrated against the penicillins, cephalosporins, aminoglycosides, erythromycin and chloramphenicol.\(^{20}\) Therapy against methicillin-resistant *S. aureus* (MRSA) requires long-term intravenous administration of vancomycin. It may be only a matter of time before vancomycin resistance emerges and becomes widespread, and currently there exists no drug to combat these highly resistant infections.

Diethyldithiocarbamate (DDTC), a metabolite of disulfiram, is a recognized chelating agent of a number of metal cations including copper, nickel, cadmium, and platinum.\(^{25,26,27}\) It is also an orphan drug approved for the treatment of nickel toxicity. However, it does not chelate calcium or magnesium.\(^10,11\) Diethyldithiocarbamate also serves as an effective antagonist of cis-platinum nephrotoxicity.\(^4,30\) A proposed mechanism for its protective effect involves chelation and subsequent removal from the kidney.\(^{30}\) Over the past two decades, Dr. F. William Sunderman, Sr. has used DDTC (Thiocarb\(^\circ\)) in the treatment of several hundred cases of acute nickel carbonyl poisoning as well as nickel eczema and dermatitis in humans.\(^{25,26,27}\)

Doses of DDTC as great as 500 mg per kg per day have been given to rodents,\(^10\) and this dose approximates the dose needed to alleviate nickel toxicity in humans. In this mode, mice tolerated chronic therapy of 100 mg per kg per day. Preliminary work in mice has shown DDTC to be effective *in vivo* against both bacterial and systemic fungal infections.\(^{31,32}\) Sabath et al\(^{22}\) have described reversal of intrinsic resistance of *S. aureus* to penicillin and cephalosporins with the chelating agents, ethylenediaminetetraacetate (EDTA), o-phenanthroline, 8-hydroxyquinoline, and diethylentriamine (DETA). This study was undertaken by us to examine the antimicrobial effects of the thiocarbamate chelators diethyldithiocarbamate (DDTC) and its structural analog, dimethyldithiocarbamate (DMTC), on MRSA and other common bacteria.

**Materials and Methods**

**Organisms**

Organisms were obtained from the University of Arkansas for Medical Sciences Clinical Microbiology Laboratory and maintained on cystine tryptcase agar (CTA).\(^*\) Bacteria to be tested were subcultured from CTA onto tryptcase soy agar containing five percent sheep blood\(^*\) and incubated at 35°C for 18 hours. *Staphylococcus aureus* susceptibility to penicillins had been determined in the Clinical Microbiology Laboratory by a commercial microtiter system.\(^\dagger\) Methicillin-resistant *S. aureus*
(MRSA) was defined as those organisms with an oxacillin minimal inhibitory concentration (MIC) $\geq 4 \mu g\ per\ ml$.

**ANTIMICROBIALS**

Diethyldithiocarbamate sodium salt (DDTC), dimethyldithiocarbamate (DMTC), and gentamicin sulfate were obtained. Solutions of DDTC, DMTC, and gentamicin were prepared fresh daily in 0.9 percent saline.

**AGAR DISK DIFFUSION**

Agar disk diffusion susceptibility tests were performed according to standard methods. A standardized inoculum was swabbed onto Mueller-Hinton agar supplemented with 25 $\mu g$ per ml magnesium and 50 $\mu g$ per ml calcium. Blank paper disks, 0.24 inch diameter were inoculated with freshly prepared DMTC or DDTC in saline for a final concentration of 10 $\mu g$ per disk or 100 $\mu g$ per disk. The paper disks containing DMTC or DDTC were then placed onto the inoculated Mueller-Hinton agar plates. After incubation at 35°C for 18 hours, the diameter of the zone of inhibition was measured. Each concentration of antimicrobial was tested in triplicate on at least two separate occasions.

**MIC DETERMINATIONS**

Microdilution broth susceptibility tests were performed according to standard methods. Mueller-Hinton broth supplemented with 25 $\mu g$ per ml magnesium and 50 $\mu g$ per ml calcium was used as the growth medium and diluent. Serial two-fold dilutions of DMTC were made in Linbro sterile tissue culture 96 well plates using a digital multichannel pipet. A standard inoculum (5 $\times$ 10$^4$ CFU) was added to each well. The microtiter plates were incubated for 18 hours at 35°C and examined for turbidity. The MIC was defined as the lowest concentration of DMTC which inhibited visible growth of the test organism.

**DRUG COMBINATION ANTIMICROBIAL ACTIVITY**

The checkerboard technique was used to test for the interaction of gentamicin and DMTC. Doubling dilutions of one drug were tested in combination with various concentrations of the other antimicrobial. A standard inoculum of 5 $\times$ 10$^4$ organisms per well was used. The microtiter plates were incubated 18 hours at 35°C. The interaction was defined as synergism if the sum of the fractional inhibitory concentration (FIC) or a checkerboard combination was $\leq 0.70$, and it was defined as additive for FIC of 0.71 to 1.29 and as antagonism for FIC $\geq 1.3$. Organisms were tested on at least two separate occasions.

**Results**

In table I are compared the *in vitro* antimicrobial activity of DDTC and DMTC against a number of common bacteria. Diethyldithiocarbamate at 10 $\mu g$ per disk produced zone sizes ranging from 12 to 21 mm against 13 isolates of *Staphylococcus* species, including those that were methicillin resistant. Dimethyldithiocarbamate appeared to be slightly more effective at 10 $\mu g$ per disk.

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* BBL Microbiological Systems, Becton Dickinson Co., Cockeysville, MD.
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** Difco, Detroit, MI.

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TABLE I

Microorganism Susceptibility of Diethyldithiocarbamate and Dimethyldithiocarbamate by Agar Diffusion

<table>
<thead>
<tr>
<th>Organism (No. of isolates)</th>
<th>Diethyldithiocarbamate</th>
<th>Zone Sizes (mm)</th>
<th>Dimethyldithiocarbamate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 µg/disk mean range</td>
<td>100 µg/disk mean range</td>
<td>10 µg/disk mean range</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus,</strong> methicillin-resistant (6)</td>
<td>16 12-21</td>
<td>29 26-34</td>
<td>21 16-28</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus,</strong> methicillin-sensitive (3)</td>
<td>21 19-25</td>
<td>32 28-35</td>
<td>23 20-28</td>
</tr>
<tr>
<td><strong>Staphylococcus species,</strong> coagulase-negative (4)</td>
<td>13 11-15</td>
<td>27 20-40</td>
<td>18 11-27</td>
</tr>
<tr>
<td><strong>Enterococcus (1)</strong></td>
<td>12 10-12</td>
<td>19 16-25</td>
<td>6 6</td>
</tr>
<tr>
<td><strong>Proteus mirabilis (3)</strong></td>
<td>6 6</td>
<td>6 6</td>
<td>6 6</td>
</tr>
<tr>
<td><strong>Proteus vulgaris (1)</strong></td>
<td>6 6</td>
<td>21 15-25</td>
<td>6 6</td>
</tr>
<tr>
<td><strong>Klebsiella pneumoniae (2)</strong></td>
<td>8 6-12</td>
<td>23 21-25</td>
<td>6 6</td>
</tr>
<tr>
<td><strong>Escherichia coli (3)</strong></td>
<td>8 6-14</td>
<td>25 21-27</td>
<td>6 6</td>
</tr>
<tr>
<td><strong>Enterobacter cloacae (1)</strong></td>
<td>13 11-14</td>
<td>26 24-26</td>
<td>7 6-11</td>
</tr>
<tr>
<td><strong>Enterobacter aerogenes (1)</strong></td>
<td>6 6</td>
<td>23 22-24</td>
<td>6 6</td>
</tr>
<tr>
<td><strong>Salmonella species (2)</strong></td>
<td>6 6</td>
<td>20 18-23</td>
<td>6 6</td>
</tr>
<tr>
<td><strong>Serratia marcescens (2)</strong></td>
<td>6 6</td>
<td>22 19-24</td>
<td>6 6</td>
</tr>
<tr>
<td><strong>Citrobacter freundii (2)</strong></td>
<td>6 6</td>
<td>22 19-23</td>
<td>6 6</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa (4)</strong></td>
<td>6 6-7</td>
<td>7 6-10</td>
<td>6 6-9</td>
</tr>
</tbody>
</table>

than was DDTC, since DMTC produced zone sizes of 16 to 28 mm against these same isolates. At 100 µg per disk, DMTC was also slightly more effective against S. aureus than DDTC with zone sizes of 24 to 42 mm. The antimicrobial activity of both DDTC and DMTC was as effective against MRSA as against other isolates of S. aureus which were methicillin-susceptible.

An attempt was made to perform an MIC with DDTC; however, there was significant turbidity in the absence of organism, possibly owing to the precipitation of metals in the media. The turbidity interfered with the determination of growth or no growth in each microtiter well. The DMTC caused no such problem with turbidity and it was possible to perform an MIC determination which is presented in table II. The MIC data support the agar diffusion data with DMTC being most effective against S. aureus at 6 µg per ml while MIC values approximated 128 µg per ml for Enterobacter cloacae, Enterobacter aerogenes, Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia coli, and Citrobacter freundii. The DMTC was not effective against Pseudomonas aeruginosa, Proteus vulgaris, Proteus mirabilis or Serratia marcescens since the MIC was greater than or equal to 256 µg per ml.

In order to determine if there existed a synergistic or additive effect with DMTC and gentamicin, a fractional inhibitory index was performed (table III) according to the method of Hamilton-Miller.13 The DMTC was synergistic with gentamicin against MRSA, coagulase-negative Staphylococcus species, Enterobacter cloacae, Klebsiella pneumoniae and Pseudomonas aeruginosa. The DMTC displayed additive antimicrobial properties with gentamicin against Staphylococcus aureus, which was methicillin-susceptible.

Discussion

Current antimicrobial therapy against MRSA remains vancomycin with or without additional non-β-lactam antimi-
IN VITRO ANTIMICROBIAL ACTIVITY OF DDTC AND DMTC

TABLE II
MIC Values of Dimethyldithiocarbamate Against Various Gram Negative Bacteria

<table>
<thead>
<tr>
<th>Organism (No. of isolates)</th>
<th>MIC (µg/ml)</th>
<th>mean</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus, methicillin-resistant (9)</td>
<td>6</td>
<td>2-8</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus, methicillin-sensitive (3)</td>
<td>7</td>
<td>4-8</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus species, coagulase-negative (4)</td>
<td>6</td>
<td>4-8</td>
<td></td>
</tr>
<tr>
<td>Enterobacter cloacae (1)</td>
<td>96</td>
<td>64-128</td>
<td></td>
</tr>
<tr>
<td>Enterobacter aerogenes (1)</td>
<td>128</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae (2)</td>
<td>128</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Klebsiella oxytoca (2)</td>
<td>128</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Proteus vulgaris (1)</td>
<td>256</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>Proteus mirabilis (3)</td>
<td>256</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa (4)</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli (3)</td>
<td>85</td>
<td>64-128</td>
<td></td>
</tr>
<tr>
<td>Citrobacter freundii (2)</td>
<td>112</td>
<td>64-128</td>
<td></td>
</tr>
<tr>
<td>Serratia marcesens (2)</td>
<td>256</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>Salmonella species (2)</td>
<td>128</td>
<td>128</td>
<td></td>
</tr>
</tbody>
</table>

Recent studies on the mechanism of resistance of MRSA have examined the PBP which are thought to be transpeptidases and are essential to cell wall peptidoglycan synthesis. Thus, PBP1 appears to be essential for growth although its function is unknown. Also, PBP2 and PBP3 are primary transpeptidases and are the primary binding sites for β-lactam antibiotics in S. aureus and a unique 78,000 dalton PBP2' has been isolated in MRSA. In addition, PBP4 is a secondary transpeptidase responsible for a high degree of peptidoglycan crosslinking in S. aureus.36 Hartman and Tomasz reported that methicillin-resistant and -susceptible strains were found to contain PBPs of the same number and electrophoretic mobilities although there existed a decreased affinity of 3H-penicillin for PBPs in the resistant strain as compared to the susceptible strain.

The ability of DDTC to chelate metals may be important in its role as an antimicrobial, perhaps by chelation of a metal cofactor necessary to an enzyme. DDTC is known to inhibit the activity of superoxide dismutase (SOD), thereby increasing the susceptibility to oxygen toxicity. Other possibilities include inhi-

TABLE III
Fractional Inhibitory Indices (FIC) of Dimethyldithiocarbamate and Gentamicin Against Various Bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>FIC Index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus - methicillin resistant</td>
<td>0.65 ± 0.25f</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>0.85 ± 0.15</td>
</tr>
<tr>
<td>Staphylococcus species - coagulase negative</td>
<td>0.25 ± 0.09</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>0.63 ± 0.20</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>0.66 ± 0.20</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.63 ± 0.10</td>
</tr>
</tbody>
</table>

*Synergy is defined as an FIC index less than or equal to 0.70, additivity as an FIC index between 0.71 and 1.29, antagonism as an FIC index of greater than or equal to 1.3. fMean ± 1 standard deviation for each isolate (n=4 for each organism).
bition of other enzymes perhaps by interacting with cofactors or enzymes containing sulphydryl groups. *S. aureus* contains SOD which utilizes manganese as a cofactor, and this may explain part of its antimicrobial activity. Since DDTC and DMTC were more effective against *S. aureus* than other bacteria, the thiocarbamates may interfere with an enzyme present in higher quantity in *S. aureus*, e.g., PBP4.

Other chelating agents have been shown to act as antibiotics against *S. aureus*. Ethylenediaminetetraacetate (EDTA) showed the best results, lowering the MIC of methicillin from 100 to 1600 μg per ml in the absence of EDTA, to 1.6 to 6.2 μg per ml in the presence of EDTA. Sabath et al. also tested four other chelating agents, o-phenanthroline, 8-hydroxyquinoline, diethylenetriamine (DETA), and triethylenetriamine (TETA). These chelators did not affect the methicillin-resistant strain significantly; however, the o-phenanthroline caused a 256-fold decrease in the MIC of methicillin in the methicillin-resistant strain when used at 24 hours.

Our results also indicate that chelators are effective against *S. aureus*; however, similar antimicrobial activity was noted by us in both methicillin-resistant and -susceptible strains. The chelators, DDTC and DMTC, may prove to be effective antimicrobials since preliminary data have been shown by the present authors that these chelators are effective in vivo.

Treatment of MRSA with DDTC in vivo is possible since plasma levels of 0.3 to 1.2 mM (44 to 178 μg per ml) have been attained in animal models. These plasma levels are several times greater than the MIC necessary to be effective against *S. aureus*, which ranged from 2 to 8 μg per ml. Metals may play an essential role in growth of *S. aureus* and these results justify further work in the mechanism of action of metals with *S. aureus* enzyme mechanisms. Chelators alone or in combination with other antimicrobials may hold promise as an antibiotic in the therapy against MRSA.

References


IN VITRO ANTIMICROBIAL ACTIVITY OF DDTC AND DMTC


