A novel agent effective against infection with *Clostridium difficile*

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Running title: Protection of hamsters from *Clostridium difficile*
Abstract

N²-(3,4-Dichlorobenzyl)-7-(2-[1-morpholinyl]ethyl)guanine (MorE-DCBG, 362E) is a synthetic purine that selectively inhibits the replication-specific DNA polymerase of Clostridium difficile (CD). MorE-DCBG and analogs strongly inhibited the growth of a wide variety of CD strains. When administered orally in a hamster model of CD-specific colitis, 362E was as effective as oral vancomycin, the current agent of choice for treating severe forms of the human disease.
*Clostridium difficile* (CD) is an anaerobic, spore-forming, Gram-positive bacterium. It is the causative agent of CD-associated disease (CDAD) an increasingly common, life-threatening disease (5,6,8). New anti-CDAD agents are needed to supplement those in current use. We have developed a series of 7-substituted-N²-(3,4-dichlorobenzyl)guanines (DCBGs) that selectively inhibit the DNA polymerase IIIC (pol IIIC) which CD requires to replicate its DNA (9). One of these derivatives, MorE-DCBG (362E, see structure in Figure 1) displays excellent potential for further development as a clinical agent. Its properties are described below.

**Inhibitors and antibacterial agents.** Compound 362E and other DCBGs (see structures in Figure 1) were synthesized and purified as described (10,11).

Vancomycin and metronidazole were from Sigma (St. Louis, MO) and clindamycin from Spectrum (New Brunswick, NJ). For use in antimicrobial assays, compounds were prepared as 40 mM stock solutions in reagent grade dimethyl sulfoxide (DMSO). Bacterial growth was not affected by DMSO concentrations up to 5%.

**Assays of antibacterial activity.** Assays were performed by Micromyx LLC (Kalamazoo, MI) and R.M. Alden Research Laboratory (Culver City, CA), using media and methods recommended by the Clinical and Laboratory Standards Institute for susceptibility testing of anaerobes (3).

Four derivatives of a larger number of N²-substituted purines (9 and data not shown) which displayed potent inhibitory activity against CD pol IIIC (9) were assessed for their MICs vs 23 different CD strains. These included one ATCC strain and 22 clinical isolates, several of which displayed different sensitivities to vancomycin and metronidazole. The results, summarized in Table 1, show that the ethyl analog, 362E, was clearly the most potent of the new compounds, displaying...
MIC$_{50}$ and MIC$_{90}$ values (2 and 4 µg/ml) close to those found for the comparators, vancomycin and metronidazole. The weaker 7-morpholinylalkyl-DCBGs were approximately equipotent. None of the vancomycin- and metronidazole-resistant strains used in this MIC assay displayed cross-resistance to any of the four DCBGs (data not shown).

Orally, 362E and 359E are poorly absorbed and apparently non-toxic. Given the results of the MIC experiments, we chose 362E and 359E for assessment of efficacy in the hamster CDAD model. Before proceeding, we examined their toxicity in hamsters and their absorption from the hamster GI tract. **Toxicity:** The results (not shown) indicate that oral doses of either compound as high as 1000 mg/kg caused no obvious toxicity. **GI absorption:** This parameter is an important determinant of an agent's potential for achieving a high local concentration at the site of CD infection in the colon. The approach, LC-MS quantification and classical "area-under-the curve" pharmacokinetics, indicated that less than 5% of an oral dose of 75 mg/kg was absorbed in both cases (results not shown).

**Assay of drug efficacy in vivo.** The Golden Syrian hamster-based model of CD-induced colitis described Kokkotou et al. (7) was used. In this model 80-90 g female Golden Syrian hamsters are injected subcutaneously with a single dose (15 mg/kg) of clindamycin hydrochloride, and, 24 hours later, one ml of CD spore suspension (ATCC strain 43255; 0.5-1 x 10$^7$ cfu/ml; prepared by us or by R.M. Alden Research Laboratories) is administered by oral gavage to each animal. In the absence of treatment, the infected animal soon develops diarrhea and proceeds to die within 48-66 hours of infection. The development of the CD-induced disease state depends on pretreatment with clindamycin; with no pretreatment, the animals remain healthy and do not develop CDAD.

To examine the efficacy of an agent in the model, suspensions of the agent in 1% carboxymethylcellulose (CMC) were administered by oral gavage to groups of 6 animals, starting 17 hours after spore administration and continuing twice daily for 3
days (or more than 3 days, if indicated). The experiment also included a negative control group receiving only vehicle and a positive control group receiving oral vancomycin in the same regimen (7).

To assess recurrent infection following various treatment periods, animals were maintained in their cages for up to 34 days after initial infection. Cages and their contents were changed every 7 days. The colonic contents of all animals that died were tested for the presence of CD toxins A and/or B (Xpect® CD Toxin A/B test kit, Remel Inc., Lenexa, KS) per manufacturer’s instructions.

Given the strong anti-CD properties of 362E and 359E, their favorable toxicity profiles, and their marginal GI absorption, we examined their efficacy in the hamster CDAD model. The results, summarized in Table 2, indicate that oral treatment with vancomycin, 362E or 359E at a dose of 50 mg/kg twice daily for three days completely protected infected animals for a period of up to 5 days. In these conditions, 362E appeared to be more potent than 359E - for example, 6.25 mg/kg of 362E was superior to the same dose of 359E (p< 0.001; one way ANOVA), whereas 6.25 mg/kg of 362E was not significantly different from 12.5 mg/kg of 359E (p> 0.05; Bonferroni multiples comparison).

Prolongation of the treatment period reduces recurrence of CDAD. Although drug treatment at 50 mg/kg twice daily completely protected animals (Table 2), this protection did not persist when the observation period went beyond 5 days. Recurrent, lethal disease was observed in both the 362E and vancomycin groups - 67% of treated animals died when treatment was limited to 3 days post-infection (Figure 2). However, treatment for 7 days with 362E at 50 mg/kg twice daily reduced the recurrence rate to 40% and delayed death when the disease recurred, and when the same treatment regimen was continued for a total of 14 days, there was no recurrence observed during the remainder of the 34 day observation period (Figure 2). (Note: intestinal exudates of all animals that died were positive for toxins A and/or B, and those of all survivors were negative.) These results show that the efficacy of
362E in CDAD, like that of vancomycin, depends on the length of the period during which drug is administered.

In sum, our lead compound, 362E, is poorly absorbed from the GI tract and essentially non-toxic when given orally. These properties and the results presented in Tables 1 and 2 and Figure 2 establish 362E as a worthy candidate for continued development as a new oral agent for the treatment of human CDAD.

Why develop another anti-CD agent when there are other agents available, i.e. vancomycin, metronidazole, and, most recently, fidaxomicin (4)? There are at least two significant reasons. First, 362E selectively hits pol IIIC, a target heretofore unexploited in CD drug development. This property gives 362E strong potential for bypassing the resistance that will eventually emerge in CD during the prolonged application of the agents in current use. Second, undesirable clinical issues frequently develop with prolonged use of any given therapy. For example, the widespread use of vancomycin, the current choice for therapy of severe CDAD, and metronidazole, the agent of choice for less severe disease, greatly increases the potential for patient shedding of both vancomycin-resistant enterococci and staphylococci (1,2).

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References


Table 1. Activity of 7-Morpholinylalkyl-DCBGs and comparators against 23 CD strains.1

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>359E</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>258D</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>363A</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>362E</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>vancomycin</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>metronidazole</td>
<td>1</td>
<td>4</td>
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</table>

1 Experiments were conducted by Alden Research Lab. with 22 clinical isolates and an ATCC strain.

Table 2. Activity of test compounds on CD infection model in Golden Syrian hamsters.2

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>Treatment (PO, bid, for 3 days), mg/kg</th>
<th>Survivors at:</th>
<th>% survivors at 120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>1</td>
<td>- (neg control)</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>vancomycin, 50</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>359E, 50</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>359E, 25</td>
<td>6</td>
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</tr>
<tr>
<td>5</td>
<td>359E, 12.5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>6†</td>
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<td>362E, 50</td>
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<td>8</td>
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<td>9</td>
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<td>6</td>
</tr>
<tr>
<td>10</td>
<td>362E, 6.25</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

2 All animals were pretreated with clindamycin hydrochloride (15 mg/kg, SC) 24 h before oral infection with ca. 10<sup>6</sup> cfu CD spores (ATCC 43255). 3 Treatments were begun 16-18 h post-infection. 4 n=3.
Figure 1. Structures of 7-(morpholinyl)alkyl-DCBGs

Figure 2. Acute cures and recurrences of CDAD in hamsters treated with 362E or vancomycin. Oral infection was on day 0 with $0.5 \times 10^7$ cfu of CD strain ATCC 43255. The set-up for the basic experimental protocol is described in the text. On day 1, 362E (■) or vancomycin (●) were given twice daily at 50 mg/kg/dose by oral gavage from days 1-3, and, in separate groups, 362E was given by oral gavage from days 1-7 (▲) or 1-14 (□). Untreated control animals (●).