Inhibitors of lipopolysaccharide biosynthesis impair the virulence potential of *Escherichia coli*

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1. SUMMARY

Inhibition of 3-deoxy-**manno**-octulosonate cytidylytransferase (CMP-KDO transferase; EC 2.7.7.38) by 8-amino-2,6-anhydro-3,8-dideoxy-**p**-glycer-**p**-talo-octonic acid (NH<sub>2</sub>dKDO) halts the growth of Gram-negative bacteria by depriving the cells of the 3-deoxy-**p**-**manno**-2-octulosonate required for the biosynthesis of the core region of the lipopolysaccharide components of the outer membrane. Low levels of this inhibitor increase the vulnerability of *Escherichia coli* to hydrophobic antibiotics, detergents, the complement-mediated antibacterial activity of serum, phagocytosis, and enhance the rate at which bacteria are cleared from the mouse bloodstream.

2. INTRODUCTION

The outer membrane of Gram-negative bacteria contains the unique molecule lipopolysaccharide (LPS), which consists of three covalently linked regions. Integrated into the outer membrane is the lipid A moiety, an acylated glucosamine disaccharide, extending outwards from which is a short oligosaccharide, the core. In virtually all Gram-negative bacteria the core oligosaccharide is linked to lipid A by a 3-deoxy-**p**-**manno**-2-octulosonate (KDO) dimer [1–3]. In so called ‘smooth’ strains a long carbohydrate polymer, the O-side chain, extends from the core region [4]. KDO is recognized as a promising target for the development of novel antibacterials acting against Gram-negatives as the inability to produce KDO is a lethal event [5]. Additionally, since KDO is not found in mammalian cells, agents directed against its biosynthesis should possess high selective toxicity.

The LPS component of the outer membrane allows Gram-negative bacteria to persist in vivo and thereby directly contributes to their virulence [6,7]. Inhibitors of LPS should therefore have an advantage over other antibacterials in that, even at sub-inhibitory concentrations, the ability of the bacteria to successfully evade host defence mechanisms will be reduced. The diazaborane Sa84474 [8] appears to inhibit LPS-biosynthesis by block-
ing the incorporation of lipids into lipid A. Although this compound has proved too toxic for use in chemotherapy it has been shown to reduce the virulence properties of *E. coli* [9]. This study was undertaken to determine if the same held true for KDO-inhibitors.

3. MATERIALS AND METHODS

3.1. Bacterial strains

Type strains were obtained from the American Type Culture Collection, Rockville, MD. *S. typhimurium* AG701i50 (*kdsA* A11) was kindly supplied by M.J. Osborn. *E. coli* C10 (O7:K1) and D120 (K1) were originally isolated from neonatal meningitis patients [10].

3.2. Antibacterials

Peptide derivatives of NH₃dKDO were synthesized [3] in the laboratories of Astra AB, Sodertalje, Sweden. Sa84474 was supplied by the Sandoz Forschungsinstitut, Vienna. Antibiotics were obtained from Sigma.

3.3. Minimum inhibitory concentration (MIC)

Two-fold serial dilutions of the antibacterials were made in the peptide-free medium of Gold- man and Leive [11] or using MacConkey Broth (Oxoid) in the wells of a microtitre plate giving a final volume of 100 µl. Bacteria (10⁵) from an overnight culture were inoculated into each well and the plate incubated at 37°C overnight. The MIC was determined as the lowest drug concentration at which no turbidity could be detected.

3.4. Serum bactericidal assay

The survival of bacterial strains in pooled normal human serum was determined as described previously [6]. Bacteria were incubated with the drug in minimal medium for 1 h before exposure to the serum.

3.5. Phagocytosis assay

Bacteria grown at 37°C in nutrient broth until mid-exponential phase, were harvested by centrifugation, washed twice in phosphate-buffered saline pH 7.3 (PBS), incubated with or without drug for 1 h at 37°C, and recentrifuged. The bacteria were opsonized by resuspending the pellet in 20% human serum in PBS, incubating at 37°C for 20 min, washing twice more in PBS and finally resuspending in PBS.

Human polymorphonuclear leukocytes (PMNLs) were prepared from fresh heparinised human blood by sedimentation through Dextran (6% w/v in 0.9% saline)-Isopaque 440 (45% in distilled water) for 45 min at 37°C. The leukocyte rich fraction was diluted 1:1 in PBS and then the PMNLs separated by a gentle centrifugation (400 × g, 25 min) through Ficoll (0.9% w/v)-Isopaque (45%). The pellet was suspended in lysing solution (0.15 M NH₄Cl, 0.01 M NaHCO₃, and 0.1 mM EDTA at 4°C, washed in PBS and resuspended in Minimal Eagles Medium (Flow Laboratories) supplemented with 18% AB serum at a PMNL concentration of 10⁷/ml.

100 µl of opsonized bacteria, 100 µl of 10⁻⁴ M luminol (Sigma) in DMSO were mixed in a cuvette at 37°C and chemiluminescence monitored using a LKB 1250 luminometer. 25-µl aliquots were withdrawn from the cuvette at the beginning and end of the experiment, the PMNLs lysed by serial dilution in ice-cold distilled water and the number of viable bacteria determined by plating on nutrient agar.

3.6. Virulence determination (*LD₅₀*)

The virulence of *E. coli* C10 after exposure to the antibacterial was determined in mice; groups of five Charles River mice received the respective bacterial challenge suspended in 5% hog gastric mucin (Sigma). The 50% lethal dose was calculated by the method of Spearman and Karber [12].

3.7. Bacterial clearance

*E. coli* D120 was grown overnight in Brain Heart Infusion, centrifuged (4000 × g, 20 min) and resuspended in normal saline at 10⁸/ml. LPS inhibitors were added to the suspension and after 1 h 0.1 ml of the suspensions injected i.v. into mice (five mice per sample), which were bled at set time-intervals and the number of viable bacteria determined by serial dilution in saline followed by plating upon nutrient agar.
4. RESULTS AND DISCUSSION

NH₂dKDO is a potent, specific inhibitor of CMP-KDO synthetase, but because it cannot penetrate to the bacterial cytoplasm it has little antibacterial activity itself. Linking a short peptide to NH₂dKDO produces compounds that are active against most Gram-negative bacteria, but not Gram-positives [1-3]. Because these antimicrobials utilize peptide permeases to cross the bacterial cytoplasmic membrane to reach their target [1], their antimicrobial effect is greatest in peptide-free free medium (Table 1). Repeating the MIC determinations in peptide-rich medium, e.g. iso-sensitest or nutrient broth gives values at least one order of magnitude higher (data not shown). However, if MICs are performed in MacConkey broth, which contains 0.4% bile salts and crystal violet, the MIC is markedly lower (Table 1). This was not true for ampicillin. One vital function of the Gram-negative outer membrane is to provide a protective barrier against hostile environments: presumably LPS-inhibitors disturb the ordered biosynthesis of this membrane, rendering it more permeable to chaotrophic agents such as those present in bile salts and to crystal violet. Similarly, bacteria pre-treated with dipeptide-substituted NH₂dKDOs became sensitive to hydrophobic antibiotics such as nafcillin, spiramycin, novobiocin or cerulenin (data not shown). Similar results were seen with the diazaborane Sa84474. Presumably these drugs, by decreasing the amount of LPS, increase the lipophilicity of the outer membrane and thereby promote the action of detergents and facilitate the penetration of hydrophobic antibiotics.

An intact outer membrane is also a vital part of the bacterium's resistance to the complement-mediated bactericidal action of serum [7] and its ability to resist the attentions of professional phagocytes [13]. E. coli D120 is highly resistant to serum, being able to grow and divide normally in neat human serum. Bacteria treated with Ala-alanine substituted NH₂dKDO (or any other of the dipeptide-lin-

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<th>Table 1</th>
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<td><strong>Antibacterial activity (MICs) of three dipeptide substituted NH₂dKDO's in peptide-free medium [11] in comparison with the diazaborane Sa84474 and with ampicillin</strong></td>
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<tr>
<td><strong>Minimum inhibitory concentration (MIC) (μg/ml)</strong></td>
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<tr>
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<tr>
<td><strong>E. coli ATCC11303</strong></td>
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<td><strong>E. coli D120</strong></td>
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<td><strong>E. coli C10</strong></td>
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<td><strong>S. typhimurium AG70150</strong></td>
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<td><strong>S. typhimurium ATCC19585</strong></td>
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Values in parentheses show equivalent determinations made in MacConkey broth.
Fig. 2. The chemiluminescence response of human PMNLs generated while ingesting *E. coli* C10 and after exposure of the bacteria for 30 min to Ala-ala-NHSDKDO or Sa84474.

Fig. 3. Effect of treatment with LPS inhibitors upon bacterial clearance in vivo. *E. coli* D120 exposed to 1 mM Ala-ala-NHSDKDO (▼) or Pro-met-NHSDKDO (▲), or 5 µg Sa84474/ml (■) before i.v. injection into mouse. Blood samples removed and the number of bacteria/ml determined. No drug control (●).

Dipeptide derivatives of NH$_2$dKDO possess insufficient metabolic stability to maintain therapeutic blood-levels in experimental animals. It appears that the N-terminal amino acid is lost in the animal producing a mono-aminoacylated inhibitor, which, because it is not transported into the bacterium [1], has no antibacterial activity. Hence any in vivo experiments must be performed using bacteria treated in vitro before injection. Under these limited conditions low concentrations of LPS inhibitors also increased the rate at which *E. coli* D120 was cleared from the bloodstream (Fig. 3).

LPS is certainly not the only, or even the dominant, determinant of virulence on the surface of *E. coli*, indeed the possession of capsular layers (e.g. K1 or K5) [6,7,14] are probably more important. However, inhibiting LPS biosynthesis does appear to reduce the ability of *E. coli* cells to survive the bactericidal activity of serum, to avoid the attentions of phagocytes and causes the bacterial cells to be cleared more readily from the

### Table 2

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<th>Treatment</th>
<th>LD$_{50}$ in mouse</th>
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<tr>
<td>None</td>
<td>$7.5 \times 10^2$</td>
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<tr>
<td>Ala-ala-NHSDKDO (0.5 mM)</td>
<td>$2.1 \times 10^4$</td>
</tr>
<tr>
<td>Ala-ala-NHSDKDO (1 mM)</td>
<td>$1.6 \times 10^5$</td>
</tr>
<tr>
<td>Pro-met-NHSDKDO (1 mM)</td>
<td>$8.1 \times 10^4$</td>
</tr>
<tr>
<td>Sa84474 (5 µg/ml)</td>
<td>$3.3 \times 10^4$</td>
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Mid-exponential cells ($5 \times 10^8$) treated with drug in minimal medium for 1 h, centrifuged free of drug and resuspended in 5% hog gastric mucin before challenge.
body (Fig. 3, Table 2). Whether these phenomena are the direct result of a reduced LPS content of caused indirectly by consequent changes in other envelope polymers or virulence factors remains to be established.

ACKNOWLEDGEMENTS

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REFERENCES