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# Discovery and Synthesis of a Gram-Negative-Active Cationic Lipopeptide Antibiotic Inspired by Primary Sequences from Underexplored Gram-Negative Bacteria

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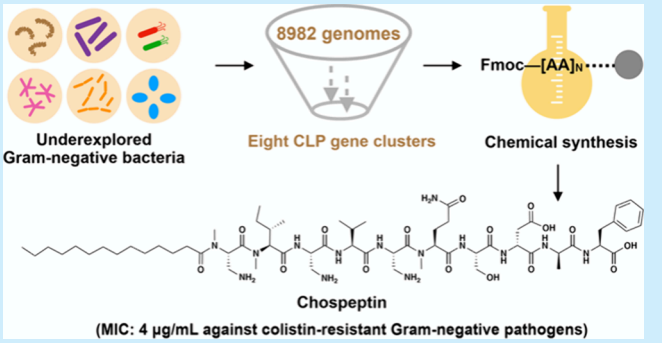
**ABSTRACT:** The emergence of multidrug-resistant Gram-negative pathogens poses a serious threat to global health. Gram-negative bacteria have become increasingly recognized as underexplored sources of Gram-negative-active cationic lipopeptide (CLP) antibiotics. We systematically screened 8982 sequenced genomes from 42 underexplored Gram-negative bacterial genera and identified eight potential CLP biosynthetic gene clusters. Their predicted products were rapidly accessed by solid-phase total synthesis, which led to the novel antibiotic chospeptin with good activities against clinically isolated colistin-resistant Gram-negative pathogens.






Infection caused by multidrug-resistant (MDR) pathogens is now recognized as a major threat to human health.[1] The increase in the incidence of MDR Gram-negative bacteria is of particular concern because ∼75% of deaths from antibiotic-resistant infections are due to Gram-negative pathogens.[2,3] Many clinically used Gram-negative-active antibiotics (i.e., colistin and gramicidin) were developed from bacterial natural products (NPs), and colistin is now used as the last therapy line against MDR Gram-negative pathogen-generated infections.[4−6] However, long-term and extensive use of these antibiotics has resulted in a rapid increase in the number of MDR bacterial strains worldwide, which necessitates the development of Gram-negative-active antibiotics with novel structures and (or) modes of action.[7,8]

Cationic lipopeptides (CLPs) make up a unique family of N-acylated lipopeptide compounds that contain at least two positively charged amino acids (i.e., 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, arginine, lysine, and ornithine).[9,10] Many CLP antibiotics are found to be potently active against MDR Gram-negative pathogens due to their unique chemical properties. They could not only penetrate highly impermeable bacterial outer membranes but also interact with different anionic intracellular targets in Gram-negative bacteria.[10,11] *Brevibacillus* and *Paenibacillus* spp. from Firmicutes are traditionally rich sources of CLP antibiotics (Table S1), but they have been overmined with high rediscovery rates.[12,13] In recent years, Gram-negative bacteria from uncommon genera, including *Chitinophaga*, *Gynuella*, *Lysobacter*, *Pedobacter*, *Pseudoalteromonas*, and *Teredinibacter*, have become increasingly recognized as underexplored sources of CLP antibiotics with therapeutic potential (Table S2).[14−16] In particular, with development of advanced genome sequencing technologies and cultivation-independent approaches, the untapped biosynthetic potential of difficultly isolated and previously overlooked Gram-negative bacterial species has been further revealed and explored for novel CLP antibiotic discovery.[17−19]

In this work, we developed a global genome mining approach to rapidly access novel CLP biosynthetic gene clusters (BGCs) from underexplored Gram-negative bacteria combined with total chemical synthesis, followed by structural prediction of unidentified CLPs. By screening 8982 Gram-negative bacterial genomes for the potential biosynthesis of CLPs, we identified one novel antibiotic with good activities against clinically isolated colistin-resistant Gram-negative pathogens.

Compared to Gram-positive actinobacteria, Gram-negative bacteria have attracted relatively little attention and been increasingly recognized as rich and underexplored sources for antimicrobial compound discovery.[14−16] Therefore, we focused on the discovery of Gram-negative-active antibiotics from Gram-negative bacteria with uncommon, diverse phylogenetic genera. In total, 8982 sequenced genomes from 42 underexplored genera of Gram-negative bacteria, which belong to five phyla (Bacteroidetes, Chloroflexi, Firmicutes, Tectomicrobia,

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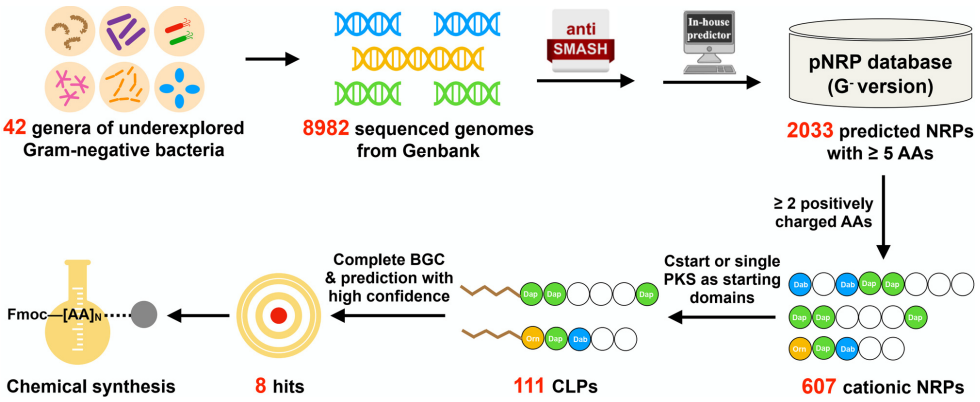
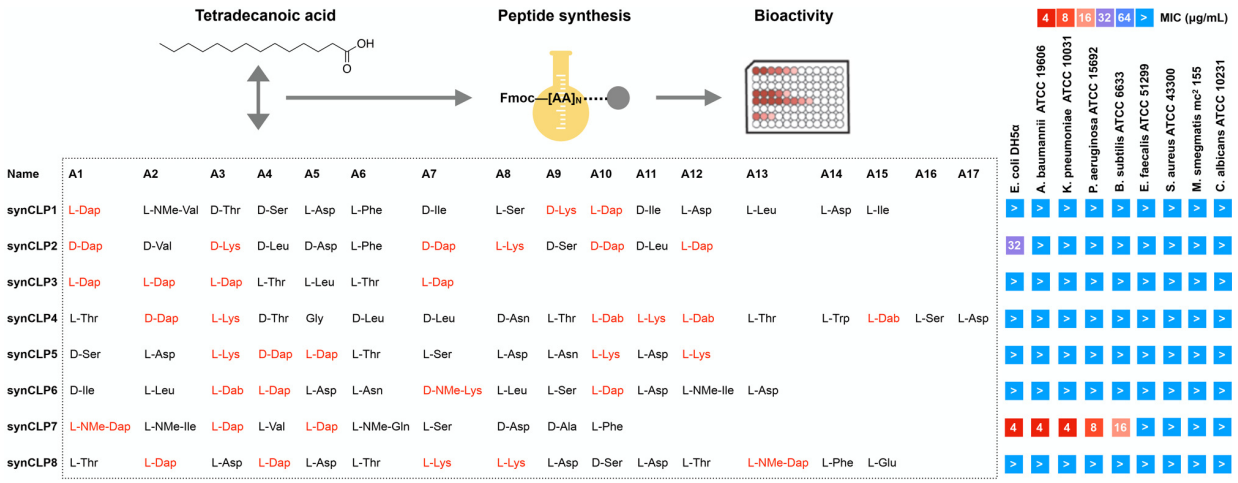



**Figure 1.** Targeted genome mining pipeline of novel cationic lipopeptides (CLPs) from underexplored Gram-negative ($G^-$) bacteria. Eight potential CLPs predicted from 8982 Gram-negative bacterial genomes were selected for total solid-phase synthesis. Abbreviations: AAs, amino acids; PKS, polyketide synthase.




| Name | A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | A9 | A10 | A11 | A12 | A13 | A14 | A15 | A16 | A17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| synCLP1 | L-Dap | L-NMe-Val | D-Thr | D-Ser | L-Asp | L-Phe | D-Ile | L-Ser | D-Lys | L-Dap | D-Ile | L-Asp | L-Leu | L-Asp | L-Ile | | |
| synCLP2 | D-Dap | D-Val | D-Lys | D-Leu | D-Asp | L-Phe | D-Dap | L-Lys | D-Ser | D-Dap | D-Leu | L-Dap | | | | | |
| synCLP3 | L-Dap | L-Dap | L-Dap | L-Thr | L-Leu | L-Thr | L-Dap | | | | | | | | | | |
| synCLP4 | L-Thr | D-Dap | L-Lys | D-Thr | Gly | D-Leu | D-Leu | D-Asn | L-Thr | L-Dab | L-Lys | L-Dab | L-Thr | L-Trp | L-Dab | L-Ser | L-Asp |
| synCLP5 | D-Ser | L-Asp | L-Lys | D-Dap | L-Dap | L-Thr | L-Ser | L-Asp | L-Asn | L-Lys | L-Asp | L-Lys | | | | | |
| synCLP6 | D-Ile | L-Leu | L-Dab | L-Dap | L-Asp | L-Asn | D-NMe-Lys | L-Leu | L-Ser | L-Dap | L-Asp | L-NMe-Ile | L-Asp | | | | |
| synCLP7 | L-NMe-Dap | L-NMe-Ile | L-Dap | L-Val | L-Dap | L-NMe-Gln | L-Ser | D-Asp | D-Ala | L-Phe | | | | | | | |
| synCLP8 | L-Thr | L-Dap | L-Asp | L-Dap | L-Asp | L-Thr | L-Lys | L-Lys | L-Asp | D-Ser | L-Asp | L-Thr | L-NMe-Dap | L-Phe | L-Glu | | |

**Figure 2.** Peptide sequences and antimicrobial bioactivities of the eight cationic lipopetides (synCLPs). All eight synCLPs were made by solid-phase peptide synthesis (SPPS) using tetradecanoic acid as the fatty acid tail. The positively charged amino acids (AAs) in synCLPs are colored in red.

and Proteobacteria), were downloaded from GenBank (Table S3) and BGCs from these genomes were analyzed by the antiSMASH pipeline.[20] The A-domain substrate binding pockets from NRPS BGCs in these genomes of Gram-negative bacteria were compared to a manually curated list of A-domain signature sequences from characterized BGCs that we developed previously.[21] On the basis of the A-domain substrate predictions that arose from this analysis, we generated a database of linear peptides that contains 2033 predicted NRPs with at least five amino acids (Figure 1). In total, 607 cationic peptides of 2033 predicted NRPs contained two or more positively charged amino acids (i.e., 2,3-diaminopropionic acid, lysine, and ornithine), which were mainly distributed in Bacteroidetes and Proteobacteria. Usually, the *N*-terminal acylation of CLPs is achieved by coupling a fatty acid to the first amino acid of the peptide by the condensation starter (Cs) domain or a single module of polyketide synthase.[9,10] On this basis, 111 of 607 cationic peptides were predicted to be potential CLPs. We then manually analyzed these potential CLP BGCs following the three standards: (1) dissimilar BGC, (2) complete BGC, and (3) structural prediction with a high degree of confidence. The detailed screening pipeline is described in the Supporting Information. Finally, eight potential CLP BGCs of interest were chosen for further study, which are from two underexplored genera, including Chitinophaga and Pedobacter (Figure S1).

Traditional NP discovery pipelines rely on microbial fermentation, which are usually hindered by a lack of BGC expression under standard laboratory conditions.[22,23] Even though BGCs of interest are activated, it is still time-consuming and labor-intensive to isolate and structurally characterize NPs from microbial fermentation broths. In cases in which a BGC-encoded final product can be bioinformatically predicted with a high degree of confidence, it will be more straightforward to access the product by total chemical synthesis.[24−26] In all eight selected potential CLP BGCs, the expression of all genes in each BGC was found to follow the same direction, which possibly form a single operon in each BGC and make structural prediction of BGC-encoded products highly accurate (Figures S2−S9). Meanwhile, A-domain substrate specificity analysis allowed us to predict the amino acid or organic acid incorporated by every A domain found in these BGCs (Tables S4−S11).[27] The linear peptide encoded by each BGC was predicted to be the direct precursor to the final peptide. To synthesize NP-inspired CLPs (synCLPs), we elected to design each synCLP to be N-acylated with tetradecanoic acid, a fatty acid commonly observed in NRPs.[28] In total, eight synCLPs were designed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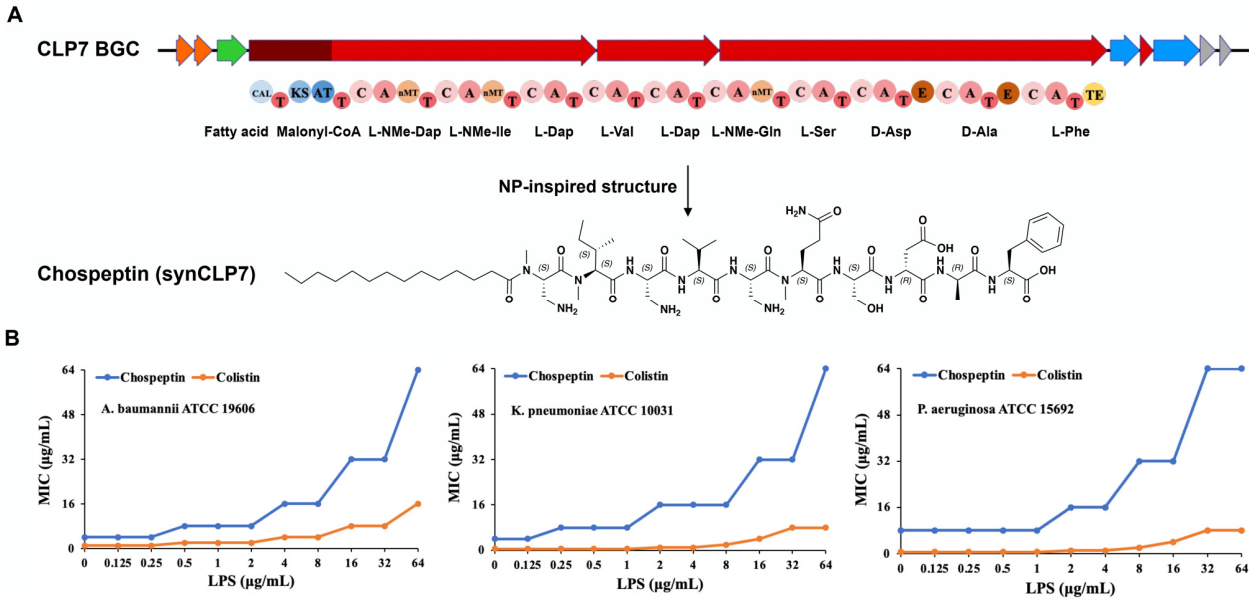


**Figure 3.** Biosynthetic gene cluster (BGC), structure, and mode of action of chospeptin. (A) Chospeptin BGC from *C. hostae* ACCC 61757 and chemical structure of chospeptin. Abbreviations: PKS, polyketide synthase; NRPS, nonribosomal peptide synthase. (B) Activities of chospeptin against three Gram-negative pathogens were determined in the presence of different concentrations of LPS ($n$ = 2). Colistin was used as the positive control.

and then synthesized on the basis of solid-phase peptide synthesis (SPPS) using standard Fmoc chemistry (Figure 2 and Figure S10). The identity of each synCLP was then confirmed by high-performance liquid chromatography (HPLC) and high-resolution mass spectrometry (MS) (Figures S11−S18 and Table S12).

The eight synCLPs that we synthesized here are expected to have Gram-negative activities because both of them contain at least two positively charged AAs, which might interact with lipopolysaccharides (LPS) in the outer membrane of Gram-negative pathogens. Therefore, we initially tested each synCLP against four Gram-negative pathogens (*Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*) and found that synCLP7 showed good Gram-negative antibacterial activities with minimum inhibitory concentrations (MICs) from 4 to 8 μg/mL (Figure 2). synCLP2 showed very weak activity against *E. coli* DH5α, and the other six synCLPs were inactive against the four tested Gram-negative pathogens (Figure 2). Furthermore, four common Gram-positive bacteria (*Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Mycobacterium smegmatis*) and one common pathogenic fungus (*Candida albicans*) were meanwhile used for the test of antimicrobial activities for each synCLP. Except for the case in which synCLP7 showed weak activity against *B. subtilis* ATCC 6633, all other seven synCLPs were inactive against the five strains listed above (Figure 2). These results indicated that synCLP7, which we named chospeptin, had a relatively narrow antimicrobial spectrum. The structure of chospeptin was further confirmed using a combination of HPLC, high-resolution MS, tandem MS and one- and two-dimensional nuclear magnetic resonance (NMR) data (Tables S12 and S13 and Figures S19 and S25).

Although the bioinformatics prediction of the peptide sequence of chospeptin appeared to be robust, the second building block in chospeptin might be L-NMe-Val instead of L-NMe-Ile (Table S10) and furthermore cyclic versions of chospeptin might be more potent. Therefore, four chospeptin analogues, including chospeptin-Val, chospeptin-C1, chospeptin-C3, and chospeptin-C5, were synthesized (Figures S26−S30 and Table S12). Compared to chospeptin, chospeptin-Val showed slightly decreased activities against most of the tested Gram-negative pathogens. In addition, the three cyclic versions of chospeptin showed significantly decreased activities against all tested Gram-negative pathogens compared to that of chospeptin (Table S14). These results indicate that chospeptin is likely to be closest to the natural product that the CLP7 BGC encodes. It is noteworthy that there is a MBL fold metallo-hydrolase encoded by the CLP7 BGC, which possibly functions as a diiron monooxygenase to introduce a β-hydroxy group into the CLP7 BGC-encoded natural product. (Figure S8).[29] The postmodification might further enhance the bioactivities of chospeptin against Gram-negative pathogens, which could be explored in the future.

The CLP7 BGC whose sequence was used as inspiration to synthesize chospeptin was found in the genome of *Chitinophaga hostae* ACCC 61757. Chitinophaga was found to be the most talented genus in terms of BGC abundance and diversity in Bacteroidetes and produced a kind of known antimicrobial CLPs, chitinopeptins.[16,30] In addition to the three same positively charged AAs [2,3-diaminopropionic acid (Dap)], chospeptin also contains three *N*-methylated amino acids (*N*-Me-Dap, *N*-Me-Ile, and *N*-Me-Gln), which might enhance the metabolic stability and hydrophobicity of chospeptin by reducing the number of hydrogen-bond donors (Figure 3A).[31]

In addition to the four types of Gram-negative pathogens that we tested above, chospeptin showed good activities against *Salmonella typhimurium* and *Shigella castellani* with a MIC of 8 μg/mL (Table 1), which indicate that chospeptin was broadly active against diverse Gram-negative bacteria. Furthermore, for a panel of clinically isolated *A. baumannii* strains, chospeptin was also active with a MIC from 4 to 8 μg/mL (Table 1). It is noteworthy that chospeptin could effectively inhibit the growth of two clinically isolated


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**Table 1. Activities of Chospeptin against Microorganisms and Human Cells (MIC, minimum inhibitory concentration)**

| type | pathogens or human cells | chospeptin | colistin |
|---|---|---|---|
| Gram-negative bacteria | *E. coli* DH5α | 4 | <0.125 |
| | *E. coli* BL21(DE3) | 8 | 64 |
| | *E. coli* 15322 | 4 | 2 |
| | *A. baumannii* ATCC 19606 | 4 | 1 |
| | *A. baumannii* 15122 | 8 | 1 |
| | *A. baumannii* P60 | 4 | >64 |
| | *A. baumannii* P57 | 8 | 1 |
| | *A. baumannii* P53 | 4 | 1 |
| | *A. baumannii* P47 | 8 | 2 |
| | *A. baumannii* 1104008 | 8 | 1 |
| | *K. pneumoniae* ATCC 10031 | 4 | 0.5 |
| | *K. pneumoniae* ATCC 13883 | 8 | 4 |
| | *K. pneumoniae* NCTC 5056 | 4 | 64 |
| | *P. aeruginosa* ATCC 15692 | 8 | 0.5 |
| | *P. aeruginosa* ATCC 9027 | 8 | 0.125 |
| | *P. aeruginosa* PAO1 | 8 | 0.5 |
| | *S. typhimurium* ATCC 14028 | 8 | 8 |
| | *S. castellani* LXX | 8 | 8 |
| Gram-positive bacteria | *B. subtilis* ATCC 6633 | 16 | >64 |
| | *E. faecalis* ATCC 51299 | >64 | >64 |
| | *S. aureus* ATCC 43300 | >64 | >64 |
| | *M. smegmatis* $mc^2$ 155 | >64 | >64 |
| fungi | *C. albicans* ATCC 10231 | >64 | >64 |
| | *S. cerevisiae* BY4741 | >64 | >64 |
| human cells | HeLa | >64 | >64 |
| | MDA-MB-231 | >64 | >64 |

colistin-resistant Gram-negative pathogens, including *A. baumannii* P60 and *K. pneumoniae* NCTC 5056 with a MIC of 4 μg/mL (Table 1). Even at the highest concentration we tested (64 μg/mL), chospeptin did not show cytotoxicity to the two human cell lines, HeLa and MDA-MB-231 (Table 1).

Considering that chospeptin as a synthetic CLP antibiotic might target LPS in the outer membrane of Gram-negative bacteria, we assayed the ability of LPS to suppress chospeptin to explore the mode of action of chospeptin. When LPS was added to the assay medium, the MIC of chospeptin increased in a dose-dependent manner for three different Gram-negative pathogens, including *A. baumannii* ATCC 19606, *K. pneumoniae* ATCC 10031, and *P. aeruginosa* ATCC 15692. The similar phenomena were also observed for the positive control, colistin (Figure 3B). The results indicate that chospeptin could target LPS, and its Gram-negative antibacterial activity was dependent on LPS. Interestingly, chospeptin was active against the two tested colistin-resistant pathogens, indicating that chospeptin should bind to a different site of LPS from colistin.

Infections caused by MDR Gram-negative bacteria, in particular colistin-resistant pathogens, are threatening to overwhelm healthcare practices worldwide.[1−3] Underexplored Gram-negative bacteria exhibit large chemical diversity for antimicrobial compound discovery.[14−16] Here we systematically screened >8900 sequenced genomes from 42 underexplored Gram-negative bacterial genera for unknown BGCs that we predicted would encode novel CLPs. Combined with chemical synthesis followed by structural prediction of eight CLPs, chospeptin was rapidly identified to be a novel antibiotic against multiple Gram-negative pathogens, especially the two clinically isolated colistin-resistant *A. baumannii* and *K. pneumoniae* strains. Considering the other seven synCLPs in addition to chospeptin were inactive, their BGC-encoded products are predicted to be cyclic peptides through either the hydroxyl group of the fatty acid (cFA) or a nucleophilic amino acid side chain (cSC). In addition, the predicted linear or cyclic peptides might need to be postmodified for bioactivity. For example, there is a dioxygenase encoded by the CLP8 BGC and a MBL fold metallo-hydrolase encoded by the CLP1 or CLP6 BGCs (Figures S2, S7, and S9). In the future, the predicted linear or cyclic peptides with or without post-modification could be further accessed for the discovery of novel antibiotics. Nevertheless, identification of chospeptin adds to the arsenal of NP-inspired structures that are available to explore in the effort to develop novel CLP-based therapeutics.

## ■ ASSOCIATED CONTENT

### Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.3c04232.

Experimental materials and methods, known CLP antibiotics, underexplored Gram-negative bacteria, CLP-domain prediction tables, CLP BGC analysis, synCLP HPLC spectra, and chospeptin NMR spectra (PDF)

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## Notes

The authors declare no competing financial interest.

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