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Therapeutic CMP-Nonulosonates against Multidrug-Resistant *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae **deploys a unique immune evasion strategy wherein the lacto-*N*-neotetraose termini of lipooligosaccharide (LOS) are "capped" by a surface LOS sialyltransferase (Lst), using extracellular host-derived CMP-sialic acid (CMP-Neu5Ac in humans). LOS sialylation enhances complement resistance by recruiting factor H (FH; alternative complement pathway inhibitor) and also by limiting classical pathway activation. Sialylated LOS also engages inhibitory Siglecs on host leukocytes, dampening innate immunity. Previously, we showed that analogues of CMP-sialic acids (CMP-nonulosonates [CMP-NulOs]), such as CMP-Leg5,7Ac$_2$ and CMP-Neu5Ac9N$_3$, are also substrates for Lst. Incorporation of Leg5,7Ac$_2$ and Neu5Ac9N$_3$ into LOS results in *N. gonorrhoeae* being fully serum sensitive. Importantly, intravaginal administration of CMP-Leg5,7Ac$_2$ attenuated *N. gonorrhoeae* colonization of mouse vaginas. In this study, we characterize and develop additional candidate therapeutic CMP-NulOs. CMP-ketodeoxynonulosonate (CMP-Kdn) and CMP-Kdn7N$_3$, but not CMP-Neu4,5Ac$_2$, were substrates for Lst, further elucidating gonococcal Lst specificity. Lacto-*N*-neotetraose LOS capped with Kdn and Kdn7N$_3$ bound FH to levels ∼60% of that seen with Neu5Ac and enabled gonococci to resist low (3.3%) but not higher (10%) concentrations of human complement. CMP-Kdn, CMP-Neu5Ac9N$_3$, and CMP-Leg5,7Ac$_2$ administered intravaginally (10 μg/d) to *N. gonorrhoeae*–colonized mice were equally efficacious. Of the three CMP-NulOs above, CMP-Leg5,7Ac$_2$ was the most pH and temperature stable. In addition, Leg5,7Ac$_2$-fed human cells did not display this NulO on their surface. Moreover, CMP-Leg5,7Ac$_2$ was efficacious against several multidrug-resistant gonococci in mice with a humanized sialome (*Cmah*$^{-/-}$ mice) or humanized complement system (FH/C4b-binding protein transgenic mice). CMP-Leg5,7Ac$_2$ and CMP-Kdn remain viable leads as topical preventive/therapeutic agents against the global threat of multidrug-resistant *N. gonorrhoeae*.**
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Neisseria gonorrhoeae is the causative agent of the sexually transmitted infection gonorrhea, the second most common worldwide sexually transmitted bacterial infection (chlamydia is the most common), with 86.9 million new cases estimated to occur annually by the World Health Organization (WHO) (1). The incidence of gonorrhea is increasing globally. In the United States, 583,405 cases were reported to the Centers for Disease Control and Prevention in 2018, which represents a 63% increase since 2014 and an 82.6% increase since the historic low in 2009 (https://www.cdc.gov/std/stats18/gonorrhea.htm). Gonorrhea commonly manifests as cervicitis, urethritis, proctitis, and conjunctivitis. Infections at these sites, if left untreated, can lead to local complications, including endometritis, salpingitis, tubo-ovarian abscess, bartholinitis, peritonitis, and perihepatitis in women, periurethritis and epididymitis in men, and ophthalmia neonatorum in newborns. Disseminated gonococcal infection is an uncommon event whose manifestations include skin lesions, tenosynovitis, septic arthritis, and rarely, endocarditis or meningitis (2, 3).

N. gonorrhoeae has demonstrated a remarkable capacity to become resistant to almost every antimicrobial used for its treatment (4). The worldwide emergence of strains resistant to third-generation cephalosporins and azithromycin (5–11), the recommended first-line agents for treatment, has ushered in an age of potentially untreatable gonorrhea. In public health efforts to stem the tide, the first-line treatment regimen was updated in 2016 to include both ceftriaxone (cephalosporin) and azithromycin (i.e., combination therapy) (12). But already by March of 2018, reports were being issued of "superbugs" resistant to the combination therapy (13, 14). In addition, the pipeline for new gonorrhea treatments is relatively "empty," with only three new candidates (solithromycin, zoliflodacin, and gepotidacin) in clinical development. Solithromycin failed to meet noninferiority criteria when compared with the

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Abbreviations used in this article: AUC, area under the curve; C4BP, C4b-binding protein; CE, capillary electrophoresis; FH, factor H; Kdn, ketodeoxynonulosonate; *lgtD*, LOS glycosyltransferase D; LNnT, lacto-*N*-neotetraose; LOS, lipooligosaccharide; Lst, LOS sialyltransferase; MIC, minimum inhibitory concentration; MS, mass spectrometry; Neu5Ac, *N*-acetylneuraminic acid; NHS, normal human serum; NJ-60, UMNJ60_06UM; NulO, nonulosonate; ST, sequence type; WHO, World Health Organization.

first-line recommended regimen of ceftriaxone plus azithromycin in a recent phase III trial (15). Zoliflodacin and gepotidacin appear promising for the treatment of uncomplicated urogenital infections, but failures to eradicate oropharyngeal infection in men who have sex with men and commercial sex workers have been reported (16–18). Thus, the possibility of untreatable gonorrhea is imminent. As such, vaccines and immunotherapeutics to prevent and treat disease caused by multidrug-resistant gonorrhea are urgently needed (19).

Targeting bacterial virulence mechanisms represents a novel way to combat antimicrobial resistance because resistance to such drugs would result in the attenuation of the microbe, thereby compromising its ability to cause disease. Sialic acids, belonging to the nonulosonate (NulO) class of monosaccharides, are negatively charged nine-carbon backbone molecules that contribute to the virulence of several pathogens, including *N. gonorrhoeae* (reviewed in Refs. 20 and 21). The addition of *N*-acetylneuraminic acid (Neu5Ac), a member of the sialic acid family prominent in humans, from host CMP-Neu5Ac to *N. gonorrhoeae* lipooligosaccharide (LOS) contributes to gonococcal serum resistance (22–24), evasion of cationic antimicrobial peptides (25), and biofilm formation (26). Experimental studies in human male volunteers (27, 28) and in mice (29, 30) have emphasized the importance of LOS sialylation in mucosal colonization. As such, *N. gonorrhoeae* LOS sialylation is a virulence mechanism that is essential for both colonization and pathogenicity and can be targeted, thereby providing new avenues for effective treatment.

In a prior study, we showed that certain analogues of sialic acid, such as Leg5,7Ac_2 and Neu5Ac9N_3 (previously referred to as Leg5Ac7Ac and Neu5Ac9Az, respectively), could be incorporated into gonococcal LOS when bacteria were fed with their respective CMP salts. Incorporation of Leg5,7Ac_2 and Neu5Ac9N_3 into LOS did not enhance bacterial resistance to complement. Remarkably, the presence of these analogue NulOs on LOS concomitantly with Neu5Ac-capped LOS rendered gonococci susceptible to human complement (31). We exploited the susceptibility of NulO-coated gonococci to innate immune defenses as a preventive/therapeutic tool and showed that intravaginal administration of CMP-Leg5,7Ac_2 decreased the duration and reduced the burden of vaginal colonization of multidrug-resistant *N. gonorrhoeae* in mice (31). In this study, we further characterize and develop therapeutic CMP-NulOs that are promising topical prophylactics/therapeutics against antimicrobial-resistant *N. gonorrhoeae*. Specifically, we evaluated the following: 1) the efficacy of other CMP-NulOs, such as CMP-ketodeoxynonulosonate (CMP-Kdn), CMP-Kdn7N_3, and CMP-Neu4,5Ac_2; 2) the efficacy of CMP-NulO candidates against various multidrug-resistant *N. gonorrhoeae* isolates; 3) dose responses; 4) efficacy in humanized mouse models; 5) pH and temperature stability of CMP-NulO candidates; and 6) NulO incorporation on human cell surfaces as a safety assessment.

Materials and Methods

Bacterial strains

A mutant of *N. gonorrhoeae* strain F62 (32) that lacked expression of LOS glycosyltransferase D (*lgtD*), called F62 ΔlgtD (33), was provided by Dr. D.C. Stein (University of Maryland). LgtD adds a GalNAc residue to the terminal Gal of the HepI lacto-*N*-neotetraose (LNnT) (34). Therefore, extension of HepI of F62 ΔlgtD is limited to NulO transferred from the CMP-NulO present in growth media by LOS sialyltransferase (Lst). A spontaneous streptomycin-resistant mutant of *N. gonorrhoeae* F62 was used in mouse infection studies (35). Strain H041 (sequence type [ST] 7363; NG-MAST ST 4220), also known as WHO reference strain X, was isolated from a female commercial sex worker in Kyoto, Japan (10). This isolate is highly resistant to ceftriaxone (minimum inhibitory concentration [MIC] 2–4 μg/ml) and several other antibiotics (10). CTX-r Spain also displays high-level ceftriaxone resistance [NG-MAST ST 1407, ceftriaxone MIC >2 μg/ml (9)]. Strain SD-1 (NG-MAST ST 3158, ceftriaxone MIC 0.094 μg/ml; cefixime MIC 0.125 μg/ml) was isolated in San Diego as part of the Gonococcal Isolate Surveillance Program (36). Strain UMNJ60_06UM (NJ-60; ceftriaxone MIC 0.38 μg/ml) was isolated in Nanjing, China and belongs to NG-MAST ST 3289 and multilocus sequence type ST 1600 (37). Strain 398078 was isolated from the female contact of a male with gonorrhea. This isolate predominantly produces the P^K-like LOS [Galα(1,4)-Galβ(1,4)-Glc] from HepI (38). All strains used in mouse experiments were rendered streptomycin resistant by transformation with *rpsL* derived from streptomycin-resistant *N. gonorrhoeae* strain FA1090 as described previously (31).

Synthesis of CMP-NulOs and biotinylated NulO-LNnT glycans

The production and characterization of CMP-Neu5Ac, CMP-Neu5,9Ac_2 (also referred to as CMP-Neu5Ac9Ac), CMP-Neu5Ac9N_3, and CMP-Leg5,7Ac_2 used in this study have been described previously (31). CMP-Neu5Ac was also obtained commercially (Nacalai Tesque). CMP-Neu4,5Ac_2 was produced using similar methods to those above with Neu4,5Ac_2 obtained commercially (Carbosynth).

CMP-Kdn and CMP-Kdn7N_3 were produced using the two methods described below. Kdn (3-deoxy-D-glycero-D-galacto-nonulosonic acid) was enzymatically prepared using a *Pasteurella multocida* aldolase (39). Typically, reactions contained 100 mM Tris (pH 7.5), 20 mM mannose, 100 mM sodium pyruvate, and ∼0.15 mg/ml aldolase. Reactions were incubated at 37˚C with gentle shaking for 24–48 h, followed by the removal of enzyme by centrifugal ultrafiltration. Next, CMP activation of synthesized Kdn was achieved enzymatically using methods similar to those described previously (31). In this study, reactions typically contained 50 mM Tris (pH 8.5), 50 mM $MgCl_2$, 5 mM CTP, ∼5 mM Kdn, 4 U pyrophosphatase per mM of CTP, and ∼0.1 mg/ml of CMP-sialic acid synthetase. Reactions were incubated at 37˚C for 2 h, followed by the removal of enzyme by centrifugal ultrafiltration. The filtered CMP-Kdn was then purified using a Q Sepharose Fast Flow (GE Healthcare) column equilibrated in 1 mM NaCl. Before sample application, the CMP-Kdn preparation was diluted ∼40-fold in 1 mM NaCl. After sample application, the resin was washed with 2 column volumes of 1 mM NaCl, and purified CMP-Kdn was obtained with a 0.8 column volume 100 mM NaCl step elution. This CMP-Kdn preparation was further desalted using diafiltration, in which the sample was transferred to a diafiltration cell (Diaflo Ultrafiltration Membranes, YCO5 76 mm), and filtered using three times the volume of 1 mM NaCl at a flow rate of 32 ml/h. After 24 h, the isolated retentate contained ∼96% of the original CMP-Kdn. Kdn7N_3 (3,7-dideoxy-7-azido-D-glycero-D-galacto-nonulosonic acid) was enzymatically prepared using a *P. multocida* aldolase (39) and methods similar to those described by Khedri et al. (40). Typically, reactions contained 128 mM Tris (pH 8.8), 17.5 mM 4-azido-4-deoxy-D-mannopyranose (Sussex Research Laboratories, Ottawa, ON, Canada), 128 mM sodium pyruvate, and sufficient quantities of aldolase. Reactions were incubated at 37˚C for ∼24 h, and the enzyme was then removed by centrifugal ultrafiltration. Next, CMP activation of synthesized Kdn7N_3 was achieved enzymatically using methods similar to those described previously (31). In this study, reactions typically contained 50 mM Tris (pH 9), 50 mM $MgCl_2$, 5 mM CTP, ∼5 mM Kdn7N_3, 4 U pyrophosphatase per mM of CTP, and ∼0.68 mg/ml of CMP-sialic acid synthetase. Reactions were incubated at 37˚C for 2 h, and the enzyme was then removed by centrifugal ultrafiltration. Filtered CMP-Kdn7N_3 samples were then lyophilized and desalted/purified using a Superdex Peptide 10/300 GL (GE Healthcare) column with 10 mM ammonium bicarbonate. To achieve additional purity, elution fractions containing CMP-Kdn7N_3 were subjected to anion-exchange chromatography (Mono Q 4.6/100 PE; GE Healthcare) using an ammonium bicarbonate gradient. Quantification of CMP-Kdn and CMP-Kdn7N_3 preparations were determined using the molar extinction coefficient of CMP (ε_{260}=7400). Purified and desalted sample aliquots were then freeze dried.

For structural characterization of CMP-Kdn and CMP-Kdn7N_3, purified material was exchanged into 100% D_2O. Structural analysis was performed using either a Varian Inova 500 MHz (^{1}H) spectrometer with a Varian *z* gradient 3-mm probe or a Varian 600 MHz (^{1}H) spectrometer with a Varian 5 mm *z* gradient probe. All spectra were referenced to an internal acetone standard (δ_H 2.225 and δ_C 31.07 ppm). Results are shown in Supplemental Table I (CMP-Kdn) and Supplemental Table II (CMP-Kdn7N_3), verifying the production of each compound.

CMP-Kdn– and CMP-Kdn7N_3–prepared compounds were also characterized using mass spectrometry (MS) or capillary electrophoresis (CE)–MS analysis. For CE-MS, mass spectra were acquired using an API 3000 Mass Spectrometer (Applied Biosystems/Sciex, Concord, ON, Canada). CE was performed using a Prince CE system (Prince Technologies, Emmen, the Netherlands). CE separation was obtained on a 90-cm length

of bare fused-silica capillary (365 μm outer diameter × 50 μm inner diameter) with CE-MS coupling using a liquid sheath-flow interface and isopropanol/methanol (2:1) as the sheath liquid. An aqueous buffer comprising 30 mM morpholine (adjusted to pH 9 with formic acid) was used for experiments in the negative-ion mode. Alternatively, mass spectra were acquired using a SQD2 (Waters, Milford, MA). In this study, the spectra were collected in the negative-ion mode, and no separations were attempted. The buffer used was a mixture of 1:1 acetonitrile/water with 0.31 mg/ml of ammonium bicarbonate. Results verifying the production of each compound are shown in Supplemental Table III, in which observed *m/z* ions from MS analysis correspond accurately to the calculated masses.

Synthesis of biotinylated NulO-LNnT glycans

LNnT-PEG_3-N_3 (Galβ-1,4-GlcNAcβ-1,3-Galβ-1,4-Glcβ-PEG_3-N_3) was synthesized starting with βLac-PEG_3-N_3 (Sussex Research Laboratories) by adding sequentially β-1,3-GlcNAc and β-1,4-Gal residues using, respectively, the HP-39 *N*-acetylglucosaminyltransferase and the HP-21 galactosyltransferase. HP-39 is a recombinant version of the JHP1032 β-1,3-*N*-acetylglucosaminyltransferase from *Helicobacter pylori*. HP-21 is a recombinant version of the HP0826 β-1,4-galactosyltransferase from *H. pylori*. The product was purified by solid phase extraction using a C18 Sep-Pak cartridge (Waters) and lyophilized after each reaction.

For the addition of Leg5,7Ac_2 or Neu5Ac to 7.5 mg of LNnT-PEG_3-N_3, the reaction contained 50 mM MES (pH 6.5), 10 mM $MgCl_2$, 7.5 mM donor, and ∼1.5 U of NST-05 (a recombinant version of the Lst from *N. meningitidis*). The reaction was incubated at 30˚C and was complete after 1 h for the addition of Neu5Ac. However, the complete addition of Leg5,7Ac_2 required additional enzyme and donor (CMP-Leg5,7Ac_2) and overnight incubation.

Once again, the samples were purified by solid phase extraction using C18 Sep-Pak cartridges and eluted using a stepwise gradient of methanol. Fractions were analyzed by TLC, run in a solvent containing ethyl acetate/methano/H_2O/acetic acid (4:2:1:0.1), and then dipped in 5% H_2SO_4 and charred. The products were recovered in the 50% methanol eluate. The desired fractions were pooled and lyophilized.

The reactions for labeling with biotin were performed in 1× PBS (pH 7.4) containing 20% DMSO, 2 mg of LNnT-PEG_3-N_3 (or the derivative with either Leg5,7Ac_2 or Neu5Ac), and a 1.5× molar excess of DBCO-PEG_4-Biotin (Click Chemistry Tools, Scottsdale, AZ). The reaction mix was incubated at 37˚C for 30 min (t_{30}). The products were purified by solid phase extraction using C18 Sep-Pak cartridges and eluted using a stepwise gradient of methanol. The products were recovered in the 50% methanol eluate and dried on a SpeedVac vacuum concentrator and by lyophilization. MS in the negative mode was used to confirm the masses expected for the biotinylated products.

Abs

mAb 3F11 (mouse IgM, kindly provided by Dr. M.A. Apicella, University of Iowa) binds to the unsialylated HepI LNnT structure; any extension beyond the terminal Gal (e.g., sialylation of LOS) results in decreased mAb 3F11 binding to LOS (41). Anti–factor H (FH) mAb from Quidel (catalog no. A254 [mAb 90×]) was used in flow cytometry experiments. Neu5Gc incorporation into surface glycans on BJA-B K20 cells was detected using a Neu5Gc-specific chicken polyclonal IgY Ab (1:2000) (42) followed by FITC-conjugated donkey anti-chicken IgY secondary Ab (1:200; Jackson ImmunoResearch). Biotinylated glycans containing α2-3 linked Leg5,7Ac_2 to LNnT epitope (43) were used to purify anti-Leg5,7Ac_2 Ab from human IVIG. Briefly, biotinylated glycans were attached to streptavidin magnetic beads (Invitrogen) by incubating at room temperature for 30 min, followed by washing with PBS. Human IVIG pooled from more than 1000 individuals was initially incubated with (unsialylated) biotinylated LNnT immobilized on streptavidin beads, followed by incubation with α2-3 linked Neu5Ac-LNnT beads for 30 min at room temperature to eliminate any Abs against the underlying glycan structure. Finally, α2-3 linked Leg5,7Ac_2-LNnT containing beads were added to these clarified IVIG pools. Following incubation under the same conditions as above, the beads were washed with PBS, and the bound anti-Leg5,7Ac_2 Abs were eluted with citric acid (pH 3) and immediately neutralized with Tris-HCl (pH 8).

SDS-PAGE

Gonococcal lysates were treated with protease K (100 μg/ml) and suspended in Tris-Tricine sample buffer (Boston BioProducts). Cell lysates were separated on 16.5% Criterion Tris-Tricine gels (Bio-Rad Laboratories) using Tris-Tricine Cathode buffer (Boston BioProducts), and LOS was stained using the Bio-Rad Silver Stain Kit.

FH binding

FH binding to bacteria was performed using flow cytometry, as described previously (44). Briefly, *N. gonorrhoeae* F62 ΔlgtD harvested from chocolate agar plates was grown in liquid media that contained the specified concentration of the CMP-NulO. Bacteria were then washed with HBSS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ ($HBSS^{++}$) and incubated with 20 μg/ml of FH purified from human plasma (Complement Technology). Bound FH was detected using an anti-FH mAb (clone 90X; Quidel), followed by FITC-conjugated anti-mouse IgG (Sigma); both Abs had similar performance characteristics. All reaction mixtures were carried out in $HBSS^{++}$/1% (w/v) BSA in a final volume of 50 μl.

Flow cytometry

Flow cytometry was performed using a FACSCalibur instrument (Becton Dickinson), and data were analyzed using FlowJo (version X; Tree Star).

Normal human serum

Serum was prepared from the blood of healthy human volunteers by phlebotomy. Sera from 10 donors were pooled and stored in single use aliquots at −80˚C.

Serum bactericidal assays

Serum bactericidal assays were performed as described previously (45). Bacteria were harvested from an overnight culture on chocolate agar plates, and ∼10^5 CFU of *N. gonorrhoeae* were grown in liquid media containing the specified concentration of CMP-NulO as specified for each experiment. Bacteria were diluted in Morse A, and ∼2000 CFU of *N. gonorrhoeae* F62 ΔlgtD were incubated with pooled normal human serum (NHS) (concentration specified for each experiment). The final reaction volumes were maintained at 150 μl. Aliquots of 25 μl of reaction mixtures were plated onto chocolate agar in duplicate at the beginning of the assay (t_0) and again after incubation at 37˚C for 30 min (t_{30}). Survival was calculated as the number of viable colonies at t_{30} relative to t_0.

Mouse vaginal colonization model

Mouse infection experiments were performed using either female wild-type BALB/c mice (The Jackson Laboratory), *Cmah*$^{-/-}$ mice back-crossed into a BALB/c background (46), or transgenic mice that expressed human complement inhibitors FH and C4b-binding protein (C4BP) that were generated in a BALB/c background (47). Mice that were 6–8 wk of age and in the diestrus phase of the estrous cycle were started on treatment (that day) with 0.5 mg of water-soluble 17β-estradiol (Premarin; Pfizer) given s.c. on each of 3 d: −2, 0, and +2 d (before, the day of, and after inoculation of bacteria) to prolong the estrus phase of the cycle and promote susceptibility to *N. gonorrhoeae* infection. Antibiotics (vancomycin, colistin, neomycin, trimethoprim, and streptomycin) ineffective against *N. gonorrhoeae* were also used to reduce competitive microflora (48). Mice were then infected with *N. gonorrhoeae* (CFU stated for every experiment). Mice were treated with 10 μg CMP-NulO (1 mg/ml in sterile H_2O or ∼1.5 mM stock) daily intravaginally, whereas the control mice were given saline (vehicle control).

Statistics

Experiments that compared clearance of *N. gonorrhoeae* in independent groups of mice estimated and tested three characteristics of the data (49): time to clearance, longitudinal trends in mean $\log_{10}$ CFU, and the cumulative CFU as area under the curve (AUC) were plotted. Statistical analyses were performed using mice that initially yielded bacterial colonies on days 1 and/or 2. Median time to clearance was estimated using Kaplan–Meier survival curves; the times to clearance were compared between groups using a Mantel–Cox log-rank test. The mean AUC ($\log_{10}$ CFU versus time) was computed for each mouse to estimate the bacterial burden over time (cumulative infection); the means under the curves were compared between groups using the nonparametric rank-sum test because distributions were skewed or kurtotic.

Cell feeding assays

Human B lymphoma BJA-B K20 cells were incubated in RPMI 1640 containing 1% (v/v) Nutridoma (Roche) for 3 d to eliminate any residual sialic acid from the cell growth media. Following incubation, 3 mM Leg5,7Ac_2 or Neu5Gc (as a positive control) was added, and cells were allowed to incubate for an additional 3 d at 37˚C. Cells were then harvested (2×10^5 cells; fed or unfed), washed, and probed with either Leg5,7Ac_2-specific polyclonal human IgG Ab (see above) or Neu5Gc-specific chicken

polyclonal IgY Ab (42) for 30 min. NulO incorporation within surface glycans was detected with fluorophore-attached secondary Abs using flow cytometry.

Stability of CMP-NulOs

The stability of CMP-Neu5Ac, CMP-Kdn, CMP-Neu5Ac9N_3, and CMP-Leg5,7Ac_2 in solution was assessed by incubation in various pH and temperature conditions for various lengths of time. Specifically, freeze-dried CMP-NulOs were resuspended immediately prior to the start of the assay at a concentration of ~1 mM in either 25 mM phosphate-citrate buffer (pH 4–7) or 25 mM sodium phosphate buffer (pH 8). The samples were incubated at 4, 20, or 37°C, over a time course ranging from 4 h to 6 wk as indicated. CE analysis was performed using a P/ACE MDQ instrument (Beckman Coulter, Fullerton, CA) equipped with a photodiode array detector and capillary as described previously (50) with a 30-min run time in 25 mM sodium tetraborate buffer (pH 9.4) and detection at 271 nm. Relative quantities of intact CMP-NulO and free CMP were determined by peak integration using 32 Karat software and expressed as a percentage relative to the t_0 timepoint. The stability assays were performed in duplicate.

Ethics statement

Collection of human sera and its use were approved by the University of Massachusetts Medical School Institutional Review Board. Informed written consent was obtained from all serum donors (no. H00005614; Docket). Use of animals in this study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School (no. A-1930; Docket).

Results

Incorporation of CMP-NulOs into N. gonorrhoeae *LOS*

Previously, we showed that *N. gonorrhoeae* Lst had broad substrate specificity; in addition to CMP-Neu5Ac, the natural substrate for gonococcal Lst, CMP salts of Neu5Gc, Neu5Gc8Me, Neu5Ac9N_3, and Leg5,7Ac_2 were used by Lst (31). Only CMP-Pse5,7Ac_2 (CMP-Pse5Ac7Ac), which differs stereochemically from the other tested CMP-NulOs at C5, C7, and C8, was not used by Lst (31). To better understand the substrate specificity of gonococcal Lst and to identify additional candidate therapeutic molecules, we tested the following CMP-NulOs: CMP-Neu4,5Ac_2, CMP-Kdn, and CMP-Kdn7N_3 (see Supplemental Fig. 1 for reference to various NulO backbone structures). These three new CMP-NulOs were chosen because their NulOs when incorporated into glycans, like Leg5,7Ac_2, are resistant to sialidases (40, 51), enzymes which are present in human cervical secretions (52). This is important as the removal of the therapeutic NulO from the gonococcal surface could compromise efficacy. Further, CMP-Kdn has an advantage because free Kdn is a "self" molecule present in human tissue/cells (53, 54); therefore, it could have lower toxicity than "non-human" NulOs such as Leg5,7Ac_2. The other CMP-NulOs used in this study, such as CMP-Neu5Ac, CMP-Neu5,9Ac_2, CMP-Neu5Ac9N_3, and CMP-Leg5,7Ac_2, were chosen from our previous work (31) as benchmarks or as controls.

mAb 3F11 was used to determine NulO incorporation into LNnT LOS. mAb 3F11 binds to terminal lactosamine in the unsialylated LNnT structure; glycan extensions beyond the terminal Gal in lactosamine, for example, NulO incorporation results in decreased binding of the Ab (31, 55). mAb 3F11 binding decreased when bacteria were grown in the presence of all CMP-NulOs except CMP-Neu4,5Ac_2 (Fig. 1A), suggesting that Neu4,5Ac_2 was not added to gonococcal LNnT LOS. These results were confirmed by silver staining of *N. gonorrhoeae* LOS on 16.5% Tricine gels, which showed no change in the migration of LOS when bacteria were grown in CMP-Neu4,5Ac_2 (Fig. 1B). Because Neu4,5Ac_2 was not incorporated into LOS, CMP-Neu4,5Ac_2 was not considered further.

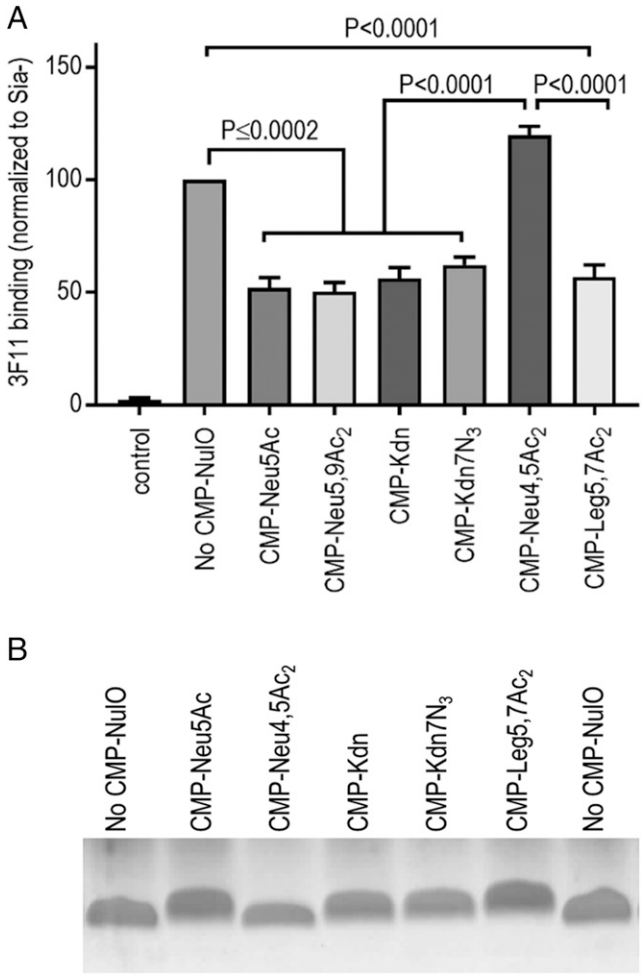

FIGURE 1. Incorporation of NulO into *N. gonorrhoeae* LOS. (**A**) mAb 3F11 binding. mAb 3F11 binds to terminal lactosamine of LNnT; extensions beyond LNnT abrogates 3F11 binding (55). *N. gonorrhoeae* F62 ΔlgtD was grown in media alone or media containing 25 μg/ml of the indicated CMP-NulO. mAb 3F11 binding was detected by flow cytometry, and median fluorescence was recorded. Binding is shown as percentage relative to 3F11 binding to unsialylated bacteria (three independent observations). Comparisons across groups, made by one-way ANOVA, showed significant differences ($F = 43.76$; $p < 0.0001$). Pairwise comparisons, made by Tukey multiple comparison test, are indicated. (**B**) Incorporation of NulO as visualized by silver-stained SDS-PAGE gels. Protease K lysates of F62 ΔlgtD grown in media alone or media containing each of the indicated CMP-NulOs (100 μg/ml) were separated on 16% Tricine gels (Bio-Rad Laboratories), and LOS was visualized by silver staining. Slower mobility relative to bacteria grown in media alone (No CMP-NulO) indicates the addition of NulO.

Effects of incorporation of NulOs into N. gonorrhoeae *LOS on serum resistance*

The effects of incorporation of the NulOs into LOS on the ability of *N. gonorrhoeae* strain F62 ΔlgtD to resist killing by NHS was next determined. The effects of adding each CMP-NulO individually on serum resistance is shown in Table I. The addition of CMP-Neu5Ac [the sialic acid Neu5Ac is scavenged by gonococci in humans that renders bacteria fully complement resistant (56, 57)] served as the positive control for serum resistance. As reported previously (31), Neu5,9Ac_2 incorporation conferred >100% survival in 3.3% NHS but not 10% NHS in which survival was <5% (31). Similarly, LOS substitution with Kdn also rendered bacteria fully resistant (>100% survival) only to 3.3% but not 10% NHS (7% survival). Bacteria with Kdn7N_3-substituted LOS showed an

Table I. Effect of CMP-NulOs on complement-dependent killing of *N. gonorrhoeae* F62 ΔlgtD

CMP-NulO Added First	CMP-NulO Added Second[a]	Percentage of Survival (Mean) In 3.3% NHS[b]	Percentage of Survival (Mean) In 10% NHS
None	None	17	3
CMP-Neu5Ac	None	126	120
CMP-Neu5,9Ac_2	None	119	4
CMP-Neu5Ac	CMP-Neu5,9Ac_2	107	12
CMP-Neu5,9Ac_2	CMP-Neu5Ac	108	14
CMP-Kdn	None	112	7
CMP-Neu5Ac	CMP-Kdn	107	107
CMP-Kdn	CMP-Neu5Ac	107	12
CMP-Kdn7N_3	None	126	34
CMP-Neu5Ac	CMP-Kdn7N_3	133	129
CMP-Kdn7N_3	CMP-Neu5Ac	126	128
CMP-Leg5,7Ac_2	None	0	0
CMP-Neu5Ac	CMP-Leg5,7Ac_2	7	6

[a]Second CMP-NulO added 15 min after first CMP-NulO.
[b]NHS, pooled NHS.

intermediate phenotype with >100% survival in 3.3% NHS and ~34% survival in 10% NHS. Consistent with our previous report (31), incorporation of Leg5,7Ac_2 rendered *N. gonorrhoeae* fully susceptible to both 3.3 and 10% NHS.

We next determined whether the addition of CMP-NulO could prevent Neu5Ac-mediated serum resistance. In these "competition" experiments, the CMP-NulO was added either 15 min before or 15 min after adding CMP-Neu5Ac to growth media (Table I). The addition of CMP-Neu5,9Ac_2 either before or after the addition of CMP-Neu5Ac to growth media rendered F62 ΔlgtD susceptible to 10% NHS but not 3.3% NHS, which simulated results with CMP-Neu5,9Ac_2 alone. In contrast, CMP-Kdn effectively prevented Neu5Ac-mediated serum resistance only when added first and, as expected, only in the presence of 10% NHS. Compared with unsialylated bacteria, CMP-Kdn7N_3 enhanced serum resistance in 10% NHS (34% survival) and was ineffective in preventing CMP-Neu5Ac–mediated serum resistance under any of the test conditions. As reported previously, CMP-Leg5,7Ac_2 (benchmark) blocked CMP-Neu5Ac–mediated serum resistance when added after CMP-Neu5Ac (31).

Effect of NulO substitution of gonococcal LOS on FH binding and complement activation

Substitution of *N. gonorrhoeae* LNnT LOS with α2,3-linked Neu5Ac enhances FH binding, which contributes to gonococcal complement resistance (58). Kdn and Kdn7N_3 incorporation resulted in FH binding (measured as fluorescence) to levels ~62% of that seen with Neu5Ac-substituted LOS (Fig. 2). As reported previously, bacteria with Neu5,9Ac_2-capped LNnT LOS bound FH with ~30% the fluorescence intensity observed with Neu5Ac-capped LOS. Bacteria with Leg5,7Ac_2 substituted LOS did not bind FH above levels seen with unsialylated bacteria, replicating prior observations (31).

Efficacy of CMP-NulOs against N. gonorrhoeae *in the mouse vaginal colonization model*

The efficacy of each of the CMP-NulOs against ceftriaxone-resistant clinical *N. gonorrhoeae* isolate H041 was studied in the mouse vaginal colonization model of gonorrhea. The efficacies of CMP-Kdn, CMP-Neu5,9Ac_2, and CMP-Neu5Ac9N_3 were evaluated; CMP-Leg5,7Ac_2 served as the benchmark for efficacy (31). Three parameters of efficacy were measured: median time to clearance, $\log_{10}$ CFU versus time, and AUC. When administered at a dosage of 10 μg intravaginally daily, CMP-Kdn and CMP-Neu5Ac9N_3 were as efficacious as CMP-Leg5,7Ac_2 in clearing gonococcal colonization, whereas CMP-Neu5,9Ac_2 was ineffective (Fig. 3).

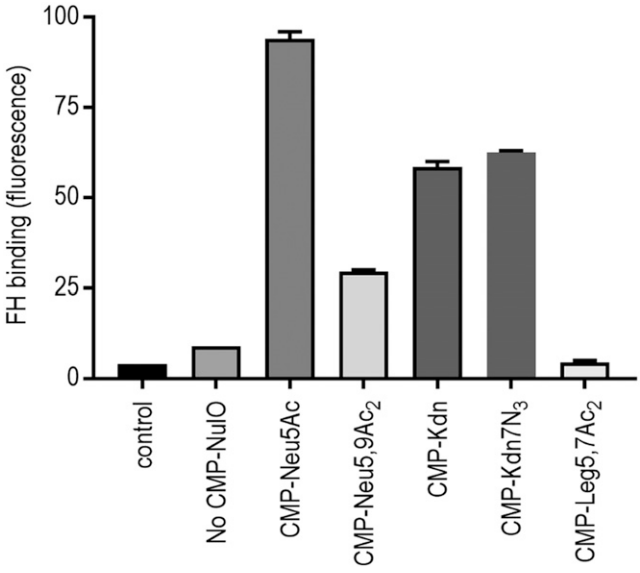

FIGURE 2. Effect of NulO incorporation into LOS on FH binding to *N. gonorrhoeae*. F62 ΔlgtD was grown in media alone or media containing each of the indicated CMP-NulOs, incubated with purified human FH (10 μg/ml), and bacteria-bound FH (measured as median fluorescence) was detected by flow cytometry using an anti-FH mAb 90× followed by anti-mouse IgG FITC. *y*-axis, mean (range) of two independent experiments. Comparisons across groups, made by one-way ANOVA, showed significant differences ($F = 1224$; $p < 0.0001$). Pairwise comparisons were made by Tukey multiple comparisons test. Pairwise differences across the control, media alone (No CMP-NulO) and CMP-Leg5,7Ac_2 groups and between CMP-Kdn and CMP-Kdn7N_3 were NS. All other pairwise comparisons were significant ($p < 0.0001$).

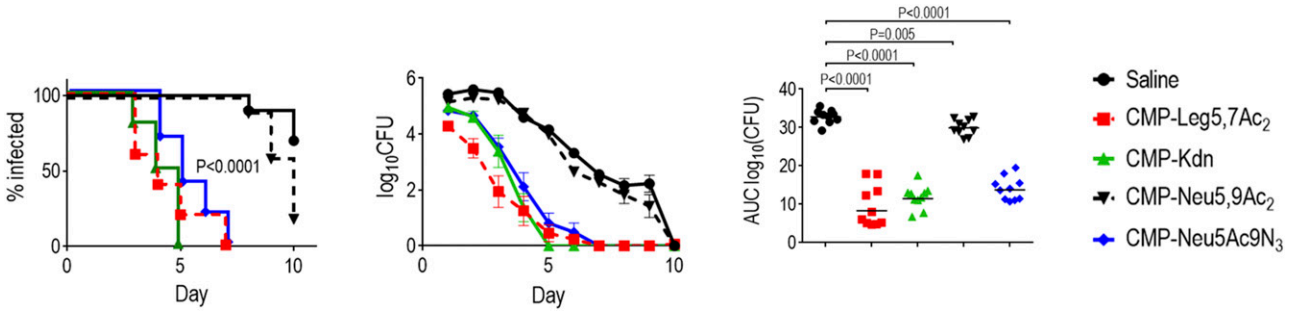

FIGURE 3. Efficacy of CMP-NulOs against multidrug-resistant *N. gonorrhoeae* H041 in the mouse vaginal colonization model. Premarin-treated wild-type BALB/c mice (n = 10/group) were infected with 10^6 CFU *N. gonorrhoeae* H041. Mice were treated daily (starting 2 h before infection) intravaginally with saline (untreated vehicle controls), with 10 μg/d of CMP-Leg5,7Ac$_2$ [positive control for clearance (31)], or with 10 μg/d of CMP-Neu5,9Ac$_2$, CMP-Kdn, or CMP-Neu5Ac9N$_3$. Vaginas were swabbed daily to enumerate *N. gonorrhoeae* CFUs. The graph on the left shows Kaplan–Meier curves, indicating time to clearance of infection. Groups were compared using the Mantel–Cox (log-rank) test. Significance was set at 0.005 (Bonferroni correction for comparisons across five groups). Pairwise comparisons between the CMP-Leg5,7Ac$_2$, CMP-Neu5,9Ac$_2$, and CMP-Kdn groups versus the saline controls or the CMP-Neu5,9Ac$_2$ group were significant ($p < 0.0001$). The middle graph shows $\log_{10}$ CFU versus time. x-axis, day; y-axis, $\log_{10}$ CFU. Comparisons of the CFU over time between each treatment group and the saline control was made by two-way ANOVA and Dunnett multiple comparison test. Significantly lower counts on day 1 were seen with the CMP-Leg5,7Ac$_2$–treated group ($p < 0.01$), on day 2 with the CMP-Leg5,7Ac$_2$, CMP-Kdn, and CMP-Neu5Ac9N$_3$ groups ($p < 0.0001$, $p < 0.05$, $p < 0.05$, respectively), and from days 3 through 9 with all the three aforementioned groups ($p < 0.0001$). The graph on the right shows bacterial burdens consolidated over time (AUC [$\log_{10}$ CFU] analysis). The five groups were compared by one-way ANOVA using the nonparametric Kruskal–Wallis equality of populations rank test. The χ^2 with ties (4 df) was 24.6 ($p < 0.0001$). Pairwise AUC comparisons across groups was made with Dunn multiple comparison test.

Stability of CMP-NulOs

Stability of the CMP-NulOs is an important consideration for shelf-life, drug formulation, safety, and efficacy in vivo. Although CMP-NulO sugars are very stable in solid dry form, they are acid and heat labile and can hydrolyze into CMP and NulO under acidic conditions typical of the human vagina (59). Hydrolysis of therapeutic CMP-NulOs could have adverse consequences for treating *N. gonorrhoeae* infections as only the intact CMP-NulO can be used by *N. gonorrhoeae* Lst. Furthermore, free NulOs have the potential to traverse host cell membranes and become incorporated into host glycans and elicit autoimmune Abs (60, 61).

The stabilities of the three CMP-NulOs (CMP-Leg5,7Ac$_2$, CMP-Neu5Ac9N$_3$, and CMP-Kdn) that showed efficacy in the mouse model of gonorrhea were tested at temperatures ranging from 4 to 37˚C and pH ranges between 4 and 7; CMP-Neu5Ac was used as a comparator. These conditions were selected to mimic human vaginal secretions (acidic pH and 37˚C) or those similar to shelf-life/storage conditions in solution (4 or 20˚C and neutral pH). CMP-Leg5,7Ac$_2$ was the most stable molecule under all the tested conditions (Figs. 4, 5). Greater than 90% of CMP-Leg5,7Ac$_2$ remained intact even after 6 wk at 4˚C with neutral pH 7 as well as after 3 d at 20˚C (at pH 7) (Fig. 4). Remarkably, ∼54% of CMP-Leg5,7Ac$_2$ remained intact after a 24-h incubation at 37˚C (pH 5); none of the other CMP-NulOs remained intact under similar conditions (Fig. 5). At the lowest pH tested (pH 4), >50% of CMP-Leg5,7Ac$_2$ remained intact after 1 h compared with <35% for all other CMP-NulOs (data not shown). By 4 h of incubation at pH 4, CMP-Leg5,7Ac$_2$ was the only CMP-NulO left intact in any appreciable amount (4%) (Fig. 5). Taking all the test conditions into consideration, CMP-Leg5,7Ac$_2$ is the most stable, followed in descending order by CMP-Neu5Ac9N$_3$, CMP-Neu5Ac, and CMP-Kdn.

Lack of Leg5,7Ac$_2$ incorporation into glycans on the surface of host cells

Based on the efficacy and stability data presented above, CMP-Leg5,7Ac$_2$ was the top-performing antigonococcal therapeutic candidate. To note, Leg5,7Ac$_2$ is a nonhuman bacterial sugar. In addition, CMP-NulOs do not typically cross mammalian cell membranes. However, Leg5,7Ac$_2$ that results from hydrolysis of CMP-Leg5,7Ac$_2$, as well as the intact nucleotide sugar, could enter human cells via macropinocytosis and be delivered to the lysosome (the intact nucleotide-sugar would likely be hydrolyzed by low pH in the lysosomes), followed by export to the cytosol by the sialic acid transporter sialin (62). If free NulOs in the cytosol get converted back to their CMP-bound form, they could potentially enter the Golgi apparatus and become incorporated into newly synthesized cell surface–associated host glycans, elicit an immune response, and result in complement-mediated tissue damage. A well-documented example of such a process with the nonhuman sialic acid Neu5Gc that can be incorporated into human tissues occurs as a result of consuming foods such as red meat that are rich in Neu5Gc (63, 64).

Human anti-Leg5,7Ac$_2$ was purified from pooled human IVIG by affinity chromatography over biotinylated Leg5,7Ac$_2$-LNnT linked to streptavidin magnetic beads. The ability of human anti-Leg5,7Ac$_2$ to detect surface-bound Leg5,7Ac$_2$-substituted glycans was validated using *N. gonorrhoeae* F62 ΔlgtD grown in CMP-Leg5,7Ac$_2$–containing media (Fig. 6A). We could not detect any Leg5,7Ac$_2$ on hyposialylated human B lymphoma BJA-B K20 cells fed with a concentration of Leg5,7Ac$_2$ as high as 3 mM (Fig. 6B). In contrast, Neu5Gc that was used as a positive control for uptake and display of a nonhuman NulO by BJA-B K20 cells was readily detected with chicken anti-Neu5Gc (Fig. 6C).

Efficacy of CMP-Leg5,7Ac$_2$ against N. gonorrhoeae *in Cmah$^{-/-}$ mice*

Among related mammals, humans are unusual in being genetically deficient in the enzyme CMP-Neu5Ac hydroxylase that converts CMP-Neu5Ac to CMP-Neu5Gc (65). Thus, mouse glycans display both Neu5Ac and Neu5Gc, but human glycans possess only Neu5Ac. Differences in NulO profiles between humans and mice may affect the activity of the CMP-NulO therapeutic. Therefore, we evaluated the efficacy of CMP-Leg5,7Ac$_2$ in *Cmah*$^{-/-}$ mice that express only Neu5Ac (i.e., not Neu5Gc) on their glycans [akin to the human sialome (46, 60, 66–68)] to simulate conditions more aligned with the human genital tract. The efficacy of CMP-Leg5,7Ac$_2$ against *N. gonorrhoeae* strains F62 ΔlgtD and

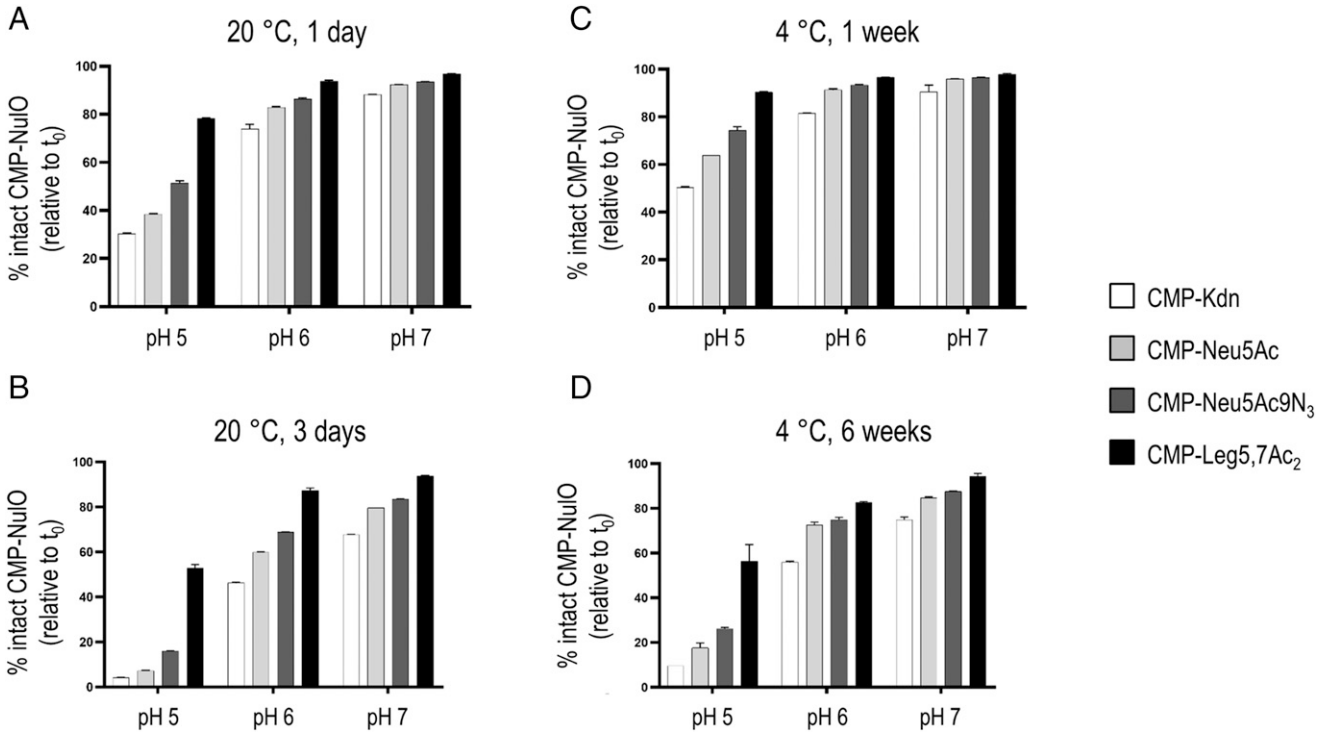

FIGURE 4. Effect of pH on CMP-NulO stability at storage temperatures. CMP-NulOs, as indicated, were resuspended in pH 5, 6, or 7 solutions and incubated for 1–3 d at 20˚C (**A** and **B**) or for 1–6 wk at 4˚C (**C** and **D**). The percentage of intact CMP-NulO after incubation relative to t_0 is indicated. Values represent the mean of two independent measurements.

H041 was tested (Fig. 7). Wild-type BALB/c mice were used as comparators. The duration and burden of gonococcal infection in the control (saline-treated) groups of the two mouse strains is shown in Supplemental Fig. 2. The median times to clearance in the *Cmah*$^{-/-}$ mice was a day longer than wild-type mice with saline-treated negative controls, and the AUC was significantly higher in the *Cmah*$^{-/-}$ mice. CMP-Leg5,7Ac$_2$ was effective against both strains of *N. gonorrhoeae* in *Cmah*$^{-/-}$ mice (Fig. 7), evidenced by more rapid rates of clearance and lower bacterial burdens compared with saline-treated mice. The efficacy of CMP-Leg5,7Ac$_2$ in *Cmah*$^{-/-}$ mice was similar to that seen in wild-type mice (data with wild-type mice is shown in Supplemental Fig. 3).

Efficacy of CMP-Leg5,7Ac$_2$ in Cmah$^{-/-}$ mice against diverse gonococcal isolates

The efficacy of CMP-Leg5,7Ac$_2$ against four additional strains of *N. gonorrhoeae* was next tested in the mouse vaginal colonization model using *Cmah*$^{-/-}$ mice (Fig. 8). Three clinical isolates (CTX-r Spain, NJ-60, and SD-1) were chosen because they are resistant to third-generation cephalosporins (either cefixime and/or ceftriaxone). The fourth isolate, 398078, was chosen because it produces the P^{K}-like LOS (Galα1-4Galβ1-4Glc) from HepI, which is sialylated through an α2,6 linkage, as opposed to LNnT, which is sialylated through an α2,3 linkage (38). As shown in Fig. 8, CMP-Leg5,7Ac$_2$ administered topically at a dosage of 10 μg daily significantly shortened the duration and burden of gonococcal colonization in all four instances. Unlike the other strains in which 100% of saline-treated animals remained colonized at the end of 7 d, strain 398078 (P^{K}-like LOS) colonized saline-treated (control) mice for only 3 d. We were unable to detect 398078 in any of the CMP-Leg5,7Ac$_2$–treated animals even on day 1 (i.e., swabs taken 24 h postinfection); hence, the AUC in this group was zero. Collectively, these data suggest that CMP-Leg5,7Ac$_2$ is effective against antibiotic-resistant clinical strains of *N. gonorrhoeae* obtained from diverse geographic locations.

Efficacy of CMP-Leg5,7Ac$_2$ in human FH/C4BP transgenic mice

Several factors contribute to the host restriction of gonococcal infection, including its ability to resist human but not nonhuman complement (reviewed in Ref. 48). Binding of human, but not nonhuman, complement inhibitors FH and C4BP is at least in part responsible for the ability of gonococci to evade killing exclusively by human complement (69, 70). Given the importance of LOS Neu5Ac in virulence both in humans and in mice, its role in counteracting bacteriolysis by complement, and the fact that the therapeutic CMP-NulO candidates (e.g., CMP-Leg5,7Ac$_2$) counteracted serum resistance mediated by CMP-Neu5Ac, we tested the efficacy of CMP-Leg5,7Ac$_2$ in human FH/C4BP dual transgenic mice. Three doses of intravaginally administered CMP-Leg5,7Ac$_2$ were tested (10, 5, and 1 μg/d). As shown in Fig. 9, the efficacy of CMP-Leg5,7Ac$_2$ was dose responsive; the lowest tested dosage (1 μg/d) was ineffective, whereas the 5 and 10 μg/d doses showed progressively increasing efficacy.

Discussion

We previously exploited the central role for LOS Neu5Ac in gonococcal pathogenesis to design novel CMP-NulO immunotherapeutic molecules to fight multidrug-resistant gonorrhea (31). Specifically, CMP-Leg5,7Ac$_2$ and CMP-Neu5Ac9N$_3$ could counteract serum resistance mediated by CMP-Neu5Ac. CMP-Leg5,7Ac$_2$ was effective in attenuating gonococcal colonization in mice (31), and in this report, we extend the findings in vivo to CMP-Neu5Ac9N$_3$ and CMP-Kdn.

Using three new CMP-NulOs, CMP-Neu4,5Ac$_2$, CMP-Kdn, and CMP-Kdn7N$_3$, this study provides further insights into the substrate specificity of the gonococcal Lst enzyme and the functional

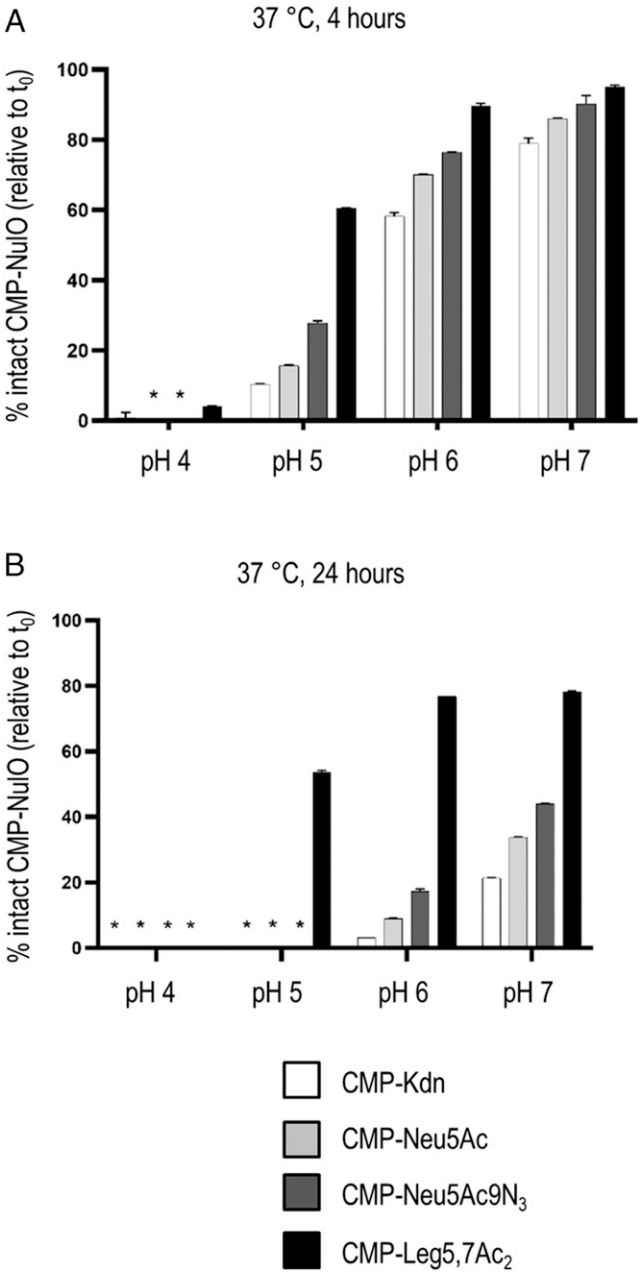

FIGURE 5. Effect of pH on CMP-NulO stability at physiological temperature. CMP-NulOs, as indicated, were resuspended in pH 4, 5, 6, or 7 solutions and incubated for up to 24 h at 37˚C. Measurements of intactness were taken at 4 h (**A**) and 24 h (**B**). The percentage of intact CMP-NulO after incubation relative to t_0 is indicated. Values represent the mean of two independent measurements. *Values reported for these measurements are 0% as the CMP-NulO indicated was hydrolyzed by this time point (data not shown for earlier time points).

consequences of NulO substitutions on FH binding and complement evasion. Although CMP-Kdn and CMP-Kdn7N$_3$ both served as substrates for Lst, Neu4,5Ac$_2$ was not added to LNnT. The interaction between *Neisseria* Lst and CMP-Neu5Ac is stabilized by several interactions (71). Specifically, an Arg residue at position 282 (numbering based on the amino acid sequence of *N. meningitidis* Lst, which is also conserved across *N. gonorrhoeae* Lst sequences) forms a hydrogen bond with the hydroxyl at the C4 position of Neu5Ac (71). Therefore, replacing this hydroxyl with *O*-acetyl at the C4 position likely prevents the binding of CMP-Neu4,5Ac$_2$ to Lst and subsequent enzymatic transfer of Neu4,5Ac$_2$ to LOS. In contrast, the substitution of NH acetyl at the C5 position of Neu5Ac with a hydroxyl to yield Kdn or subsequent N$_3$ (azido) substitution at the C7 position of the exocyclic side chain did not interfere with the ability of gonococcal Lst to transfer the NulO moiety from the respective CMP-NulOs to LNnT (i.e., both CMP-Kdn and CMP-Kdn7N$_3$ were transferred).

The exocyclic chain of Neu5Ac (C7–C9; see Supplemental Fig. 1) is important for inhibition of the alternative pathway of complement by sialoglycans (72, 73) and for interactions of Neu5Ac with FH domain 20 (74, 75). Thus, alterations of the exocyclic chain have a profound impact on the binding of FH to sialylated gonococci, as evidenced by the lack of detectable FH binding when Leg5,7Ac$_2$ (deoxy and methyl at the C9 position, in addition to C7 NH acetyl), Neu5Gc8Me (*O*-methyl at the C8 position), and Neu5Ac9N$_3$ (deoxy and N$_3$ at the C9 position) capped LNnT LOS (31), and there was a ∼70% decrease in FH binding fluorescence with Neu5,9Ac$_2$ (*O*-acetyl at the C9 position). Changes in the cyclic region of NulOs have either no impact (for example, Neu5Gc, which differs from Neu5Ac in a single oxygen atom at the C5 position; see Supplemental Fig. 1) (31) or only a modest (∼40%) decrease in binding with Kdn (NH acetyl at the C5 position in Neu5Ac replaced with OH; see Fig. 2). Of note, Kdn7N$_3$ substituted LOS-bound similar amounts of FH as LOS capped by Kdn (Fig. 2), suggesting that alterations at the C7 position in the exocyclic side chain are better tolerated than changes at the C8 or C9 positions. These data are consistent with findings of Blaum et al. (74) who showed that the C8 and C9 hydroxyl groups of the exocyclic moiety of Neu5Ac that was α2,3 linked to lactose formed hydrogen bonds with the amide and carbonyl groups, respectively, of the W1198 residue in FH domain 20.

Similar to Neu5,9Ac$_2$, the addition of Kdn or Kdn7N$_3$ to LOS enhanced resistance of *N. gonorrhoeae* F62 ΔlgtD to complement-dependent killing by 3.3%, but not 10%, NHS. This is in accordance with reduced FH binding seen with Kdn or Kdn7N$_3$-substituted LOS. It is worth noting that differences in FH binding alone may not account for differences in serum resistance. For example, bacteria with Kdn-substituted LOS shows a 2-fold greater fluorescence than Neu5,9Ac$_2$-coated gonococci yet shows similar serum resistance profiles. Kdn and Kdn7N$_3$ on LOS both result in similar FH binding, but Kdn7N$_3$ resulted in greater serum resistance. Sialylation of gonococcal LOS also regulates the classical pathway by modulating IgG binding (31, 76), and it is likely that the various NulOs may differ in their ability to inhibit the classical pathway, which could also factor into the differences seen in their complement-regulating properties.

Interestingly, despite their similar effects on resistance to complement when added to media singly and the observation that CMP-Kdn counteracted the protective effects conferred by CMP-Neu5Ac against complement only when added prior to CMP-Neu5Ac, only CMP-Kdn but not CMP-Neu5,9Ac$_2$ was efficacious in vivo. A possible explanation is that hydrolysis of CMP-Neu5,9Ac$_2$ to CMP-Neu5Ac may occur over time in vivo, thereby negating its activity (i.e., because of esterase activity that may remove the C9 *O*-acetyl group in the vaginal mucosa). Another and not mutually exclusive possibility is that Neu5Ac and Neu5,9Ac$_2$, but not other NulOs such as those that decrease colonization (Leg5,7Ac$_2$, Kdn, and Neu5Ac9N$_3$), may protect the organism against host defenses other than complement. Examples include engaging Siglec receptors that dampen inflammatory responses (77–79) and protection against cationic antimicrobial peptides (25).

Stability at the local site of delivery is an important consideration in the development of therapeutic CMP-NulOs. The normal pH of

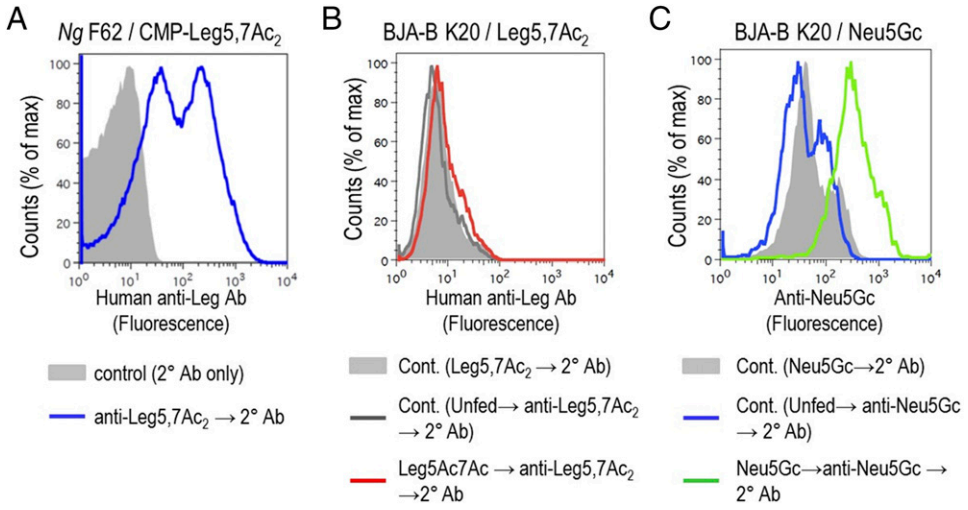

FIGURE 6. Leg5,7Ac$_2$ is not detected on the surface of hyposialylated human BJA-B K20 cells fed with Leg5,7Ac$_2$. (**A**) Validation of reactivity of human anti-Leg5,7Ac$_2$. *N. gonorrhoeae* F62 was grown in media alone or media supplemented with 30 μg/ml CMP-Leg5,7Ac$_2$. Bacteria were incubated with human anti-Leg5,7Ac$_2$ (1:10) followed by anti-human IgG conjugated to PE (blue histogram) or with fluorescent conjugate alone (gray shaded histogram). F62 grown in media alone did not show any reactivity over conjugate control levels (data not shown). *x*-axis, fluorescence on a $\log_{10}$ scale; *y*-axis, counts. (**B**) Leg5,7Ac$_2$ is not detectable on BJA-B K20 cells fed with Leg5,7Ac$_2$. Hyposialylated BJA-B K20 cells were incubated with media alone (unfed cells) or media containing 3 mM Leg5,7Ac$_2$ and incubated with human anti-Leg5,7Ac$_2$ and anti-human IgG conjugated to PE (unfed cells, solid gray line; Leg5,7Ac$_2$-fed cells, red histogram) or with secondary conjugate alone (shaded gray histogram). Axes are as in (A). (**C**) Incorporation of Neu5Gc by BJA-B K20 cells. As a positive control for NulO incorporation, BJA-B K20 cells were incubated with media alone (unfed cells) or media containing 3 mM Neu5Gc and incubated with chicken anti-Neu5Gc and anti-chicken IgY conjugated to Alexa Fluor 647 (unfed cells, solid blue line; Neu5Gc-fed cells, green histogram) or with secondary conjugate alone (shaded gray histogram). Axes are as in (A).

the human vagina ranges from 4 to 5 (80, 81). CMP-Leg5,7Ac$_2$ was the most stable of the CMP-NulOs tested across a pH range from 4 to 7 at physiological temperature 37˚C. Because its stability exceeds that of the endogenous host molecule, CMP-Neu5Ac, we expect CMP-Leg5,7Ac$_2$ to effectively outcompete CMP-Neu5Ac in the acidic human vaginal environment. Moreover, CMP-Leg5,7Ac2 was the most stable CMP-NulO when subjected to conditions representing short- and long-term storage in solution. However, in a solid dry state, all CMP-NulOs are expected to be near 100% stable. Finally, it is interesting to note that the pH stability of the various CMP-NulOs in order of highest to lowest also follows a similar order of anticipated hydrophobicity of each molecule, with CMP-Leg5,7Ac$_2$ expected to have the highest hydrophobicity due to a 9-deoxy methyl group and two other methyls associated with the two NH acetyl groups present.

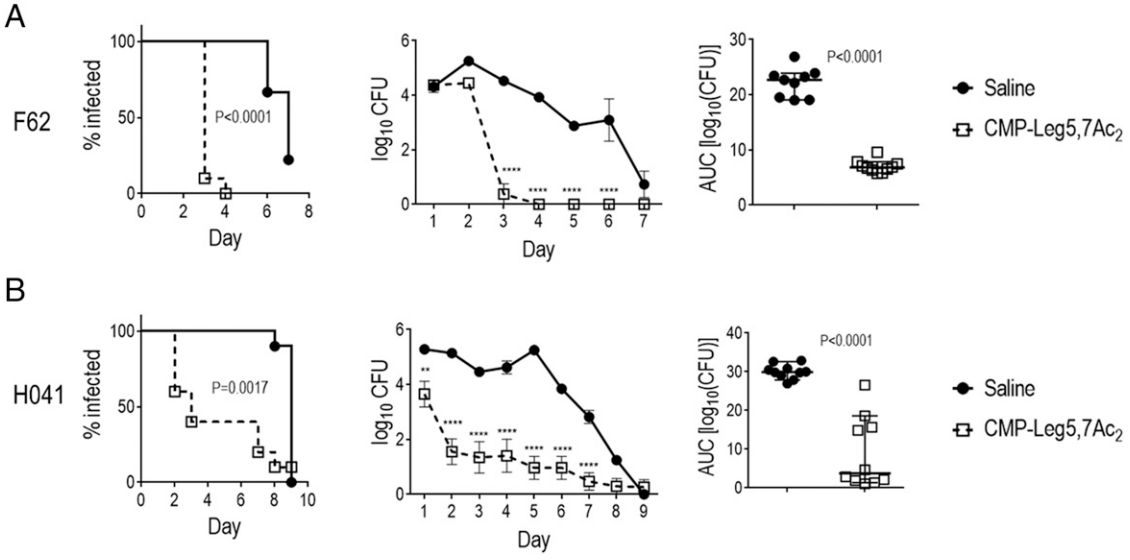

FIGURE 7. CMP-Leg5,7Ac$_2$ is efficacious in *Cmah*$^{-/-}$ mice. *Cmah*$^{-/-}$ mice that express only human-like Neu5Ac, but not nonhuman Neu5Gc seen in nonhuman primates and lower animals including mice, were treated with Premarin and infected with 4.5×10^5 CFU *N. gonorrhoeae* F62 (**A**) or 6×10^5 CFU of strain H041 (**B**). Mice were treated intravaginally with either saline (vehicle control; filled black circles) or CMP-Leg5,7Ac$_2$ 10 μg daily (open squares), starting 2 h before infection. Vaginas were swabbed daily to enumerate CFUs. Measures of treatment efficacy included Kaplan–Meier curves (left-hand column) showing time to clearance of infection (groups were compared using the Mantel–Cox [log-rank] test), $\log_{10}$ CFU versus time (middle column), and bacterial burdens consolidated over time (AUC [$\log_{10}$ CFU] analysis) (right-hand column). Pairwise AUC comparisons across groups was made with the two-sample Wilcoxon rank-sum (Mann–Whitney) test.

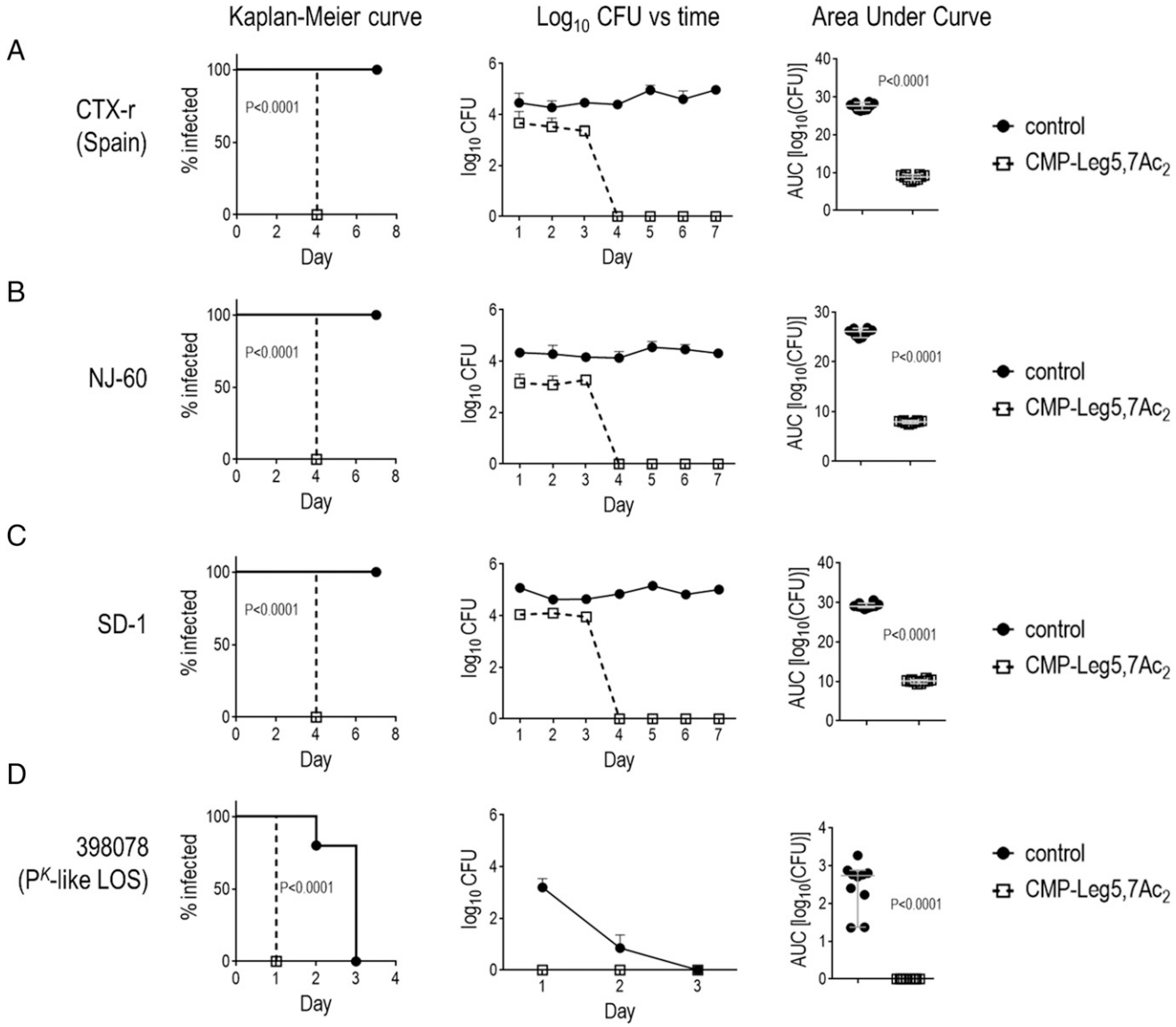

FIGURE 8. CMP-Leg5,7Ac$_2$ is efficacious against diverse clinical isolates of *N. gonorrhoeae* in *Cmah*$^{-/-}$ mice. Premarin-treated *Cmah*$^{-/-}$ (n = 10/group) were infected with *N. gonorrhoeae* strains CTX-r Spain (9.5×10^5 CFU) (**A**), NJ-60 (7.7×10^5 CFU) (**B**), *N. gonorrhoeae* SD-1 (5.1×10^5) (**C**), and 398078 (P^K-like globotriose LOS; 5.5×10^5 CFU) (**D**) and treated intravaginally with either saline (vehicle control; black circles) or CMP-Leg5,7Ac$_2$ 10 μg daily (open squares), starting 2 h before infection. Vaginas were swabbed daily to enumerate CFUs. Measures of treatment efficacy included Kaplan–Meier curves (left-hand column) showing time to clearance of infection (groups were compared using the Mantel–Cox [log-rank] test), $\log_{10}$ CFU versus time (middle column), and bacterial burdens consolidated over time (AUC [$\log_{10}$ CFU] analysis) (right-hand column). $\log_{10}$ CFU over time between the saline and CMP-Leg5,7Ac$_2$ groups were compared by two-way ANOVA and Sidak multiple comparisons test. The differences in CFUs between the saline- and CMP-Leg5,7Ac$_2$–treated groups were significant ($p < 0.0001$) on all days (days 1 through 7) for strains CTX-r Spain, NJ-60, and SD-1 and on days 1 and 2 for strain 398078. Pairwise AUC comparisons across groups was made with the two-sample Wilcoxon rank-sum (Mann–Whitney) test. Note that infection with strain 398078 lasted only 2 d even in untreated mice.

Leg5,7Ac$_2$ is a nonhuman glycan that is expressed by several microbes that colonize or infect humans such as *Legionella pneumophilia* (82), *Campylobacter jejuni* (83, 84), *Acinetobacter baumannii* (85, 86), *Enterobacter cloacae* (87), and *Cronobacter turicensis* (88). It is therefore no surprise that human serum contains antilegionaminic Abs (89). If Leg5,7Ac$_2$ is displayed on host cells following CMP-Leg5,7Ac$_2$ treatment, then binding of such Abs to host tissues could cause complement-mediated damage. We are encouraged by the observation that the incubation of BJA-B K20 cells with free Leg5,7Ac$_2$ at concentrations as high as 3 mM did not result in surface expression of this NulO. Only NulOs, but not their CMP salts, can be taken up and metabolically incorporated into mammalian cells. Thus, in the event CMP-Leg5,7Ac$_2$ is hydrolyzed at the mucosal surface and is taken up by cells or is hydrolyzed in the lysosomal compartment after macropinocytosis, our data suggest that host cell glycans are unlikely to be capped by Leg5,7Ac$_2$ and be targeted by antilegionaminic Abs. Another consideration with topical treatment with CMP-Leg5,7Ac$_2$ is the ability of human ST6Gal-I to enzymatically transfer Leg5,7Ac$_2$ to select glycans (50). However, we were unable to detect ST6Gal-I in human cervical lavage samples by Western blotting (data not shown), suggesting that if transfer of NulO to cell surface glycans were to occur, it would be at extremely low levels. Kdn is a host molecule (53, 54); therefore, CMP-Kdn will also be considered for further development to circumvent any toxicity issues of CMP-Leg5,7Ac$_2$, if they were to arise. In contrast, pre-existing human Abs against Leg5,7Ac$_2$ glycans could contribute to the efficacy of CMP-Leg5,7Ac$_2$ by

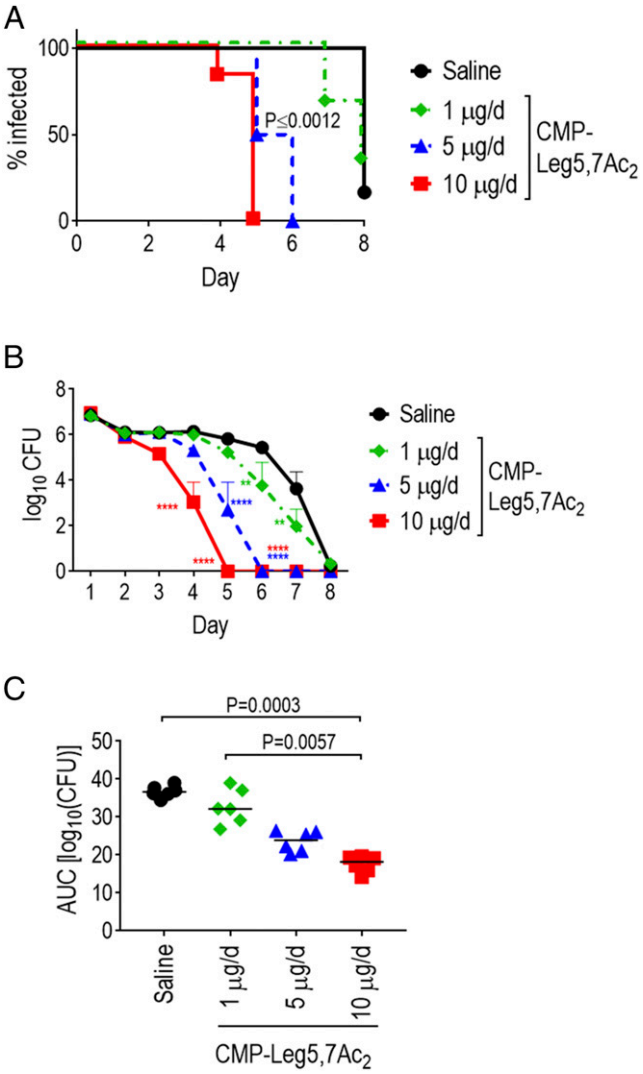

FIGURE 9. Efficacy of CMP-Leg5,7Ac$_2$ in human FH/C4BP transgenic mice. Transgenic mice in a BALB/c background that express the human complement inhibitors, FH and C4BP, were treated with Premarin and infected intravaginally with multidrug-resistant *N. gonorrhoeae* strain H041. Mice (n = 6/group) were treated with either saline (vehicle control) or CMP-Leg5,7Ac$_2$ at doses of 10, 5, or 1 μg daily intravaginally, commencing 2 h before infection. Vaginas were swabbed daily to enumerate CFUs. (**A**) Kaplan–Meier curves showing time to clearance of infection. Pairwise comparisons were made using the Mantel–Cox (log-rank) test. Significance was set at 0.008 (Bonferroni correction for comparisons across four groups). The groups that received the 10 and 5 μg/d doses of CMP-Leg5,7Ac$_2$ cleared infection significantly faster than the saline or 1 μg/d groups ($p \leq 0.0012$). (**B**) Log_{10} CFU versus time. Comparisons of the CFU over time between each treatment group and the saline control was made by two-way ANOVA and Dunnett multiple comparison test. $^{**}p < 0.01$, $^{****}p < 0.0001$. The color of the asterisks corresponds to the graph of the corresponding color. (**C**) Bacterial burdens consolidated over time (AUC [$\log_{10}$ CFU] analysis). The four groups were compared by one-way ANOVA using the nonparametric Kruskal–Wallis equality of populations rank test. The χ^2 with ties (3 df) was 19.98 (p = 0.0002). Pairwise AUC comparisons across groups was made with Dunn multiple comparison test.

binding to Leg5,7Ac$_2$-coated gonococci and enhancing complement activation.

Another advantage of CMP-Leg5,7Ac$_2$ and CMP-Kdn as therapeutics is the resistance of Leg5,7Ac$_2$- and Kdn-terminating glycans to the effects of several bacterial, viral, and mammalian sialidases (40, 51). As such, Leg5,7Ac$_2$ or Kdn will remain linked to the gonococcal surface even in the presence of sialidases/neuraminidases elaborated by microflora concomitantly present in the cervical secretions of women with gonorrhea (52) and render gonococci susceptible to clearance by host defenses.

In conclusion, CMP-Leg5,7Ac$_2$ is efficacious against diverse strains of *N. gonorrhoeae* in mice that express human-like sialic acid and human complement inhibitors. At physiological temperature, it is stable over pH ranges that are encountered in the human female genital tract, it is not incorporated into host cell glycans, and it is resistant to sialidases. Furthermore, CMP-Kdn, a human NulO representative anticipated to have low toxicity, was shown to have efficacy in a mouse vaginal colonization model that is on par with CMP-Leg5,7Ac$_2$. In addition, there are low-cost methods for both CMP-Leg5,7Ac$_2$ and CMP-Kdn production (43, 90). These qualities together make CMP-Leg5,7Ac$_2$ and CMP-Kdn our best lead antigonococcal therapeutic CMP-NulO compounds.

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Disclosures

The authors have no financial conflicts of interest.

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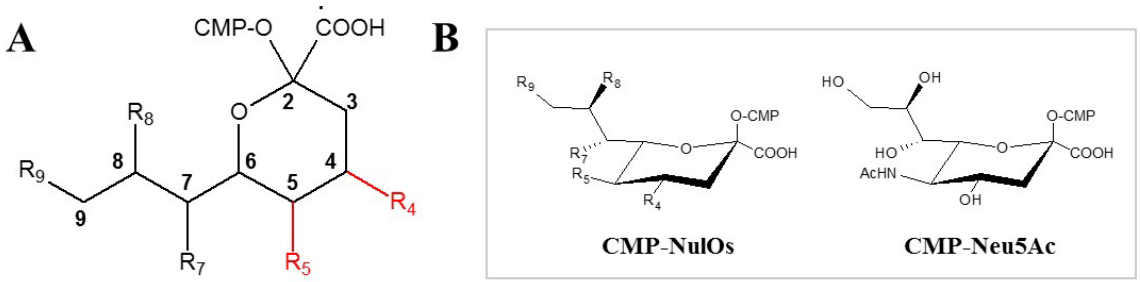

CMP-NulO	R_4	R_5	R_7	R_8	R_9
CMP-Neu5Ac	OH	NH-Acetyl	OH	OH	OH
CMP-Neu5Gc	OH	NH-Glycolyl	OH	OH	OH
CMP-Neu5,9Ac_2	OH	NH-Acetyl	OH	OH	O-Acetyl
CMP-Neu5Ac9N_3	OH	NH-Acetyl	OH	OH	N_3
CMP-Leg5,7Ac_2	OH	NH-Acetyl	NH-Acetyl	OH	H
CMP-Neu4,5Ac_2	O-Acetyl	NH-Acetyl	OH	OH	OH
CMP-Kdn	OH	OH	OH	OH	OH
CMP-Kdn7N_3	OH	OH	N_3	OH	OH

Fig. S1. Structures of the CMP-nonulosonate (CMP-NulO) analogs used in this study. **A** General chemical structure, and **B** $^2C_5$ chair chemical structure of CMP-nonulosonates used with reference to CMP-Neu5Ac. All NulO sugars have the same stereochemistry (D-*glycero*-D-*galacto* configuration). CMP-NulOs used as benchmarks/reference of for follow-up studies (from previous work) are shown in dark shading, whereas CMP-NulOs newly investigated in this study are shown in light shading. For reference, the nine carbon atoms of the NulOs are numbered in **A,** and the NulO structural moieties further investigated here are highlighted in red.

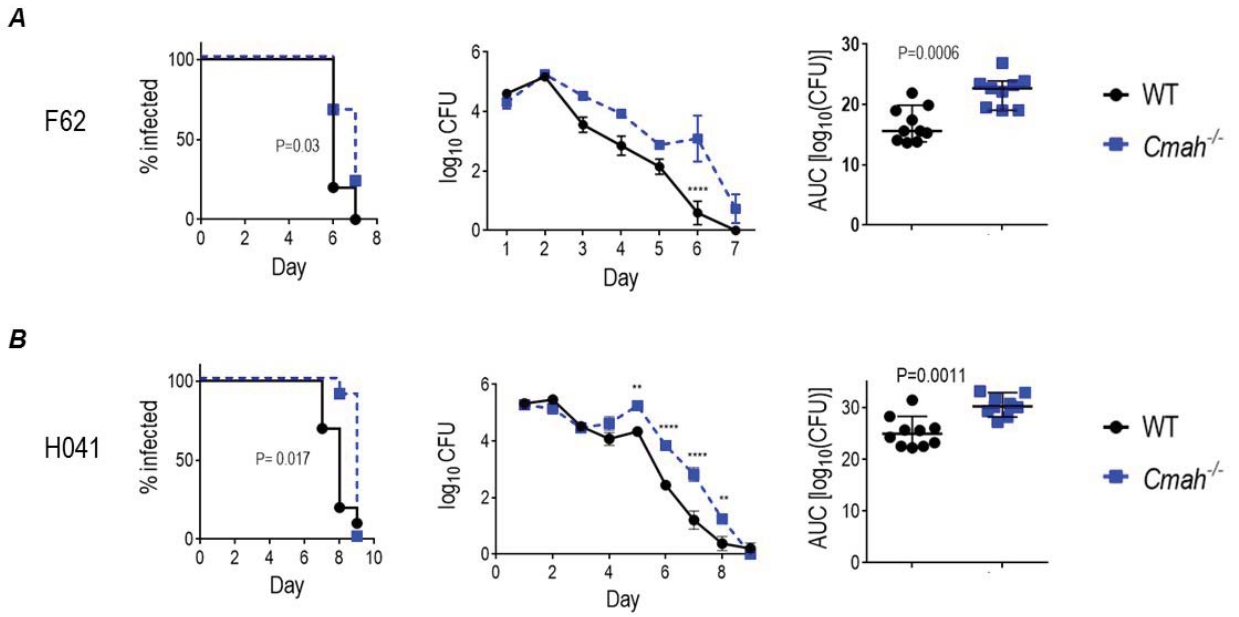

Fig. S2. Comparison of the duration and burden of gonococcal infection in saline-treated wild-type (WT) and *Cmah*$^{-/-}$ mice. The top row shows data with *N. gonorrhoeae* strain F62 (n=10 mice in WT group and n=9 in the *Cmah*$^{-/-}$ group), while the bottom row shows data with *N. gonorrhoeae* H041 (n=10 mice in WT and *Cmah*$^{-/-}$ groups). The left hand column shows times to clearance (Kaplan-Meier curves; comparisons made with Mantel-Cox log-rank test), the middle column shows $\log_{10}$ CFU versus time (comparisons made by two-way ANOVA and Sidak's multiple comparsions test; **, P<0.01; ****, P<0.0001) and the right hand column Area Under Curve analysis (comparisons made by Mann-Whitney's non-parametric test).

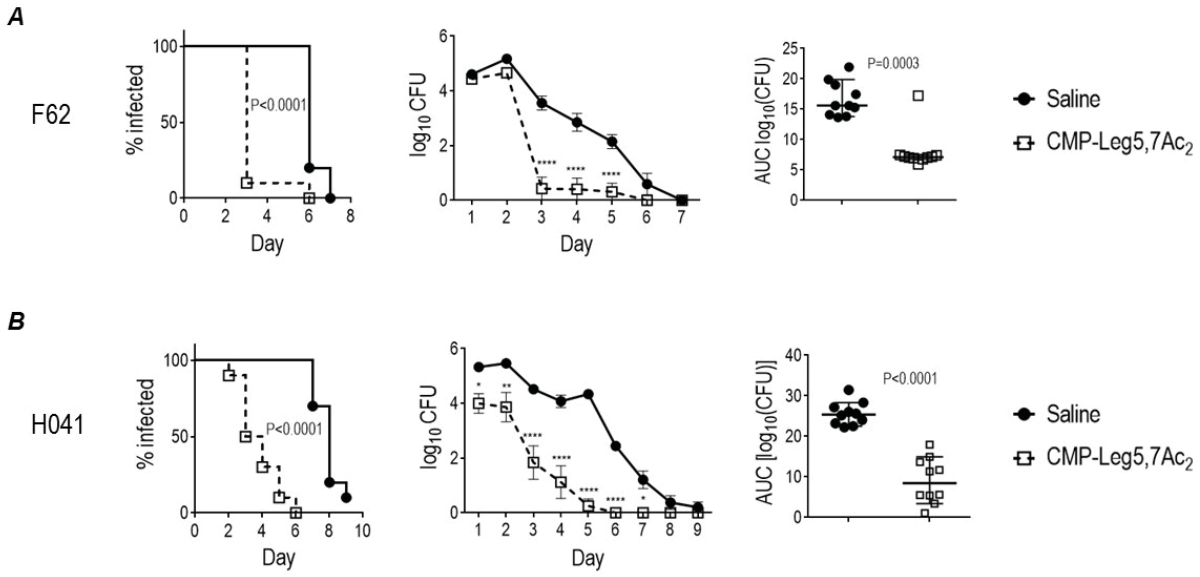

Fig. S3. Efficacy of CMP-Leg5,7Ac$_2$ in wild-type mice. The top row shows data with *N. gonorrhoeae* strain F62 (n=10 mice in the saline and CMP-Leg5,7Ac$_2$ groups), while the bottom row shows data with *N. gonorrhoeae* H041 (n=10 mice in the saline and CMP-Leg5,7Ac$_2$ groups). The left hand column shows times to clearance (Kaplan-Meier curves; comparisons made with Mantel-Cox log-rank test), the middle column shows $\log_{10}$ CFU versus time (comparisons made by two-way ANOVA and Sidak's multiple comparsions test; *, P<0.05; **, P<0.01; ****, P<0.0001) and the right hand column Area Under curve analysis (comparisons made by Mann-Whitney's non-parametric test).

Table S1. NMR chemical shifts δ (ppm) for CMP-3-deoxy-D-glycero-D-galacto-nonulosonic acid (CMP-Kdn).

H3ax	1.61		
H3eq	2.44	C3	42.1
H4	4.03	C4	69.7
H5	3.60	C5	71.1
H6	4.09	C6	74.1
H7	3.75	C7	69.7
H8	3.94	C8	70.9
H9	3.67; 3.92	C9	64.3

Table S2. NMR chemical shifts δ (ppm) for CMP-3,7-dideoxy-7-azido-D-glycero-D-galacto-nonulosonic acid (CMP-Kdn7N_3).

H3ax	1.67		
H3eq	2.48	C3	42.0
H4	4.03	C4	69.7
H5	3.61	C5	72.2
H6	4.20	C6	73.8
H7	3.82	C7	62.3
H8	4.06	C8	69.7
H9	3.77; 3.94	C9	64.1

Table S3. MS data for CMP-3-deoxy-D-glycero-D-galacto-nonulosonic acid (CMP-Kdn) and CMP-3,7-dideoxy-7-azido-D-glycero-D-galacto-nonulosonic acid (CMP-Kdn7N_3).

Compound	Observed ***m/z***	Calculated mass	Formula (M)	Comments
CMP-Kdn	572.4	573.4	$C_{18}H_{28}O_{16}N_3P$	$[M-H]^-$
CMP-Kdn7N_3	597.1	598.4	$C_{18}H_{27}O_{15}N_6P$	$[M-H]^-$