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Published in final edited form as:
*J Immunol*. 2020 June 15; 204(12): 3283–3295. doi:10.4049/jimmunol.1901398.

# Therapeutic CMP-nonulosonates against multidrug-resistant *Neisseria gonorrhoeae*

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## Abstract

*Neisseria gonorrhoeae* deploys a unique immune evasion strategy wherein the lacto-*N*-neotetraose (LNnT) termini of lipooligosaccharide (LOS) are capped by a surface sialyltransferase (Lst), utilizing 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 analogs of CMP-sialic acids (CMP-nonulosonates or 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. Here, we characterize and develop additional candidate therapeutic CMP-NulOs. CMP-ketodeoxynonulosonate (Kdn) and CMP-Kdn7N$_3$, but not CMP-Neu4,5Ac$_2$, were substrates for Lst, further elucidating gonococcal Lst specificity. LNnT 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/day) 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/

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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*.

## Introduction

*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 U.S, 583,405 cases were reported to the Centers for Disease Control and Prevention (CDC) 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 ‘super-bugs’ 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 non-inferiority criteria when compared to the 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 (MSM) 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 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 virulence of several pathogens, including *N. gonorrhoeae* (reviewed in (20, 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 analogs 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 analog nonulosonates (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). Here, we further characterize and develop therapeutic CMP-NulOs that are promising topical prophylactics/ therapeutics against antimicrobial resistant *N. gonorrhoeae*. Specifically, we evaluated 1) efficacy of other CMP-NulOs such as CMP-Kdn, CMP-Kdn7N$_3$ and CMP-Neu4,5Ac$_2$, 2) 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 lipooligosaccharide glycosyltransferase D *(lgtD)*, called F62 ΔlgtD (33), was provided by Dr. Daniel C. Stein (University of Maryland). LgtD adds GalNAc to the terminal Gal of the HepI lacto-*N*-neotetraose (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 *Ng* 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 (WHO X), was isolated from a female commercial sex-worker in Kyoto, Japan (10). This isolate is highly resistant to ceftriaxone (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; Ref. (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 (GISP) (36). Strain UMNJ60_06UM (called NJ-60 in this study; ceftriaxone MIC 0.38 μg/ml) was isolated in Nanjing, China and belongs to NG-MAST sequence type 3289 and MLST sequence type 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 Hep I (38). All strains used in mouse experiments were rendered streptomycin-resistant by transformation with *rpsL* derived from streptomycin resistant *Ng* strain FA1090 as described previously (31).

### Synthesis of CMP-NulOs.

The production and characterization of CMP-Neu5Ac, CMP-Neu5,9$Ac_2$ (also referred to as CMP-Neu5Ac9Ac), CMP-Neu5Ac9$N_3$ (also called CMP-Neu5Ac9Az) and CMP-Leg5,7$Ac_2$ (also called CMP-Leg5Ac7Ac) used in this study have been described previously (31). CMP-Neu5Ac was also obtained commercially (Nacalai). CMP-Neu4,5$Ac_2$ was produced using similar methods to those above with Neu4,5$Ac_2$ obtained commercially (Carbosynth).

CMP-Kdn and CMP-Kdn7$N_3$ were produced using the 2 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 approximately 0.15 mg/mL aldolase. Reactions were incubated at 37°C with gentle shaking for 24-48 h, followed by removal of enzyme by centrifugal ultrafiltration. Next, CMP-activation of synthesized Kdn was achieved enzymatically using methods similar to those described previously (31). Here, reactions typically contained 50 mM Tris pH 8.5, 50 mM $MgCl_2$, 5 mM CTP, approximately 5 mM Kdn, 4 units pyrophosphatase per mM of CTP and approximately 0.1 mg/mL of CMP-sialic acid synthetase. Reactions were incubated at 37°C for 2 hours, followed by 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 approximately 40-fold in 1 mM NaCl. After sample application, the resin was washed with 2 CV of 1 mM NaCl and purified CMP-Kdn was obtained with a 0.8 CV 100 mM NaCl step elution. This CMP-Kdn preparation was further desalted using diafiltration, where the sample was transferred to a diafiltration cell (Diaflo ultrafiltration membranes, YCO5 76 mm), and filtered using 3 times the volume of 1 mM NaCl at a flow rate of 32 ml/h. After 24 hours, the isolated retentate contained approximately 96% of the original CMP-Kdn. Kdn7$N_3$ (3,7-dideoxy-7-azido-D-*glycero*-D-*galacto*-nonulosonic acid) was enzymatically prepared using a *Pasteurella 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 Inc.), 128 mM sodium pyruvate, and sufficient quantities of aldolase. Reactions were incubated at 37°C for approximately 24 hours, and enzyme was then removed by centrifugal ultrafiltration. Next, CMP-activation of synthesized Kdn7$N_3$ was achieved enzymatically using methods similar to those described previously (31). Here, reactions typically contained 50 mM Tris pH 9, 50 mM $MgCl_2$, 5 mM CTP, approximately 5 mM Kdn7$N_3$, 4 units pyrophosphatase per mM of CTP and approximately 0.68 mg/mL of CMP-sialic acid synthetase. Reactions were incubated at 37°C for 2 hours, and enzyme was then removed by centrifugal ultrafiltration. Filtered CMP-Kdn7$N_3$ 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-Kdn7$N_3$ were subjected to anionexchange chromatography (Mono Q 4.6/100 PE, GE Healthcare) using an ammonium bicarbonate gradient. Quantification of CMP-Kdn and CMP-Kdn7$N_3$ preparations were determined using the molar extinction coefficient of CMP ($\varepsilon_{260}$=7,400). Purified and desalted sample aliquots were then freeze dried.

For structural characterization of CMP-Kdn and CMP-Kdn7$N_3$, 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 ($\delta_H$ 2.225 ppm and $\delta_C$ 31.07 ppm). Results are shown in Table S1 (CMP-Kdn) and Table S2 (CMP-Kdn7$N_3$) verifying the production of each compound.

CMP-Kdn and CMP-Kdn7$N_3$ prepared compounds were also characterized using mass spectrometry (MS) or CE-MS analysis. For CE-MS, mass spectra were acquired using an API3000 mass spectrometer (Applied Biosystems/Sciex, Concord, ON, Canada). CE was performed using a Prince CE system (Prince Technologies, Netherlands). CE separation was obtained on a 90 cm length of bare fused-silica capillary (365 μm OD x 50 μm ID) 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). Here, 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 Table S3, where 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 Inc., Canada) 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 Corp., Milford, MA) and lyophilized after each reaction.

For the addition of Leg5,7$Ac_2$ or Neu5Ac to 7.5 mg of LNnT-$PEG_3$-$N_3$, the reaction contained 50 mM MES pH 6.5, 10 mM MgCl2, 7.5 mM donor and approximately 1.5 units of NST-05 (a recombinant version of the LOS α−2,3-sialyltransferase (Lst) from *Neisseria meningitidis*). The reaction was incubated at 30$^o$C and was complete after 1 h for the addition of Neu5Ac. However, the complete addition of Leg5,7$Ac_2$ required additional enzyme and donor (CMP-Leg5,7$Ac_2$) and over-night 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), 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 labelling with biotin were performed in 1X 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 1.5X molar excess of DBCO-PEG$_4$-Biotin (Click Chemistry Tools, Scottsdale, AZ). The reaction mix was incubated at 37$^{o}$C for 30 min. 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, dried on a SpeedVac vacuum concentrator and by lyophilization. Mass spectrometry in the negative mode was used to confirm the masses expected for the biotinylated products.

### Antibodies.

mAb 3F11 (mouse IgM, kindly provided by Dr. Michael A. Apicella, University of Iowa) binds to the unsialylated HepI lacto-*N*-neotetraose structure; any extension beyond the terminal Gal, for e.g., sialylation of LOS, results in decreased mAb 3F11 binding to LOS (41). Anti-FH mAb from Quidel (catalog no. A254 (mAb 90X)) 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 (1:2,000) (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 utilized to purify anti-Leg5,7Ac$_2$ Ab from human intravenous immunoglobulins (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 a thousand 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 antibodies 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) using Tris-Tricine Cathode buffer (Boston Bioproducts) and LOS was stained using the Bio-Rad Silver Stain kit.

### Factor H binding.

Factor H (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 Hanks Balanced Salt Solution (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, Inc.). Bound FH was detected using an anti-FH mAb

(Quidel; clone 90X), 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, Inc.).

#### Normal human serum.

Serum was prepared from the blood of healthy human volunteers by phlebotomy. Sera from 10 donors was 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 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 (Jackson Laboratories), *Cmah*$^{-/-}$ mice back-crossed into a BALB/c background (46) or transgenic mice that expressed human complement inhibitors, factor H and C4b-binding protein (C4BP), that were generated in a BALB/c background (47). Mice that were 6-8 weeks 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 subcutaneously on each of three days; −2, 0 and +2 days (before, the day of and after inoculation of bacteria) to prolong the estrus phase of the cycle and promote susceptibility to *Ng* 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 approx. 1.5 mM stock) daily intravaginally while 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 vs. 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 containing 1% (v/v) Nutridoma (Roche) for 3 days to eliminate any residual sialic acid from the cell growth media. Following incubation, 3 mM Leg5,7$Ac_2$ or Neu5Gc (as a positive control) was added, and cells were allowed to incubate for an additional 3 days at 37°C. Cells were then harvested ($2 \times 10^5$ cells; fed or unfed), washed and probed with either Leg5,7$Ac_2$-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-Neu5Ac9$N_3$ and CMP-Leg5,7$Ac_2$ 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 approximately 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 weeks as indicated. Capillary electrophoresis 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 (IRB). Informed, written consent was obtained from all serum donors (Docket # H00005614). 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 (IACUC) at the University of Massachusetts Medical School (Docket # A-1930).

## Results

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

Previously, we showed that *N. gonorrhoeae* LOS sialyltransferase (Lst) had broad substrate specificity; in addition to CMP-Neu5Ac, the natural substrate for gonococcal Lst, CMP salts of Neu5Gc, Neu5Gc8Me, Neu5Ac9$N_3$ and Leg5,7$Ac_2$ were utilized 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 utilized 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 Fig. S1 for reference to various NulO backbone structures). These three new CMP-NulOs were chosen because their NulO's 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 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 lacto-*N*-neotetraose structure; glycan extensions beyond the terminal Gal in lactosamine, for example, NulO incorporation results in decreased binding of the antibody (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 Ng LOS on 16.5% Tricine gels, which showed no change in 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.

## 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 normal human serum (NHS) was next determined. The effects of adding each CMP-NulO individually on serum resistance is shown in Table 1 (rows shaded grey). 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 where survival was less than 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 intermediate phenotype with >100% survival in 3.3% NHS and approximately 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 1). 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 to unsialylated bacteria, CMP-Kdn7$N_3$ 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-Leg5,7$Ac_2$ (benchmark) blocked CMP-Neu5Ac-mediated serum resistance when added after CMP-Neu5Ac (31).

## Effect of NulO substitution of gonococcal LOS on Factor H 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 Kdn7$N_3$ 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 Neu5,9$Ac_2$-capped LNnT LOS bound FH with ~30% the fluorescence intensity observed with Neu5Ac-capped LOS. Bacteria with Leg5,7$Ac_2$ 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-Neu5,9$Ac_2$ and CMP-Neu5Ac9$N_3$ were evaluated; CMP-Leg5,7$Ac_2$ served as the benchmark for efficacy (31). Three parameters of efficacy were measured: median time to clearance, $\log_{10}$ CFU versus time and Area Under Curve (AUC). When administered at a dose of 10 μg intravaginally daily, CMP-Kdn and CMP-Neu5Ac9$N_3$ were as efficacious as CMP-Leg5,7$Ac_2$ in clearing gonococcal colonization, while CMP-Neu5,9$Ac_2$ was ineffective (Fig. 3).

## Stability of CMP-NulOs

Stability of the CMP-NulOs is an important consideration for shelf-life, drug formulation, safety and for efficacy *in vivo*. While 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 utilized by *N. gonorrhoeae* Lst. Furthermore, free NulOs have the potential to traverse host cell membranes and become incorporated into host glycans and elicit autoimmune antibodies (60, 61).

The stabilities of the three CMP-NulOs (CMP-Leg5,7$Ac_2$, CMP-Neu5Ac9$N_3$ and CMP-Kdn) that showed efficacy in the mouse model of gonorrhea were tested at temperatures ranging from 4 °C 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 ºC), or those similar to shelf-life/storage conditions in solution (4 or 20 ºC and neutral pH). CMP-Leg5,7$Ac_2$ was the most stable molecule under all the tested conditions (Fig. 4 and Fig. 5). Greater than 90% of CMP-Leg5,7$Ac_2$ remained intact even after 6 weeks at 4 °C with neutral pH 7 as well as after 3 days at 20 °C (at pH 7) (Fig. 4). Remarkably, about 54% of CMP-Leg5,7$Ac_2$ remained intact after 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) greater than 50% of CMP-Leg5,7Ac$_2$ remained intact after 1 h, compared to less than 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 anti-gonococcal therapeutic candidate. To note, Leg5,7Ac$_2$ is a non-human 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 non-human 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 non-human 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 (Cmah) 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 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 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 Fig. S2. 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 to saline treated mice. 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 Fig. S3).

### 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 dose of 10 μg daily significantly shortened the duration and burden of gonococcal colonization in all four instances. Unlike the other strains where 100% of saline-treated animals remained colonized at the end of 7 days, strain 398078 (P$^K$-like LOS) colonized saline-treated (control) mice for only three days. 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 after infection), 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 non-human complement (reviewed in Ref. (48)). Binding of human, but not non-human complement inhibitors, FH and C4b-binding protein (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/day). As shown in Fig. 9, efficacy of CMP-Leg5,7Ac$_2$ was dose responsive; the lowest tested dose (1 μg/day) was ineffective, while the 5 μg/day and 10 μg/day 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 sialyltransferase enzyme and the functional consequences of NulO substitutions on FH binding and complement evasion. While CMP-Kdn and CMP-Kdn7$N_3$ both served as substrates for Lst, Neu4,5$Ac_2$ was not added to LNnT. The interaction between Neisserial 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 binding of CMP-Neu4,5$Ac_2$ to Lst and subsequent enzymatic transfer of Neu4,5$Ac_2$ to LOS. On the other hand, 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-Kdn7$N_3$ were transferred).

The exocyclic chain of Neu5Ac (C7-C9; see Fig. S1) 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 binding of FH to sialylated gonococci, as evidenced by lack of detectable FH binding when Leg5,7$Ac_2$ (deoxy and methyl at C9-position, in addition to C7 NH-acetyl), Neu5Gc8Me (*O*-methyl at C8-position) and Neu5Ac9$N_3$ (deoxy and $N_3$ at C9-position) 'capped' LNnT LOS (31), and a ~70% decrease in FH binding fluorescence with Neu5,9$Ac_2$ (*O*-acetyl at 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 C5-position; see Fig. S1) (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, Kdn7$N_3$ 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, 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 (74).

Similar to Neu5,9$Ac_2$, the addition of Kdn or Kdn7$N_3$ 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 Kdn7$N_3$-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 two-fold greater fluorescence than Neu5,9$Ac_2$-coated gonococci, yet show similar serum-resistance profiles. Kdn and Kdn7$N_3$ on LOS both result in similar FH binding, but Kdn7$N_3$ 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., due to 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 the vagina in women 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 2 other methyls associated with the two NH-acetyl groups present.

Leg5,7Ac$_2$ is a non-human 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 anti-legionaminic antibodies (89). If Leg5,7Ac$_2$ is displayed on host cells following CMP-Leg5,7Ac$_2$ treatment, then binding of such antibodies to host tissues could cause complement-mediated damage. We are encouraged by the observation that 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 in to 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 micropinocytosis, our data suggest that host cell glycans are unlikely to be ‘capped’ by Leg5,7Ac$_2$ and be targeted by anti-legionaminic antibodies. 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. On the other hand, pre-existing human antibodies against Leg5,7Ac$_2$ glycans could contribute to the efficacy of CMP-Leg5,7Ac$_2$ by binding to Leg5,7Ac$_2$-coated gonococci and enhancing complement activation.

Another advantage of CMP-Leg5,7$Ac_2$ and CMP-Kdn as therapeutics is the resistance of Leg5,7$Ac_2$- and Kdn-terminating glycans to the effects of several bacterial, viral and mammalian sialidases (40, 51). As such, Leg5,7$Ac_2$ 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-Leg5,7$Ac_2$ 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 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-Leg5,7$Ac_2$. In addition, there are low-cost methods for both CMP-Leg5,7$Ac_2$ and CMP-Kdn production (43, 90). These qualities together make CMP-Leg5,7$Ac_2$ and CMP-Kdn our best lead anti-gonococcal therapeutic CMP-NulO compounds.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Dr. Daniel C. Stein (University of Maryland) for F62 ΔlgtD and Dr. Ann E. Jerse (Uniformed Services University of Health Sciences, Bethesda, MD) for streptomycin-resistant *N. gonorrhoeae* F62. We thank Drs. Magnus Unemo (WHO Collaborating Centre for Gonorrhoea and other STIs, Örebro University Hospital, Örebro, Sweden) and Makoto Ohnishi (National Institute of Infectious Diseases, Tokyo, Japan) for *N. gonorrhoeae* H041, Dr. Carmen Ardanuy (Department of Microbiology, Hospital Universitari de Bellvitge, Instituto de Investigación Biomédica de Bellvitge (IDIBELL) , University of Barcelona, Barcelona, Spain) for strain Ctx-r(Spain), Dr. Severin Gose (San Francisco Department of Public Health, San Francisco, CA) for strain *Ng* SD-1 and Dr. Xiao-Hong Su (Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing, P. R. China) for strain UMNJ60_06UM. We thank Nancy Nowak for technical assistance. We thank Michel Gilbert, Evgeny Vinogradov, Jianjun Li (all from the National Research Council, Ottawa), Mohamed Hassan, Chris Boddy (both from the University of Ottawa) and Dennis Whitfield (SRL) for technical assistance.

This work was supported by National Institutes of Health grants R33 AI119327, R01 AI114790 and R01 GM32373.

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**Key points**

1. NulOs displayed on gonococcal LOS renders bacteria susceptible to host complement.
2. CMP-NulOs show promise against multidrug-resistant gonorrhea in preclinical studies.
3. CMP-Leg5,7$Ac_2$ and CMP-Kdn are the current lead candidates.

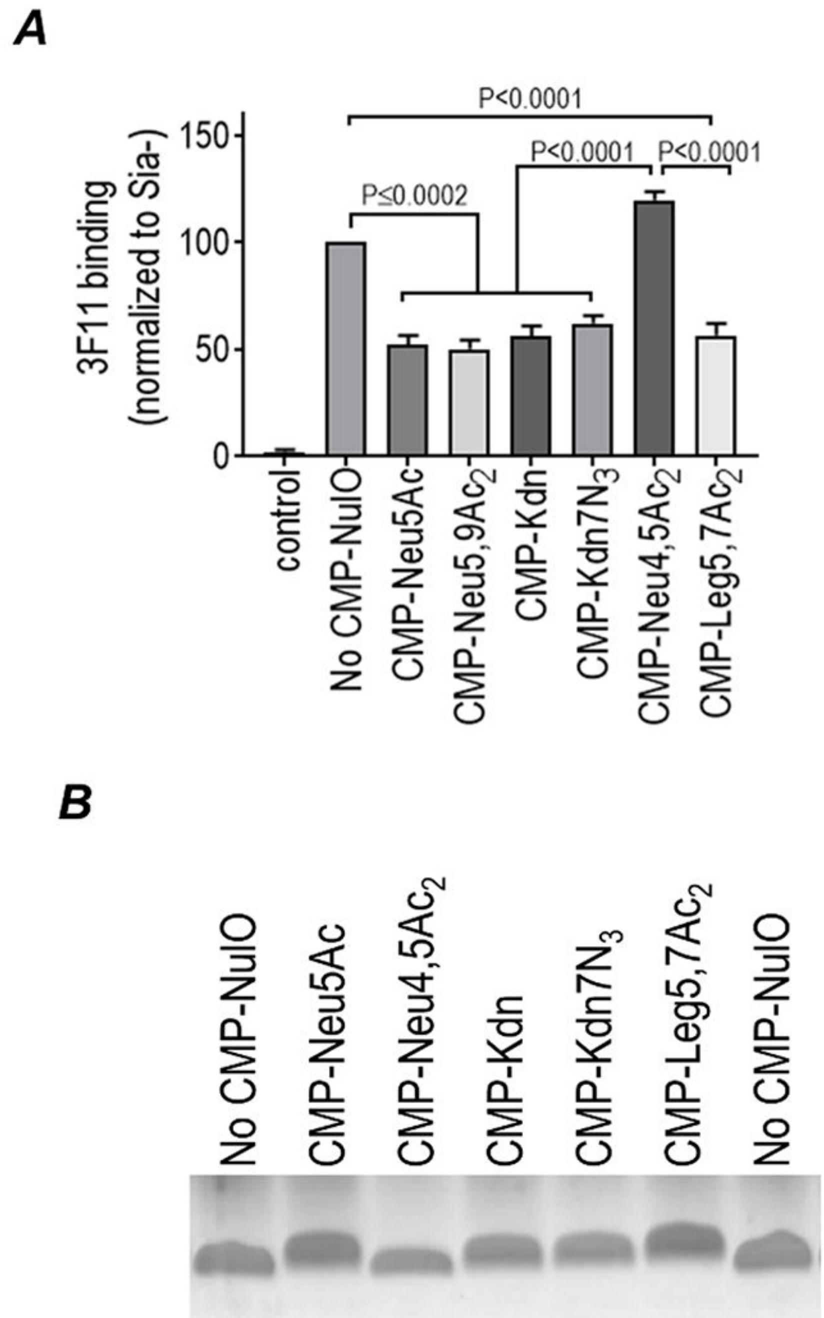


**Fig. 1.**

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 were observed (F=43.76; P<0.0001).

Pairwise comparisons, made by Tukey's multiple comparisons 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) and LOS was visualized by silver staining. Slower mobility relative to bacteria grown in media alone (No CMP-NulO) indicates addition of NulO.

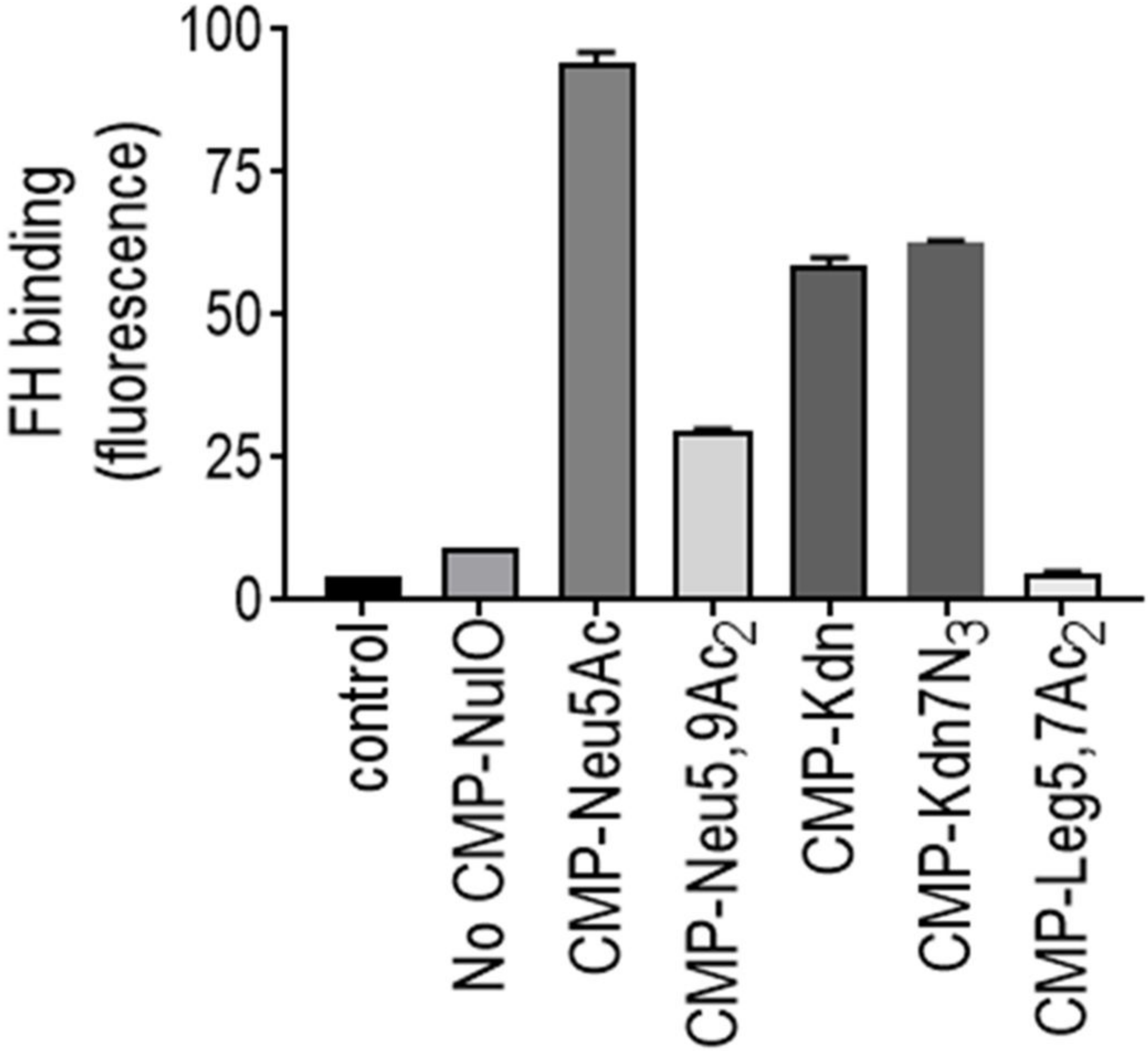


**Fig. 2.**

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 anti-FH mAb 90X 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 were observed ($F=1224$; $P<0.0001$). Pairwise comparisons were made by Tukey's multiple comparisons test. Pairwise differences across the control, 'No CMP-NulO' and CMP-Leg5,7$Ac_2$ groups and between CMP-Kdn and CMP-Kdn7$N_3$ were not significant. All other pairwise comparisons were significant ($P<0.0001$).

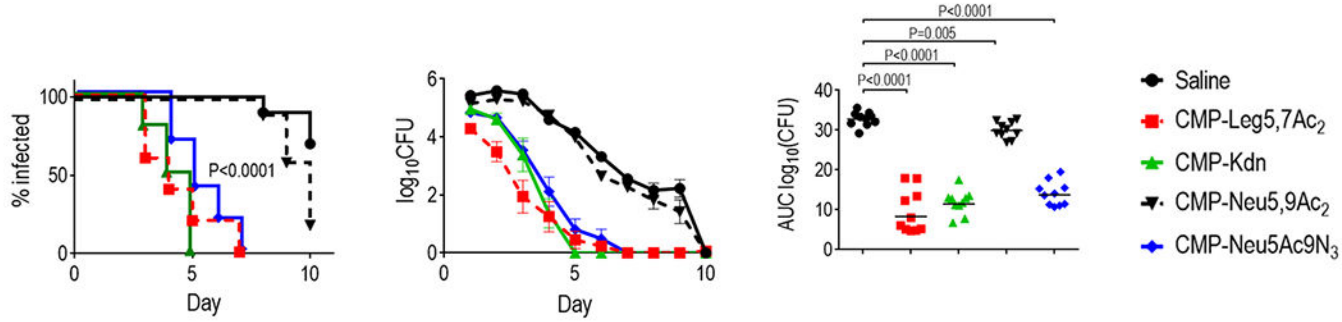


**Fig. 3.**

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; Ref. (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's 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's 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 and 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 (Area Under the Curve [log$_{10}$ CFU] analysis). The five groups were compared by one-way ANOVA using the non-parametric Kruskal-Wallis equality of populations rank test. The $\chi^2$ with ties (four degrees of freedom) was 24.6 (P<0.0001). Pairwise AUC comparisons across groups was made with Dunn's multiple comparison test.

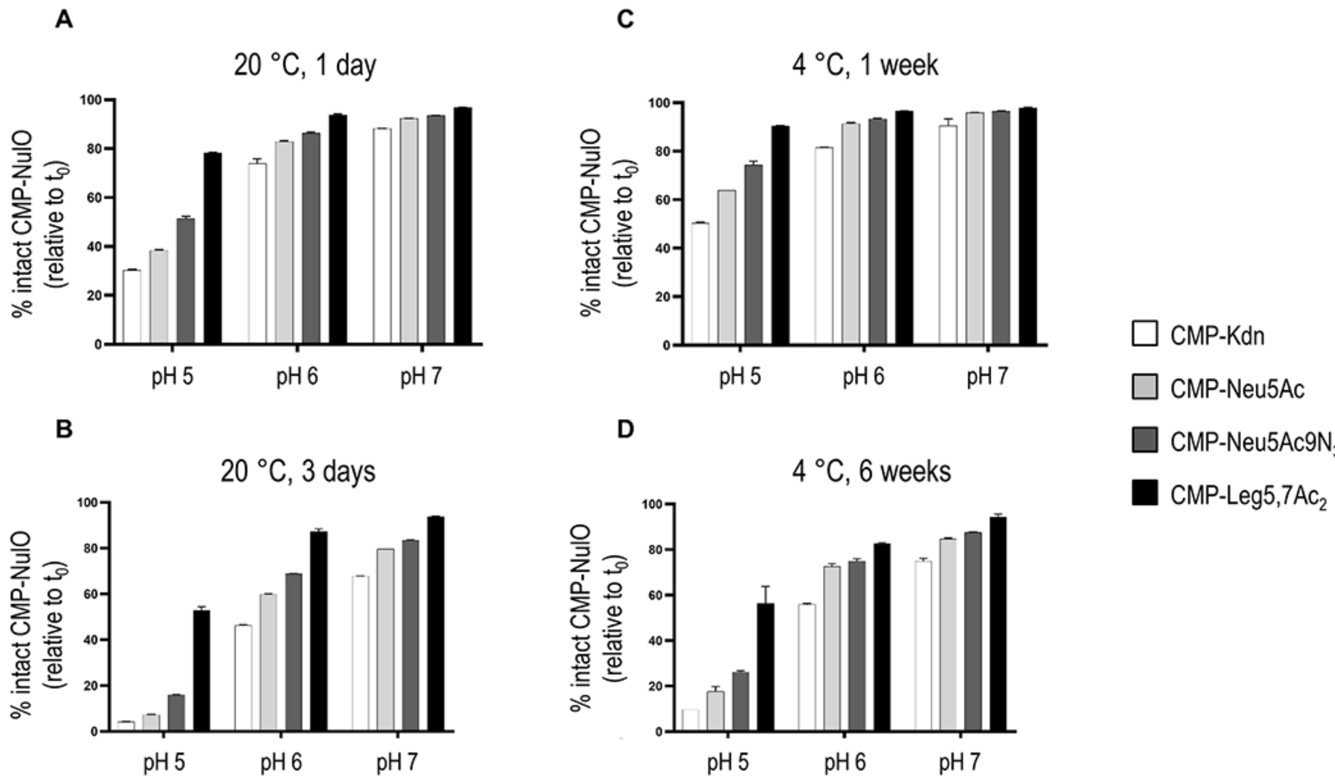


**Fig. 4.**

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 days at 20° C (panels A and B) or for 1–6 weeks at 4° C (panels C and D). The percentage of intact CMP-NulO after incubation relative to $t_0$ is indicated. Values represent the mean of 2 independent measurements.

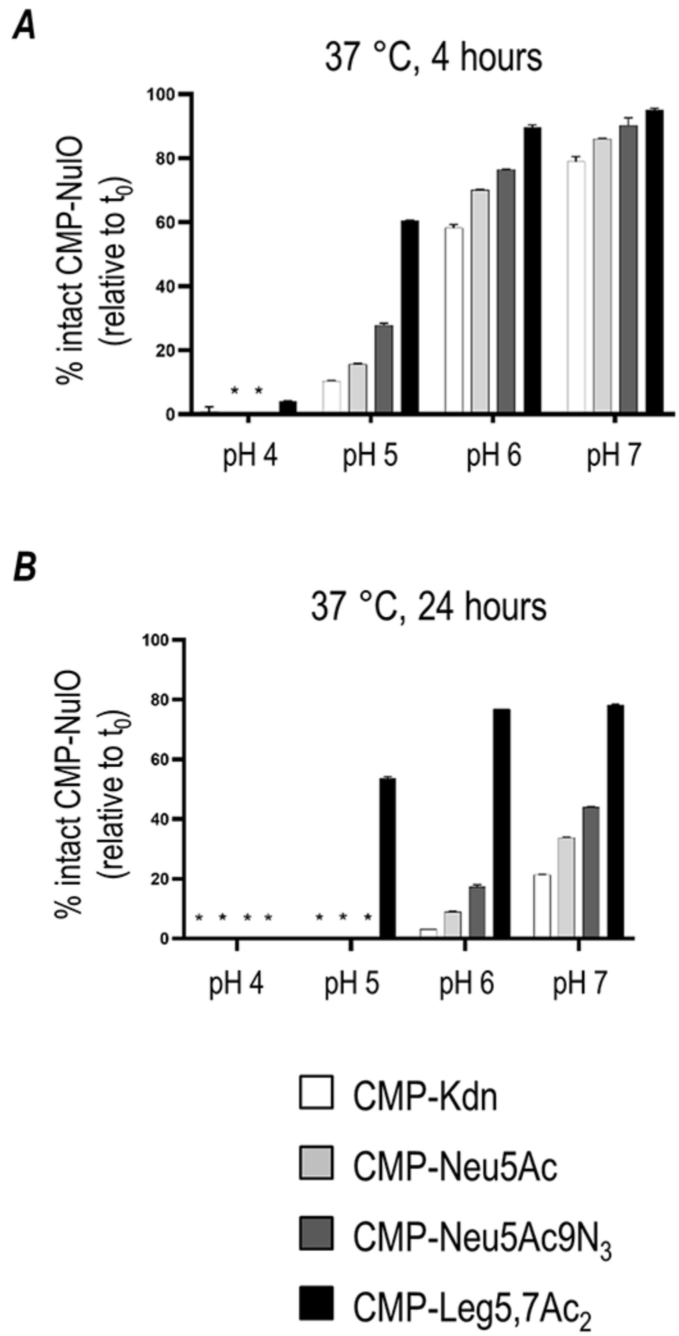


**Fig. 5.**

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 hours at 37° C. Measurements of intactness were taken at 4 hours (panel A) and 24 hours (panel B). The percentage of intact CMP-NulO after incubation relative to $t_0$ is indicated. Values represent the mean of 2 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).

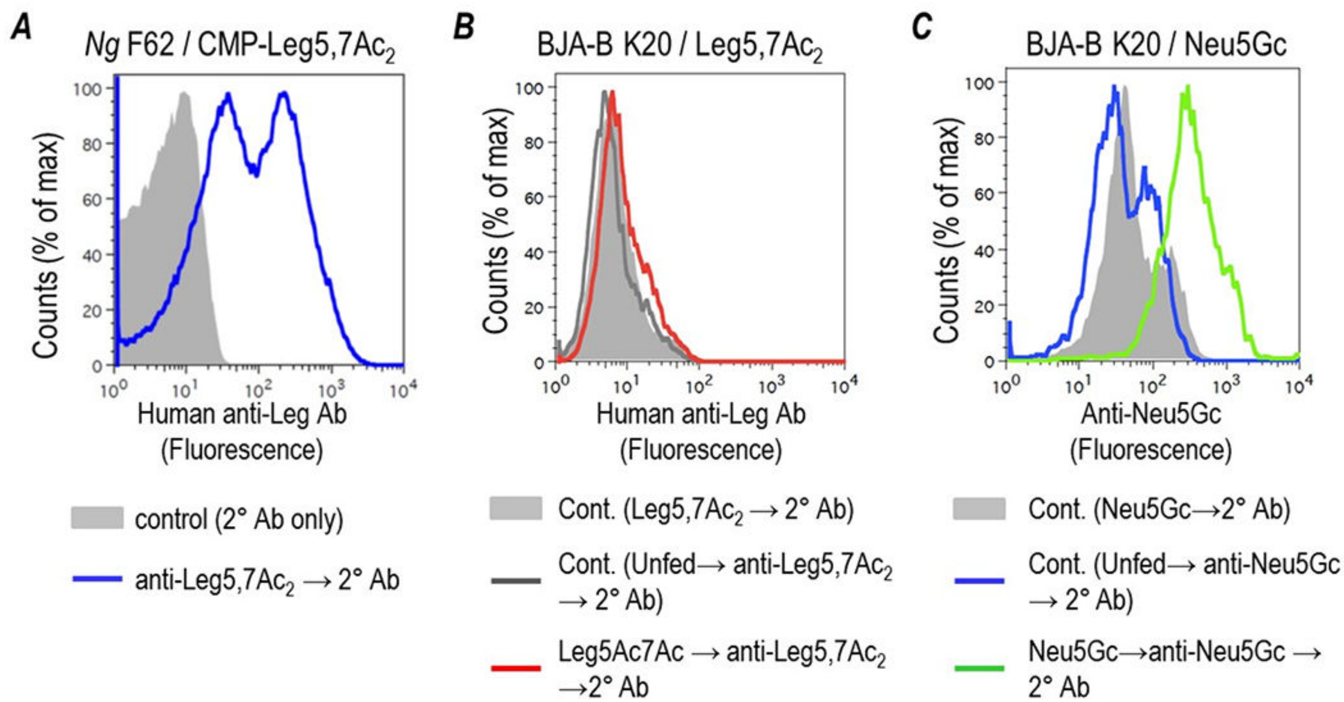


**Fig. 6.**

Leg5,7$Ac_2$ is not detected on the surface of hyposialylated human BJA-B K20 cells fed with Leg5,7$Ac_2$. ***A***. Validation of reactivity of human anti-Leg5,7$Ac_2$. *N. gonorrhoeae* F62 was grown in media alone or media supplemented with 30 μg/ml CMP-Leg5,7$Ac_2$. Bacteria were incubated with human anti-Leg5,7$Ac_2$ (1:10) followed by anti-human IgG conjugated to PE (blue histogram), or with fluorescent conjugate alone (grey shaded histogram). F62 grown in media alone did not show any reactivity over conjugate control levels (data not shown). X-axis, fluorescence on a log10 scale; Y-axis, counts. ***B***. Leg5,7$Ac_2$ is not detectable on BJA-B K20 cells fed with Leg5,7$Ac_2$. Hyposialylated BJA-B K20 cells were incubated with media alone (unfed cells) or media containing 3 mM Leg5,7$Ac_2$ and incubated with human anti-Leg5,7$Ac_2$ and anti-human IgG conjugated to PE (unfed cells, solid grey line; Leg5,7$Ac_2$-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*.

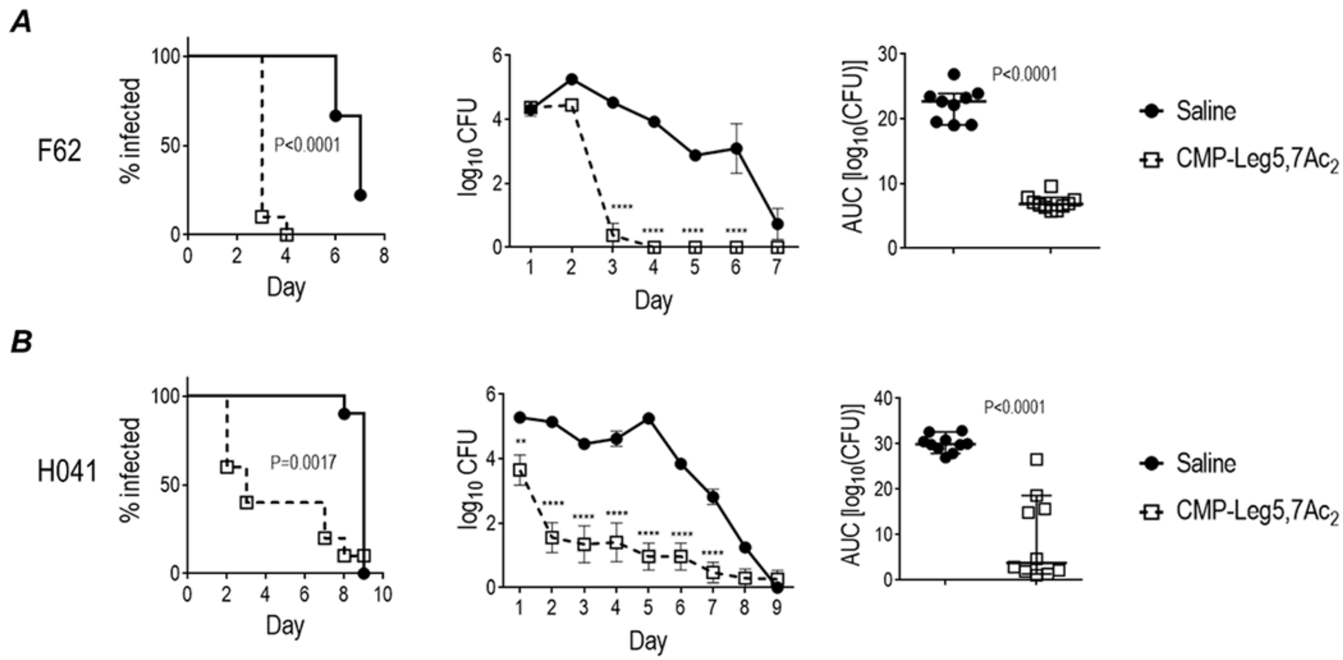


**Fig. 7.**

CMP-Leg5,7Ac$_2$ is efficacious in *Cmah*$^{-/-}$ mice. *Cmah*$^{-/-}$ mice that express only 'human-like' Neu5Ac, but not 'non-human' Neu5Gc seen in non-human primates and lower animals including mice, were treated with Premarin® and infected with $4.5 \times 10^5$ CFU *N. gonorrhoeae* F62 (top panel) or $6 \times 10^5$ CFU of strain H041 (bottom panel). 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 (Area Under the Curve [log$_{10}$ CFU] analysis) (right hand column). Pairwise AUC comparisons across groups was made with the Two-sample Wilcoxon rank-sum (Mann-Whitney) test.

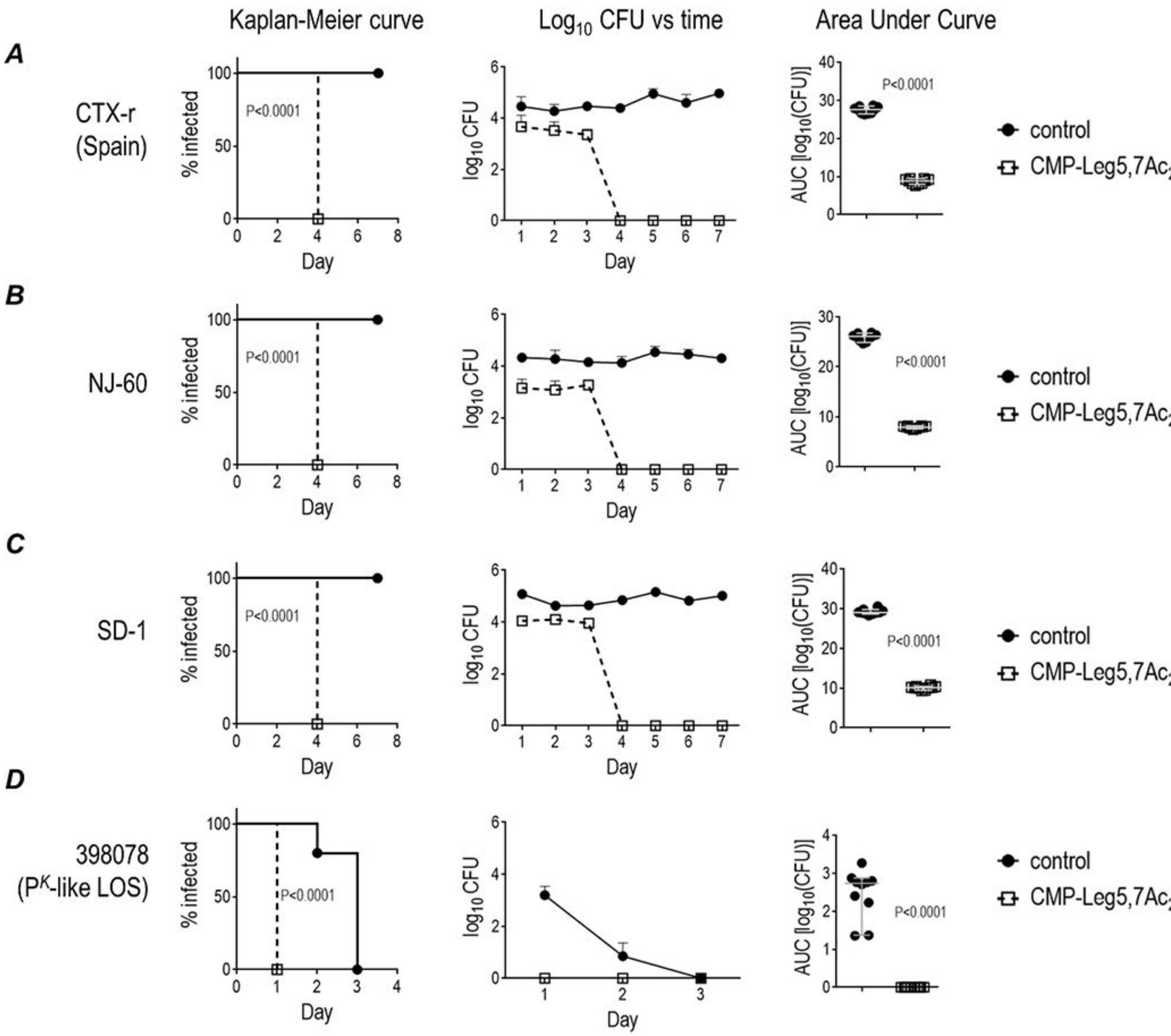


**Fig. 8.**

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 ($9.5 \times 10^5$ CFU) (panel ***A***), NJ-60 ($7.7 \times 10^5$ CFU) (panel ***B***), Ng SD-1 ($5.1 \times 10^5$) (panel ***C***) and 398078 ($P^K$-like globotriose LOS; $5.5 \times 10^5$ CFU) (panel ***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 (Area Under the Curve [$\log_{10}$ CFU] analysis) (right hand column). $\text{Log}_{10}$ CFU over time between the saline and CMP-Leg5,7Ac$_2$ groups were compared by two-way ANOVA and Sidak's multiple comparisons test. The differences in CFUs between the saline and CMP-Leg5,7$Ac_2$-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 two days even in untreated mice.

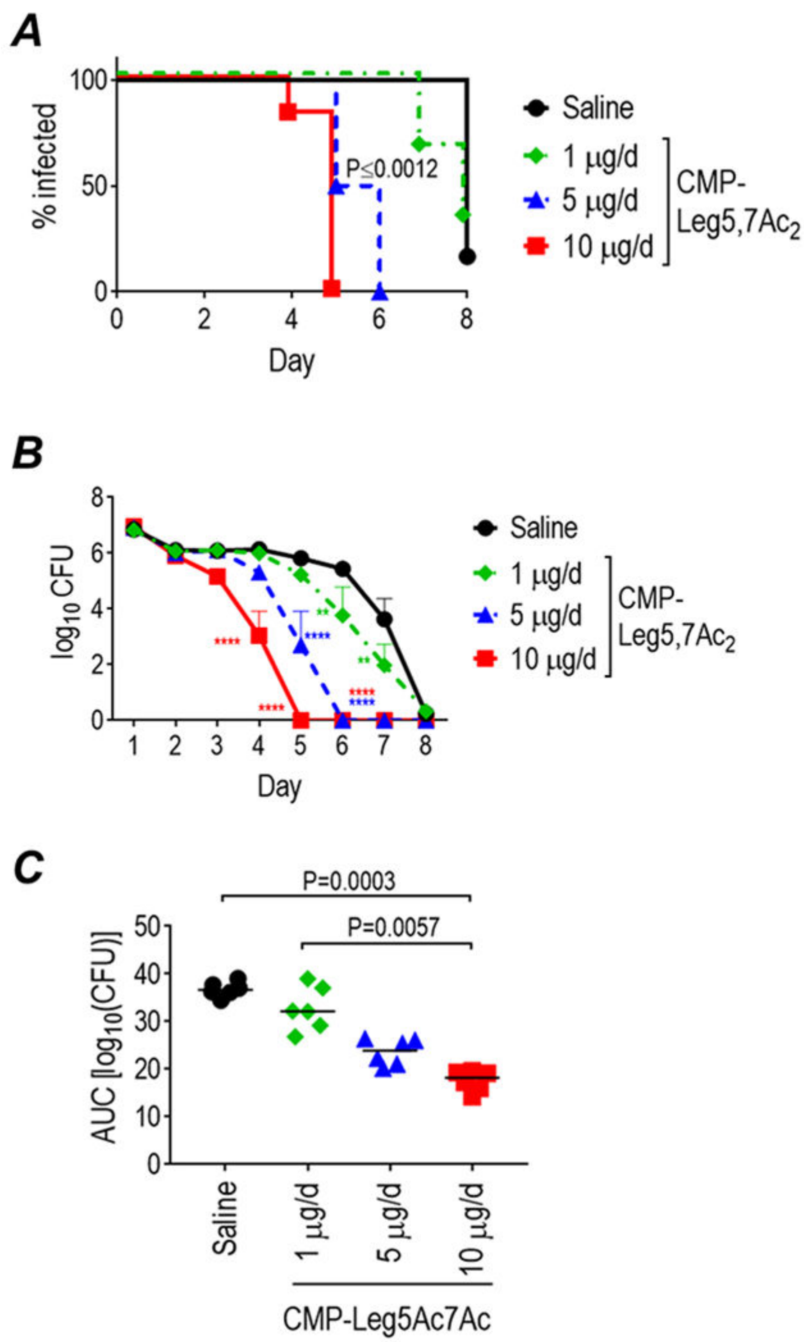


**Fig. 9.**

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's correction for comparisons across four groups). The groups that received the 10 and 5 μg/day doses of CMP-Leg5,7$Ac_2$ cleared infection significantly faster than the saline or 1 μg/day 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's 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 (Area Under the Curve [$log_{10}$ CFU] analysis). The four groups were compared by one-way ANOVA using the non-parametric Kruskal-Wallis equality of populations rank test. The $\chi^2$ with ties (three degrees of freedom) was 19.98 ($P=0.0002$). Pairwise AUC comparisons across groups was made with Dunn's multiple comparison test.

**Table 1.**

Effect of CMP-NulOs on complement-dependent killing of *Neisseria gonorrhoeae* F62 ΔlgtD

| CMP-NulO added first | CMP-NulO added second[A] | Percent survival (mean) in | |
|---|---|---|---|
| | | 3.3% NHS[B] | 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 normal human serum