CHAPTER TWELVE

Strategies in the Design and Use of Synthetic "Internal Glycan" Vaccines

Abirami Lakshminarayanan, Balakumar Vijayakrishnan, Hagan Bayley, Benjamin G. Davis[1]
University of Oxford, Oxford, United Kingdom
[1]Corresponding author: e-mail address: ben.davis@chem.ox.ac.uk

Contents

Abstract

The relative structural conservation of "internal glycans" in the cell walls of pathogens suggests that they might as target epitopes less prone to variation and hence with greater potential universality as vaccine targets. Examples of such glycans include the inner core sugars of lipopolysaccharides in Gram-negative bacteria. However, due to the buried nature of such internal epitopes, this approach has been rarely adopted. Here we briefly review and compare strategic approaches and outline practical methods associated with evaluating one synergistic strategy that combines (i) blocking of the display of "external glycans" with (ii) vaccination targeted at "internal glycans."

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1. INTRODUCTION

Since the days of Goebel and Avery (1929), some 90 years ago, we have known that conjugation of microbial glycans to protein carriers creates immunogens that may possess a potency as well as a flexibility in their conjugated design not present in the use of weakened pathogens or glycan extracts alone. This chapter does not seek to recapitulate the several recent, excellent review articles (Anish, Schumann, Pereira, & Seeberger, 2014; Astronomo & Burton, 2010; Berti & Adamo, 2013; Bhatia, Dimde, & Haag, 2014; Costantino, Rappuoli, & Berti, 2011; De Gregorio & Rappuoli, 2014; Johnson & Bundle, 2013; Jones, 2015; Terra et al., 2012; Vella & Pace, 2015) that have covered some of the striking advances in the construction and use of such glycoconjugate vaccines but instead to highlight a somewhat neglected strategy in the field. The rate of progress in glycoconjugate vaccine construction in recent decades has been greatly accelerated by both scientific and technological developments, in particular, automated glycan synthesis (Seeberger, 2015) and more precise protein conjugation chemistries (Grayson et al., 2011; Spicer & Davis, 2014; Stefanetti et al., 2015).

In principle, any glycan from a pathogen may be considered as an epitope against which an immune response may be raised. However, broadly speaking, three issues hamper the consideration of glycans. First, glycans alone have long been considered to be poor in generating long-lasting immune "memory." Second, the variation of glycans within pathogens can give rise to many serotypes, immunotypes, and associated phase variation. Third, the accessibility of glycans within pathogens must be considered; there is logic to the notion that those that are more deeply "buried," e.g., below a capsular region, are likely to be less accessible to key components of the immune system, such as antibodies raised toward them through vaccination. These concerns have led to a battery of approaches including the use of carrier proteins capable of aiding the recruitment of T-cells, the use of so-called "multivalent" vaccines (note that in the context of vaccinology this term is used to denote multiple antigen/immunogen types rather than multiple copies) directed at, e.g., several serotypes, and a focus on the outer components of the cell wall.

The targeting of what we term here "internal glycans," those that are more buried, has been more rare (Di Padova et al., 1993; Muller-Loennies, Brade, & Brade, 2007; Plested et al., 1999; Reinhardt et al., 2015), but brings with it certain advantages. Primarily, some internal glycans in pathogens show greater conservation of structure and hence potential

"universality" as epitopes across serotypes, species, and even genera. For example, in Gram-negative bacteria immediately distal to lipid A, the so-called "inner core" glycans (Fig. 1) show strong similarity in structural motifs across certain enterobacteria (e.g., *Salmonella*, *Escherichia coli*). Even in nonenteric bacteria (e.g., *Klebsiella*), a common central tetrasaccharide Hep-Hep-Kdo-Kdo exists. Not only do these partly conserved, cross-species internal glycans present a site for more general targeting, but these internal glycans appear also to be less affected by phase variation (Kahler, Datta, Tzeng, Carlson, & Stephens, 2005).

Notably, nature may already draw on the strategic advantage offered by this partial conservation via the toll-like receptor 4 (TLR4), which recognizes lipopolysaccharide (LPS) via various internal glycan-binding protein partners (e.g., LPS-binding protein, CD14). Indeed, the similarity of the structural modes of binding displayed by TLR4 and antiinternal glycan antibodies, such as WN1-222-5 (Di Padova et al., 1993), has been noted (Gomery et al., 2012). It should be noted too, in this context, that the development and use of internal glycan-binding biomolecules as therapeutics, obtained via vaccination or even used passively, may have a broader value in the context of preventing "septic shock" arising from antiendotoxin activity (Muller-Loennies et al., 2007; Song, Nguyen, Hong, & Kim, 2017) even if raised against portions of internal glycans other than lipid A (the causative agent of such "shock").

Proof-of-principle success for the idea of targeting internal glycans has been seen with certain pathogen targets. The formative work of Moxon et al. has critically demonstrated not only the accessibility of the inner core glycans of *Neisseria* to serum antibodies but also opsonic activity and protection (Plested et al., 1999, 2001, 2003). The success in certain *Neisseria* species may reflect, in part, the fact that *Neisseria* lack the O-polysaccharide found in most LPS (and this is therefore likely to improve accessibility). That said, there are limited reports of accessibility in pathogens bearing O-polysaccharide for other antiinternal glycan antibodies (e.g., WN1 222-5) (Muller-Loennies et al., 2007).

We therefore set out to develop a coordinated strategy (Kong, Almond, Bayley, & Davis, 2016; Kong et al., 2013; Kong, Vijayakrishnan, et al., 2016) that might in principle allow us to address, through the use of internal glycans, some of the issues identified above that hamper synthetic antimicrobial vaccines. Our strategy adopted three principle arms. First, the use of glycoconjugates in which glycans were constructed through target synthesis and then attached via lysine-specific chemistries to a T-helper-recruitment

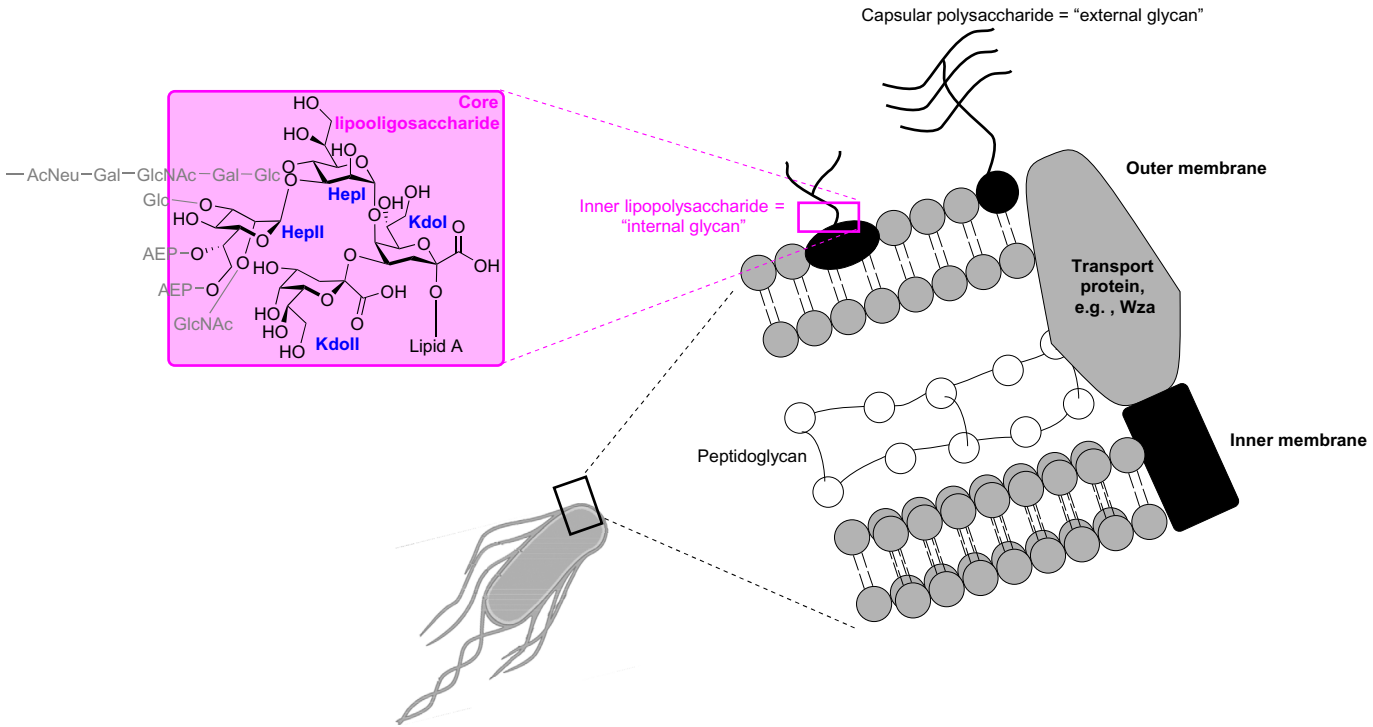

Fig. 1 Schematic of the Gram-negative bacterial cell wall. Typically, capsular polysaccharide (CPS), which is exported in type 1 pathway species by a protein complex that contains the Wza pore, “covers” lipopolysaccharide (LPS). The outer portions of the CPS and LPS are more exposed and have therefore been the typical but variable targets of glycoconjugate vaccines, despite their greater variability. The “inner glycan” considered here is the more buried portion of LPS that is just distal to the endotoxin causative agent lipid A; these glycans are typically much less variable in composition than “outer glycans.” The core tetrasaccharide Hep-Hep-Kdo-Kdo in the inner glycan, for example, is present in several pathogens including those that do and do not have extended O-polysaccharide (i.e., those that have LPS or just a shorter lipooligosaccharide, respectively).

protein carrier (Kong, Vijayakrishnan, et al., 2016), in our case a recombinantly expressed variant of diphtheria toxin (Pecetta et al., 2016). These methods, while novel and challenging synthetically, are not the primary focus of this chapter. Second, glycoconjugates bearing the internal Hep-Hep-Kdo-Kdo inner core tetrasaccharide were used in fairly standard immunization protocols to generate sera against this "universal" internal glycan motif. Third, vitally, sera were used synergistically in conjunction with blockers of capsular polysaccharide (CPS) export to allow enhanced access to these "internal glycan" epitopes. Together, therefore, these represent a proof-of-principle strategy that might imagine be applied in a two-stage process (Fig. 2): prior induction of antiinternal glycan responses through vaccination (of an "at-risk" cohort) and then, upon infection, blocking of the CPS through inhibition to allow access. It should be noted that, while we concentrate here on "internal glycan" targets from Gram-negative examples, at least in principle other pathogens can also be considered (e.g., by exploiting targets from within the lipoteichoic acids of Gram-positive bacteria). This chapter therefore focuses on three more novel aspects of methods associated with this strategy that we think may be of greatest value to the broader community: a strategy for single-molecule screening of blockers of external glycan display; testing of those blockers in bacteria; and testing of the synergistic application of sera and blockers.

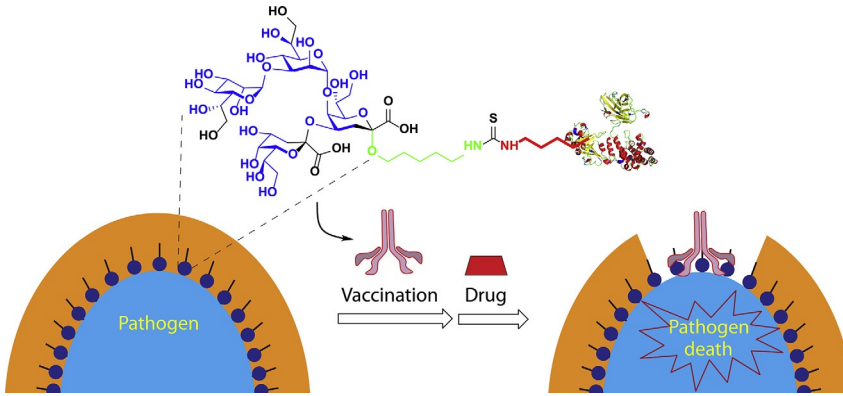

Fig. 2 Schematic of putative two-step therapeutic strategy for synergistic application of antiinternal glycan vaccines. Use of drugs that block display of "external glycans" (e.g., those that prevent display of capsular polysaccharide (CPS)) would enhance access to "internal glycans" and hence increase their viability as potential targets. *Taken from Kong, L., Vijayakrishnan, B., Kowarik, M., Park, J., Zakharova, A. N., Neiwert, L., … Davis, B. G. (2016). An antibacterial vaccination strategy based on a glycoconjugate containing the core lipopolysaccharide tetrasaccharide Hep2Kdo2.* Nature Chemistry, 8*(3), 242–249. http://dx.doi.org/10.1038/nchem.2432 with permission.*

2. SCREENING OF EXTERNAL GLYCAN DISPLAY BLOCKERS

The strategy for blocker screening described here is based on the inhibition of an *E. coli* group 1 pathway export protein, the homooctamer Wza (Dong et al., 2006; Nesper et al., 2003). The single-molecule assay described here (Kong et al., 2013) relies upon the reconstitution of Wza variants within an artificial lipid bilayer and the measurement of the current carried by ions that pass through the pore, which reflects the open or closed (blocked) state of the transporter. While the present protocol focuses on one particular variant, it could, in principle, be applied to a variety of relevant transporters such as group 1 pathway variants in other target organisms such as *Klebsiella*.

2.1 Expression of Wza

Plasmid DNA encoding the Wza gene (wild type (WT) and mutants) is subjected to in vitro transcription and translation (IVTT) using an *E. coli* T7-S30 extract system for circular DNA. Proteins expressed by IVTT undergo spontaneous oligomerization to form octameric Wza pores. The oligomers can be separated from the monomeric subunits by SDS-polyacrylamide gel electrophoresis providing sufficient material for single-channel electrical recording (Kong et al., 2013). Wza-Y110G and Wza-ΔP106-A107 are mutants, which have a more open pore structure that resembles the state of Wza during CPS transport (Collins et al., 2007).

2.1.1 Equipment

- ND100 Nanodrop spectrophotometer (Thermo Scientific) or any suitable spectrophotometer
- Criterion™ vertical Electrophoresis unit (Bio-Rad)
- Vacuum gel drier (Bio-Rad)
- X-ray film developer (Xograph Imaging Systems)
- Eppendorf Thermomixer (No. 5355 31077)
- Eppendorf Centrifuge 5417 R
- Whatman™ filter paper (Grade: 3 MM CHR, GE Healthcare)
- Gel Scanner (CanoScan LiDEF500)
- 0.2 μm Microfilterfuge tubes (Rainin)

2.1.2 Buffers and Reagents

- Plasmid DNAs encoding the Wza genes (WT or mutants) in a vector with a pT7 promoter (Kong et al., 2013)
- *E. coli* T7-S30 extract system for circular DNA (Promega, L1130)
- [^{35}S]Methionine (37.0 TBq/ mmol, concentration:10.25 mCi/mL, MP Biomedicals, UK)
- 4%–12% Bis-Tris precast gel (Criterion™ XT)
- Running buffer for SDS-PAGE: MOPS buffer (NuPAGE® MOPS) (50 m*M* MOPS, 50 m*M* Tris base, 0.1% SDS, 1 m*M* EDTA, pH 7.7)
- Precision Plus Protein™ Dual Color Standards (Bio-Rad)
- Autoradiography film: Criterion® Kodak® BioMax® MR Film (Sigma-Aldrich, Z350370)
- Rehydration buffer: 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.0
- Nuclease-free water
- Laemmli sample buffer for sample loading (Sigma-Aldrich)
- Fixer (Fixaplus 354) and developer (Devalax153) solutions for X-ray film (Champion Photochemistry)

2.1.3 Procedure

1. Measure the concentration of the plasmid DNA and prepare a stock of 100 ng/μL in nuclease-free water.
2. Add 20 μL S30 Premix, 15 μL T7 S30 extract, 5 μL amino acid mixture (1 m*M* minus Methionine), 2 μL [^{35}S]methionine, and 8 μL DNA to a 1.5 mL tube.
3. Gently mix by pipetting up and down.
4. Incubate at 37°C at 500 rpm for 3 h on a Thermomixer.
5. Centrifuge the reaction mixture at 25,000 × *g* for 10 min.
6. Resuspend the pellet in 12 μL 1 × Laemmli sample buffer and load the suspension on a 4%–12% Bis-Tris precast gel. Load the protein markers in the adjacent lane.
7. Dilute the 20 × MOPS running buffer to 1 × using deionized water.
8. Run the gel overnight using 1 × MOPS buffer at 35 V, 4°C.
9. Place the gel on a Whatman™ paper and dry under vacuum for 8 h.
10. Staple the dried gel to an autoradiography film in order to hold the film in place and expose the film for 1 h.
11. Cut or fold the right-hand corner of the film and gel to help align the gel after development of the film.

12. Develop the film using an X-ray film developer.
13. Align the developed film with the dried gel, and cut out the band corresponding to octameric Wza (~170 kDa based on the markers).
14. Transfer the filter paper to a 1.5 mL tube. Add 100 μL rehydration buffer (10 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.0) and incubate on ice for 20 min. Carefully remove the filter paper using tweezers and crush the gel pieces using a pestle.
15. Incubate on ice for a further 20 min and transfer the gel pieces to a microfilterfuge tube using a pipette.
16. Centrifuge at 25,000 × *g* for 40 min. The filtrate contains the octameric protein, which is aliquoted (15–20 μL) and stored at −80°C.

2.1.4 Notes

1. Samples are radioactive and proper use of dedicated space for handling radioactivity (with appropriate bench protection), and waste disposal in accordance with local regulations is important. Ingestion is the main hazard. The final Wza sample is only weakly radioactive, and in general, no precautions have been taken during the electrical recordings. It is possible to make nonradioactive protein, by using radiolabeled protein as a marker.
2. Samples are stable at −80°C for about 6–8 months.

2.2 Screening of Blockers

The WT and mutant Wza pores obtained by IVTT as described above are screened with blockers. For this purpose, single-channel recording (SCR) provides a method with high sensitivity, precision, and the ability to detect and distinguish individual blocking events occurring at subsecond timescales from which useful quantitative data may be derived, e.g., on and off rate constants and K_d values. A planar lipid bilayer is constructed by the Mueller–Montal approach (Maglia, Heron, Stoddart, Japrung, & Bayley, 2010; Montal & Mueller, 1972). Diluted Wza is added to the *cis* compartment of the SCR chamber so that the insertion of a single Wza pore into the bilayer (as judged by a current step) can be controlled. The pore may then be screened for current blockades by different compounds. Here two cyclodextrin derivatives, namely, $am_8\gamma CD$ and $am_7\beta CD$, which block mutant Wza pores (with K_d values of $13 \pm 2\,\mu M$, $2.9 \pm 1.8\,mM$, respectively), are illustrated (Fig. 3; Kong et al., 2013).

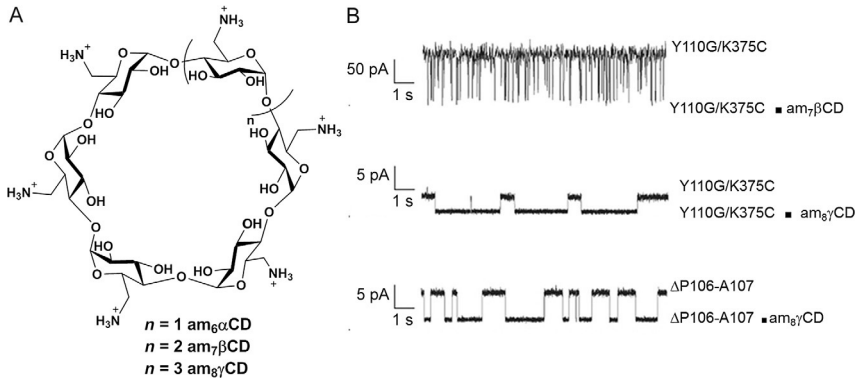

Fig. 3 Blocking of Wza. (A) Molecular structures of cyclodextrin derivatives used to block the Wza pore. (B) Representative single-channel recordings (SCR) showing current blockades of "open" Wza mutant pores by the cyclodextrins. Upper trace: Y110G/K375C: +75 mV, 2 *M* KCl, 200 μ*M* am7βCD; middle trace: Y110G/K375C: +3 mV, 2 *M* KCl, 5 μ*M* am8γCD; lower trace: ΔP106-A107: +3 mV, 2 *M* KCl, 5 μ*M* am8γCD. The blockers were added to the *trans* chamber in each of the recordings. *Taken from Kong, L., Harrington, L., Li, Q., Cheley, S., Davis, B. G., & Bayley, H. (2013). Single-molecule interrogation of a bacterial sugar transporter allows the discovery of an extracellular inhibitor.* Nature Chemistry, 5*(8), 651–659. http://dx.doi.org/10.1038/nchem.1695 with permission.*

2.2.1 Equipment

- Axopatch 200B patch-clamp amplifier (Molecular Devices) with 16-bit digitizer (Digidata 1440A, Axon Instruments)

2.2.2 Buffers and Reagents

- 20 μm PTFE film
- 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC; Avanti Polar Lipids)
- Hexadecane and pentane (Sigma-Aldrich)
- Cyclodextrins to be screened (Cyclolab Ltd., Sigma-Aldrich, Fluka, or through synthesis)
- Wza mutants expressed by IVTT
- Buffer for SCR: 2 *M* KCl, 5 m*M* HEPES, 100 μ*M* EDTA, pH 7.5 or 300 m*M* KCl, 5 m*M* HEPES, pH 7.5.

2.2.3 Procedure

1. Prepare a chamber for electrical recording and fresh Ag/AgCl electrodes by using the protocols described previously (Maglia et al., 2010).
2. Fill the compartments with buffer (2 *M* KCl, 5 m*M* HEPES, 100 μ*M* EDTA, pH 7.5) and form a planar lipid bilayer using the

hexadecane-DPhPC procedure following the Mueller–Montal method described previously (Maglia et al., 2010).

3. Add 1 μL Wza octamer stock (~200 μg/mL) to the *cis* compartment (grounded) of the chamber. Apply a voltage of +50 mV and stir the solution in the compartment to aid pore insertion. The insertion of a single Wza pore at +50 mV results in a current step of +88 ± 10 pA for Wza mutant Y110G/K375C and +130 ± 1 pA for the ΔP106-A107 mutant. The WT Wza showed a current of only +0.95 ± 0.14 pA (Kong et al., 2013).
4. Add the blocker (cyclodextrin) to the *trans* chamber, apply a voltage of +75 mV, and record the current over 30 min. Amplify the current with a patch-clamp amplifier (Axopatch 200B) using the internal low-pass Bessel filter (80 db/decade) with a corner frequency of 1 kHz.
5. Record current changes at different voltages (+3, +5, +10 mV) after the addition of a blocker to probe voltage-dependent binding as well as the kinetics of binding.
6. Analyze the data using Clampfit version 10.3 software to determine the mean dwell times of the cyclodextrin bound to the Wza pore (τ_{off}) and the mean interevent intervals (time between blockades, τ_{on}).
7. Recordings at different concentrations of the inhibitors allow the calculation of dissociation constants (K_d) with the following relationship (Gu, Braha, Conlan, Cheley, & Bayley, 1999).

$$\mathrm{P.CD} \underset{k_{on}}{\overset{k_{off}}{\rightleftarrows}} \mathrm{P} + \mathrm{CD}$$

where *P* represents the Wza pore and CD the cyclodextrin inhibitor

$$\frac{\tau_{on}}{\tau_{off}} \times [\mathrm{CD}] = \frac{[\mathrm{P}]}{[\mathrm{P.CD}]} \times [\mathrm{CD}] = \frac{[\mathrm{P}] \times [\mathrm{CD}]}{[\mathrm{P.CD}]}$$

$$\therefore k_{on} = \frac{1}{\tau_{on} \times [\mathrm{CD}]} \Rightarrow \tau_{on} = \frac{1}{k_{on} \times [\mathrm{CD}]}; \quad k_{off} = \frac{1}{\tau_{off}}$$

$$\therefore K_d = \frac{k_{off}}{k_{on}}$$

2.2.4 Notes

1. In our initial screening, the cyclodextrin derivative $am_8\gamma CD$ emerged as the strongest blocker of Wza (Kong et al., 2013) and was selected for the studies of synergism with antisera described below.

3. BLOCKING OF POLYSACCHARIDE TRANSPORT

The single-molecule screening approach described above provides a convenient platform for evaluation of blocker/pore combinations that could, in principle, be applied to any cell-wall glycan transport protein. Variation of target-protein sequence and blocker structure would allow structure–activity relationships to be determined, useful in the heuristic design of blocker variants, and the analysis of the effect of sequence variation from species to species. For an analysis of the sequence similarity of Wza-like pores and likely critical interacting residues, see Kong et al. (2013).

These methods use current flow mediated by buffer ions as a proxy for the restricted state of the pore; lowered current in the presence of blocker indicates action of the blocker. However, it should be noted that, as described, this assay is not directly measuring CPS transport but the state of the pore that mediates this transport. Methods for the measurement of the direct interactions of synthetic CPS fragments with Wza have recently been developed (Kong, Almond, et al., 2016) and may provide another mode of analysis of such blockers (e.g., in competition with substrate fragment competitors) not described here.

Altered CPS transport can also be assessed through extraction and quantification of glycans from treated bacteria. Since the method considers glycans in context, this extraction–quantification approach is usefully complementary to SCR methods and perhaps more closely linked to the likely success of any synergistic approach that would be based on increased accessibility. The method described here (Kong et al., 2013) provides a dose–response curve for CPS expression, which adapts a readily applied semipurification and "staining" procedure (Chakraborty, Friebolin, & Stirm, 1980; Hungerer, Jann, Jann, ØRskov, & ØRskov, 1967). While again this focuses on CPS, application to other "outer glycans" can be envisaged using similar principles.

Here, the inhibitory effects of $am_8\gamma CD$ as a blocker for extracellular transport are illustrated with the example of *E. coli* K30 CPS (K30-CPS). $am_6\alpha CD$ and $am_7\beta CD$, less potent inhibitors, are used as comparators.

3.1 Isolation of Cell-Wall Polysaccharide From Treated Bacteria

The quantification of capsular polysaccharides (CPS), and LPS, from bacteria treated with blockers and inhibitors, allows a measure of the export of polysaccharide to the cell surface. An extended isolation and purification of CPS has been described previously (Chakraborty et al., 1980;

Hungerer et al., 1967). The modified protocol (Kong et al., 2013) described below is an adaptation that allows more rapid isolation and hence assessment of both LPS and CPS content.

3.1.1 Equipment

- Eppendorf Centrifuge 5417 R
- Novaspec III Visible Spectrophotometer (Amersham Biosciences)
- Vortex mixer (Vortex Genie, Scientific Industries, SI-0136)
- 37°C incubator (Thermo Scientific Heraeus B6030)
- Eppendorf Thermomixer (No. 5355 31077)

3.1.2 Buffers and Reagents

- *E. coli* strains: E69 (contains Wza transporter, and expresses CPS and O9a LPS), CWG281 (Wza null, O9a LPS positive), LE392 (no surface polysaccharide layers)
- Resuspension buffer: 20 m*M* Tris–HCl, 2 m*M* $MgCl_2$, 20 m*M* NaCl, pH 8.0
- Buffer-equilibrated phenol (Sigma-Aldrich)
- M9 medium for bacterial culture (Sigma-Aldrich)
- Benzonase (250 U/μL) (Sigma-Aldrich)
- Proteinase K (10 mg/mL) (Thermofisher)
- Hexakis (6-amino-6-deoxy)-α-cyclodextrin hydrochloride $am_6\alpha CD$, heptakis (6-amino-6-deoxy)-β-cyclodextrin $am_7\beta CD$, octakis (6-amino-6-deoxy)-γ-cyclodextrin $am_8\gamma CD$ (Cyclolab Ltd.)

3.1.3 Procedure

1. Grow *E. coli* strains E69, CWG281, and LE392 in 10 mL M9 medium at 37°C for approximately 9 h on a shaking incubator until an OD_{600} of 0.5 is reached.
2. Adjust the OD_{600} to 0.4 by dilution with M9 medium and add 10 μL aliquots to 10 mL M9 media in 50 mL Falcon tubes.
3. Add a blocker to each tube at the required concentration (e.g., from 20 to 100 μ*M*).
4. Incubate the cells at 37°C on a shaking incubator for another 9 h until the OD_{600} is ~0.5. Adjust the OD to 0.4 by dilution with M9 medium. Transfer 600 μL to a 1.5 mL tube.
5. Harvest the cells by centrifugation at 3800 × *g*, 20 min, 4°C.
6. Resuspend the pellet in 750 μL resuspension buffer and add 750 μL buffer-equilibrated phenol.
7. Mix the solution using a vortex mixer and incubate at 65°C for 10 min.

8. Centrifuge the solution at $1000 \times g$ for 5 min at 4°C
9. Transfer the aqueous phase to a separate 1.5 mL tube and add 1 μL benzonase (250 U/μL) to each sample. Mix well by pipetting and incubate at 37°C for 2 h.
10. Add 2 μL (10 mg/mL) of Proteinase K to the above solution and incubate at 37°C for 16 h.

3.1.4 Notes

1. The use of benzonase and Proteinase K in the above protocol is intended to ensure that the remaining high molecular weight molecules (>30 kDa) in the solution are polysaccharides.
2. Benzonase replaces conventional DNase and RNase enzymes to digest all nucleic acids.
3. Solutions obtained after phenol extraction are stored at −80°C if they are not taken forward for enzymatic treatment immediately.

3.2 Gel Electrophoresis and Staining of Cell-Surface Polysaccharides

In order to distinguish between LPS and CPS and to compare the inhibitory effects of cyclodextrin derivatives, the polysaccharides obtained in Section 3.1 are subjected to SDS-PAGE followed by staining of carbohydrates. Carbohydrate staining is carried out in two steps (Kong et al., 2013): oxidation of the polysaccharides using periodate, followed by treatment with Pro-Q Emerald 300 glycoprotein stain (Molecular Probes). This fluorescence technique allows CPS and LPS to be distinguished based on their molecular weight difference. Image analysis provides a method to quantify the amount of each polysaccharide in each sample (Fig. 4).

3.2.1 Equipment

- SDS-PAGE electrophoresis unit (Bio-Rad)
- Gel documentation system (Gel Doc XR, Bio-Rad)
- Rocking shaker (Stuart Gyrorocker SSL3)

3.2.2 Reagents

- 10% Bis-Tris Precast gel (Criterion™ XT)
- Running buffer for SDS-PAGE: MOPS buffer (NuPAGE® MOPS) (50 m*M* MOPS, 50 m*M* Tris base, 0.1% SDS, 1 m*M* EDTA, pH 7.7)
- Precision Plus Protein™ Dual Color Standard (Bio-Rad)
- Laemmli sample-loading buffer (Bio-Rad)
- Pro-Q Emerald 300 Glycoprotein stain kit (Molecular Probes)

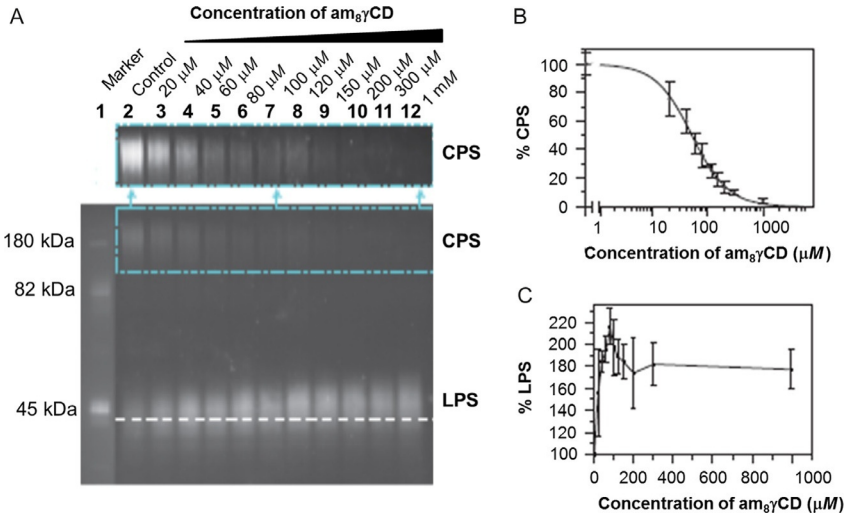

Fig. 4 Carbohydrate staining of CPS and LPS isolated from *E. coli* E69 treated with $am_8\gamma CD$. (A) SDS-polyacrylamide gel stained with the fluorescent aldehyde-reactive stain (Pro-Q Emerald 300) following periodate treatment. (B and C) Analysis of fluorescence intensities obtained from the gel as a function of the concentration of the Wza inhibitor $am_8\gamma CD$. *Taken from Kong, L., Harrington, L., Li, Q., Cheley, S., Davis, B. G., & Bayley, H. (2013). Single-molecule interrogation of a bacterial sugar transporter allows the discovery of an extracellular inhibitor.* Nature Chemistry, *5(8), 651–659. http://dx.doi.org/10.1038/nchem.1695 with permission.*

- Wash solution: 3% acetic acid in dH_2O (250 mL)
- Fixing solution: 50% methanol and 5% acetic acid in dH_2O
- Oxidation solution: 3% acetic acid in water (250 mL) added to 2.5 g of periodic acid (10 mg/mL) (e.g., from the Pro-Q Emerald 300 Glycoprotein kit, Molecular Probes)

3.2.3 Procedure

1. Add 3 μL of 2 × Laemmli sample-loading buffer to 3 μL of polysaccharide sample (isolated as described in Section 3.1.3) and load on a 10% Bis-Tris Precast gel.
2. Run the gel at 200 V at room temperature for 55 min.
3. Transfer the gel to a tray, add 100 mL fixing solution, and incubate on a rocking shaker at 50 rpm for 45 min at room temperature.
4. Wash the gel using 100 mL wash solution and incubate on rocking shaker at 50 rpm for 45 min at room temperature. The washing step is repeated twice.

5. Add 25 mL oxidation solution to the gel and incubate at room temperature for 30 min.
6. Wash the gel twice for 15 min with 3% acetic acid in water.
7. Incubate the gel in 25 mL Pro-Q Emerald 300 stain for 1 h in the dark.
8. Image the gel using a Gel Documentation system using a UV source with an excitation wavelength of 302 nm and a fluorescence emission band pass filter of 548–630 nm.
9. Analyze the image using Bio-Rad Quantity One software to obtain the relative fluorescence intensities of the bands.
10. Normalize the intensities to fluorescence values obtained from cells without any inhibitor treatment, i.e., control cells (Fig. 4B and C).

3.2.4 Notes

1. The IC_{50} value of $am_8\gamma CD$ obtained by the analysis of fluorescence intensities is $51.2 \pm 1.0\,\mu M$.
2. An interesting observation of this analysis is that the LPS level increases with decreasing CPS levels (Fig. 4C). The greater LPS exposure and, indeed, apparently increased content provide an enhanced internal glycan "target."

4. SYNERGISTIC ACTION OF SERA AND BLOCKER

Binding of $am_8\gamma CD$ to Wza results in decreased extracellular export of K30 polysaccharide, which in turn results in enhanced exposure of O9a LPS as seen by carbohydrate staining (Fig. 4A). Cells with increased LPS exposure are hence rendered prone to attack or binding by antibodies (either in sera as part of the synergistic strategy shown in Fig. 2 or those used as reagents to measure O-antigen exposure). Flow cytometry (FACS) provides a ready method that can be used, for example, to analyze the binding of fluorescently labeled O9a-LPS-specific antibodies (Clarke, Cuthbertson, & Whitfield, 2004) and in principle could be used for any antiinternal glycan antibodies with suitable secondary antibodies or other methods for labeling. This allows quantitation of the amount of exposed LPS as a function of inhibitor concentration and could be used as a complementary assay to those used to estimate external glycan reduction as described above.

Various synergistic "killing" assays can be envisaged. Here we describe the action of rabbit sera (9-week-old New Zealand white rabbits, 87-day terminal bleed) raised after vaccination with mutant diphtheria toxin-Hep-Hep-Kdo-Kdo conjugate ($(Hep_2Kdo_2)_4$-DT) (Kong, Vijayakrishnan, et al., 2016). Prior

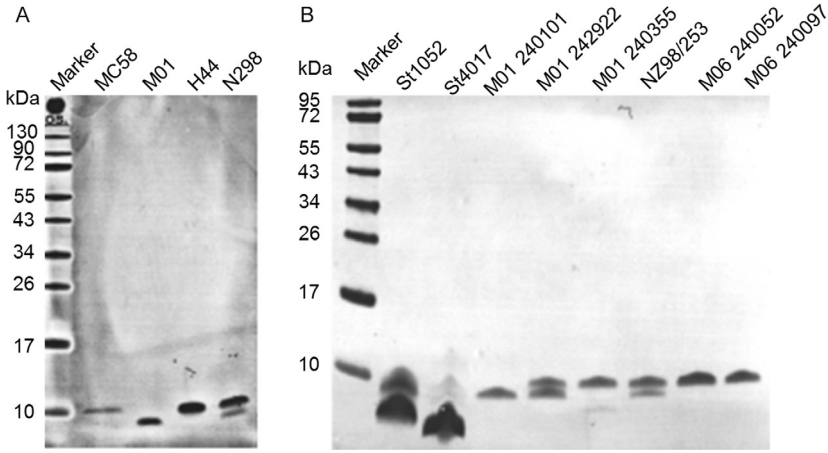

Fig. 5 Western blot analysis of carbohydrate extracts from various bacterial strains to probe internal glycan recognition by antisera obtained upon immunization (Kong, Vijayakrishnan, et al., 2016) (A) *N. meningitides* strains MC58, M01, H44, and N298. (B) *N. meningitides* strains M01 240101, M00 242922, M01 240355, NZ98/254, M06 240052, M06 240097, *E. coli* strains St1052, and *P. aeruginosa* strain St4017. *Taken from Kong, L., Vijayakrishnan, B., Kowarik, M., Park, J., Zakharova, A. N., Neiwert, L., … Davis, B. G. (2016). An antibacterial vaccination strategy based on a glycoconjugate containing the core lipopolysaccharide tetrasaccharide Hep2Kdo2.* Nature Chemistry, 8*(3), 242–249. http://dx.doi.org/10.1038/nchem.2432 with permission.*

to use in synergistic killing, antigen-binding capacity may be tested using standard ELISA methods and through Western blot-type methods against cell-wall isolates (Fig. 5).

4.1 Isolation of Glycans From Different Pathogenic Bacteria

The sera generated may be tested for their cross-reactive ability to bind, for example, LPS isolated from various pathogenic bacterial strains, viz., *Neisseria meningitidis*, *E. coli*, and *Pseudomonas aeruginosa*. Here these were used to test the binding of anti-$Hep_2(Kdo_2)_4$-DT serum by Western blot to isolated glycan cell-wall components (Fig. 5).

4.1.1 Equipment

- Innova 44 Incubator Shaker
- Eppendorf Centrifuge 5417 R
- Novaspec III Visible Spectrophotometer (Amersham Biosciences)

- Vortex mixer (Vortex Genie, Scientific Industries, SI-0136)
- 37°C incubator (Thermo Scientific Heraeus B6030)
- Eppendorf Thermomixer (No. 5355 31077)

4.1.2 Reagents and Buffers

- Pathogens: e.g., *N. meningitidis*, *E coli*, and *P. aeruginosa* (various strains, here *E. coli* E69, CWG281, and LE392)
- Resuspension buffer: 20 m*M* Tris–HCl, 2 m*M* $MgCl_2$, 20 m*M* NaCl, pH 8.0
- M9 medium for bacterial culture (Sigma-Aldrich)
- Proteinase K (10 mg/mL)
- Laemmli sample-loading buffer (Bio-Rad)

4.1.3 Procedure

1. Grow strains (here *E. coli* E69, CWG281, and LE392) in 10 mL M9 medium at 37°C for approximately 9 h on a shaking incubator until an OD_{600} of 0.5 is reached.
2. Adjust the OD_{600} to 0.4 by dilution and add 10 μL aliquots to 10 mL M9 medium in 50 mL Falcon tubes.
3. Incubate the cells at 37°C on a shaking incubator for another 9 h until OD_{600} is 2.
4. Harvest cells by centrifugation at 3800 × *g*, 20 min, 4°C.
5. Resuspend the pellet in 100 μL Laemmli sample buffer and incubate at 95°C for 10 min.
6. Add 2 μL Proteinase K solution, mix well using vortex mixer, and incubate at 60°C for 1 h.

4.2 Testing Effect of Antiserum on Glycans Isolated From Different Pathogenic Bacteria

4.2.1 Equipment

- iBlot® gel transfer device (Invitrogen)
- SDS-PAGE electrophoresis unit (Bio-Rad)
- Scanner for scanning gel (CanoScan LiDEF500)

4.2.2 Reagents and Buffers

- 4%–12% Bis-Tris precast gel (Criterion™ XT)
- Running buffer for SDS-PAGE: MOPS buffer (NuPAGE® MOPS) (50 m*M* MOPS, 50 m*M* Tris base, 0.1% SDS, 1 m*M* EDTA, pH 7.7)

- Precision Plus Protein™ Dual Color Standard (Bio-Rad)
- Laemmli sample-loading buffer (Bio-Rad)
- BCIP/NBT® Liquid Substrate (Sigma-Aldrich)
- iBlot® gel transfer stacks and nitrocellulose membrane (Invitrogen)
- Blocking solution: 3% BSA in phosphate-buffered saline (PBS)
- PBS (137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, pH 7.4)
- PBS-T buffer (137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, 0.05% Tween 20)
- Secondary antibody: alkaline phosphatase conjugated goat antirabbit immunoglobin G (IgG)F(ab′)$_2$ (Sigma-Aldrich, SAB3700833)

4.2.3 Procedure

1. Load the samples (after Proteinase K treatment) onto a 4%–12% Bis-Tris precast gel (Criterion™ XT).
2. Run at 200 mV using 1 × MOPS buffer for 50 min.
3. Transfer the gel to a PVDF membrane at room temperature, with the iBlot® transfer stack using an iBlot® transfer device.
4. Add 40 mL blocking solution and incubate the blot at room temperature for 2 h.
5. Wash the blot using 20 mL PBS-T. Repeat the wash step three times.
6. Prepare an antiserum dilution of 1:50 in PBS-T and incubate the blot in 40 mL of this solution for 3 h.
7. Wash the blot using PBS-T three times.
8. Add secondary antibody at a 1:10,000 dilution in PBS-T (40 mL) and incubate at room temperature for 2 h.
9. Wash the blot with PBS-T three times.
10. Add BCIP/NBT® Liquid substrate (Sigma-Aldrich) to the blot such that the entire blot is covered with the substrate.
11. Wait for 10 min to see band development. Once bands are visualized, place the blot in water to stop the substrate reaction.
12. Image the blot using a scanner.

4.3 Synergistic Survival Assay

Treatment of bacterial cells with $am_8\gamma CD$, an identified blocker, results in an enhanced exposure of LPS (Kong et al., 2013). The "dual action" of sera and blocker can be assessed through the adapted survival assay described here (Fig. 6).

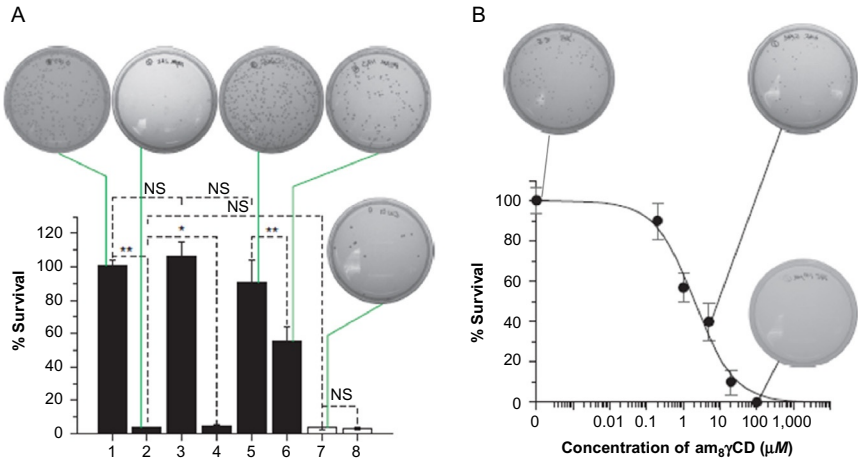

Fig. 6 Inhibition of bacterial growth in the presence of $am_8\gamma CD$ and antisera. (A) Column 1: E69, Hep_2Kdo_2 antiserum, no $am_8\gamma CD$; column 2: E69, Hep_2Kdo_2 antiserum, 100 μ*M* $am_8\gamma CD$; column 3: E69, Kdo_2 antiserum, no $am_8\gamma CD$; column 4: E69, Kdo_2 antiserum, 100 μ*M* $am_8\gamma CD$; column 5: E69 NRS, no $am_8\gamma CD$; column 6: E69 NRS, 100 μ*M* $am_8\gamma CD$; column 7: CWG281, Hep_2Kdo_2 antiserum, no $am_8\gamma CD$; column 8: CWG281, Hep_2Kdo_2 antiserum, 100 μ*M* $am_8\gamma CD$. (B) Determination of IC_{50} values of am8γCD in the presence of 35% anti-DT-$(Hep_2Kdo_2)_4$ antiserum. *Taken from Kong, L., Vijayakrishnan, B., Kowarik, M., Park, J., Zakharova, A. N., Neiwert, L., … Davis, B. G. (2016). An antibacterial vaccination strategy based on a glycoconjugate containing the core lipopolysaccharide tetrasaccharide Hep2Kdo2.* Nature Chemistry, 8*(3), 242–249. http://dx.doi.org/10.1038/nchem.2432 with permission.*

4.3.1 Equipment

- Innova 44 Incubator shaker
- 37°C incubator (Thermo Scientific Heraeus B6030)

4.3.2 Reagents and Buffers

- Antisera generated by immunization
- Normal rabbit serum (NRS)
- $am_8\gamma CD$ (Cyclolab Ltd.)
- RPMI medium (Sigma-Aldrich)
- LB agar plates

4.3.3 Procedure

1. Add 1 mL 10^3 *E. coli* cells (E69 or CWG281) to a 1.5 mL tube containing 100 μL RPMI medium supplemented with 25% (v/v) serum (antiserum or NRS).

2. Add am8γCD (0 or 100 μ*M*) to each tube and incubate at 37°C for 4 h to allow bacterial growth.
3. Spread 20 μL of the culture on LB agar plates and incubate the plates at 37°C for 10 h.
4. Count the number of colonies obtained after incubation and determine the % survival.
5. To obtain IC_{50} values, culture 10^3 E69 cells in RPMI medium in the presence of 35% antiserum.
6. Add increasing concentrations of am8γCD (0 n*M* to 100 μ*M*) and incubate the cultures at 37°C for 4 h.
7. Plate 20 μL of the culture on LB agar plates and incubate at 37°C for 4 h.
8. Count the number of colonies and plot a graph of % survival vs concentration (Fig. 6B).

4.3.4 Notes

1. The IC_{50} value of am8γCD in the presence of 35% Hep_2Kdo_2 antiserum = 2.0 ± 0.4 μ*M*.

5. SUMMARY AND CONCLUSIONS

Examples of antiinternal glycan strategies are limited and we describe here just one approach with a set of initial methods focused on the target/model organism of *E. coli* and the associated Wza transport protein system. Notably, sera raised through the use of antiinternal glycan glycoconjugate vaccines show cross-reactivity to other pathogen cell-wall glycans (Kong, Vijayakrishnan, et al., 2016) consistent with other examples of cross-reactivity shown by an antiinternal glycan antibody (Muller-Loennies et al., 2007). The primary caveat is therefore that, while aspects of this system could be (and are being) readily adapted to investigate similar internal glycans in other organisms, each will require adaptation of the three components that we have given example methods for here. The single-molecule methods used here are flexible and allow, for example, ready adaptation to sequence changes (from species variation or those that might emerge in response to treatment) by simply expressing the corresponding variant channels using IVTT. The cell-wall staining methods for outer glycans are, in principle, generic and so can be extended to other species provided electrophoresis allows separation and correct identification to then allow quantification and, hence, dose–response analyses. Finally, the synergistic use of sera with blockers can be explored through a variety of "killing"

assays; we describe here just one bespoke method, that is, in part, dictated by immunization protocols (i.e., the use of rabbit sera). A fuller evaluation of synergy would, of course, require immunization in patients.

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