Glycoarrays

DOI: 10.1002/anie.201309436

Discovery of Inhibitors of Shiga Toxin Type 2 by On-Plate Generation and Screening of a Focused Compound Library**

*Somnath Dasgupta, Pavel I. Kitov, Joanna M. Sadowska, and David R. Bundle**

Abstract: *A new microtiter-plate-based method for the rapid generation and evaluation of focused compound libraries was developed and applied to screening ligand analogues for the E. coli Shiga-like toxin Stx2a. The method is general, it mitigates the masking of intrinsic affinity gains by multivalency and enables the discovery of potential hits when starting from ligands that exhibit extremely low affinity with proteins that depend on multivalency for their function.*

Enterohemorrhagic *Escherichia coli* (EHEC), predominantly serotypes O157:H7 and O104:H4, are pathogens that have caused mass infections as a result of contamination of food or water supplies.[1] Shiga toxins type 1 and type 2 (Stx1 and Stx2) are major virulence factors of EHEC, which is the leading cause of acute pediatric kidney failure resulting from life threatening hemolytic uremic syndrome (HUS).[2] Expression of Stx is inducible by antibiotics,[3] hence supportive care is the only option available for the treatment of HUS.[4] New means of controlling EHEC infections and treating HUS remain a high priority. Targeting virulence factors rather than the bacteria themselves offers a number of advantages. Since the toxins enter the circulation and continue to inflict damage even after the bacteria have been killed and cleared from the host, detoxification would be beneficial in any therapy scenario. Nonlethal antimicrobial agents circumvent the problem of disruption of gut microbiota that typically accompanies antibiotic treatment. Such agents are also much less likely to induce drug resistance, one of the most important challenges of the 21st century.[5]

Shiga toxins are AB_5-type toxins. Five binding subunits (B_5) mediate toxin binding to cell-surface receptors and subsequent endocytosis, thereby enabling the active holotoxin, which contains an enzymatic domain (A), to disrupt cellular homeostasis.[6] To reach their intracellular target, Shiga toxins must be translocated to the cytosol through receptor-mediated retrograde endocytosis.[7] Given that in this study, the toxin is the larger binding partner and accommodates a smaller carbohydrate entity, the former will be referred to as the receptor and the latter as the ligand.

[*] Dr. S. Dasgupta, Dr. P. I. Kitov, J. M. Sadowska, Prof. Dr. D. R. Bundle
Alberta Glycomics Centre, Department of Chemistry
University of Alberta, Edmonton, Alberta T6G 2G2 (Canada)
E-mail: david.bundle@ualberta.ca
Homepage: http://www.chem.ualberta.ca/~glyco/who/index.htm

[**] This work was supported by a grant from Alberta Innovates, Technology Futures.

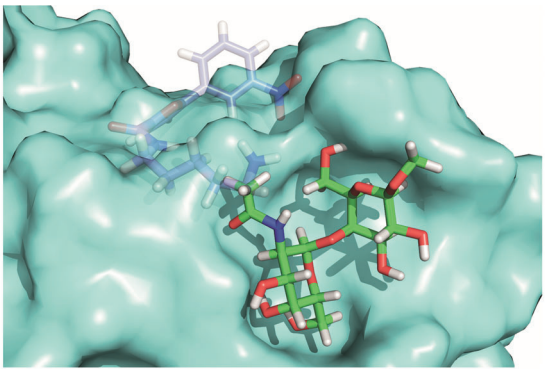
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201309436.

The glycolipid Gb_3, which contains the P^k-trisaccharide [αGal(1-4)βGal(1-4)βGlc(1-*O*)], is the ligand for Stx in humans that mediates toxin entry into the cell.[8] Despite low intrinsic affinity ($K_d \approx 1$–2 mM) for its native ligand,[9] Shiga toxins are successful in binding to the cell surface at sub-nanomolar concentrations owing to a multivalent mode of interaction, which amplifies their activity by several orders of magnitude. The most successful Shiga toxin antagonists to date have been multivalent molecules capable of simultaneously engaging several binding sites of the pentameric receptor.[10,11]

Epidemiological studies suggest that Stx2a-producing *E. coli* are associated with the development of HUS. In mice, Stx2a has an LD_{50} 400-fold lower than Stx1 and causes extensive renal pathology.[12] In primates, Stx2a but not Stx1 causes HUS.[13] We have reported potent Stx1 inhibitors that are effective in vivo[10] but inhibition of Stx2a remains elusive. Stx2a binds with lower avidity to the P^k-trisaccharide than Stx1 and it was recently discovered that it has affinity for several *E. coli* lipopolysaccharide (LPS) serotypes.[14] The structure of these LPSs resembles the galabiose portion of the P^k-trisaccharide with the galactose moieties replaced by *N*-acetyl-galactosamine. Synthetic analogues of the P^k-trisaccharide and galabiose with an *N*-acetyl group at the 2′ and 2″ positions were also found to bind Stx2a.[15]

The crystal structure of Stx2a bound to disaccharide αGalNAc(1-4)βGalOMe[16] shows that the *N*-acetyl carbonyl oxygen atom is involved in a hydrogen bond while the methyl group points outward (Figure 1). This suggested an opportunity to enhance the interaction between Stx2a and the ligand by replacement of this methyl group with a variable fragment.

To identify affinity gains in the presence of the multivalent interactions that are crucial for a toxin capture assay, we developed an iterative approach that employs polyvalent ligands with successively reduced ligand density. This idea was put into practice by using the polymer copovidone to display the ligands. Microtiter plates coated with a glycoconjugate based on poly(*N*-vinyl-2-pyrrolidone-*co*-vinyl alcohol) (poly-(NVP-*co*-VA); copovidone) remain fully active through the multiple cycles of harsh treatments (1M H_3PO_4) and washing steps required for conducting repeated solid-phase assay.[17] Here, we take advantage of this finding and extend the scope of synthetic manipulations that can be performed on an immobilized ligand followed by evaluation of activity on the same plate. Figure 2 outlines the principle scheme of the experiment.[18] The first iteration was performed at ligand density level 1 to give the **L1** polymers (payload 4.8%), the second at density level 2 to give the **L2** polymers (payload 1.2%), and the third at density level 3 to give the **L3** polymers



1 PkNAc (R=CH_3)

Figure 1. Crystal structure of Stx2a in complex with disaccharide **1** suggests a possibility for additional interactions when a variable fragment replaces the acetamido methyl group in the 2″-*N*-acetyl P^k-trisaccharide analogue (P^kNAc). The variable fragment modifications are illustrated as transparent additions.

(payload 0.4%). Three polymeric conjugates containing different amounts of the precursor trisaccharide for the compound library were obtained by Huisgen 1,3-dipolar copper(I)-catalyzed cycloaddition reaction with an azide-presenting polymer **3** followed by deprotection of the Boc-protected amino group. The coating reagent with the highest level of trisaccharide (**L1**, payload 4.8%) was prepared by condensation of alkyne **2** and azide **3**, while preparation of those with lower levels (**L2** and **L3**, payload 1.2% and 0.4% respectively) required the addition of propargyl alcohol as a diluent. Payload is expressed as percentage of modified monomers with respect to the total number of monomers, NVP and VA combined. It was calculated as previously described using integration of resonances in the ^{1}H NMR spectra for ligand and modified monomers compared to those of unmodified monomers.[17] Microtiter plates were coated by overnight incubation with the conjugated polymers (**L1**, **L2**, or **L3**, 10 μg mL^{-1} in phosphate-buffered saline).

The binding assay was conducted in a manner analogous to that described for Stx1.[17] Briefly, Stx2a[19] was added to the microtiter plate and incubated for 1 h, and then bound Stx2a was revealed by using an antiStx2a monoclonal antibody.[18] A binding assay conducted on plates coated with **L1** with unmodified amine (R = NH_2 Figure 2) revealed no interaction with Stx2a, while N-acetylation of the plate with acetic anhydride in methanol prior to ELISA allowed us to obtain

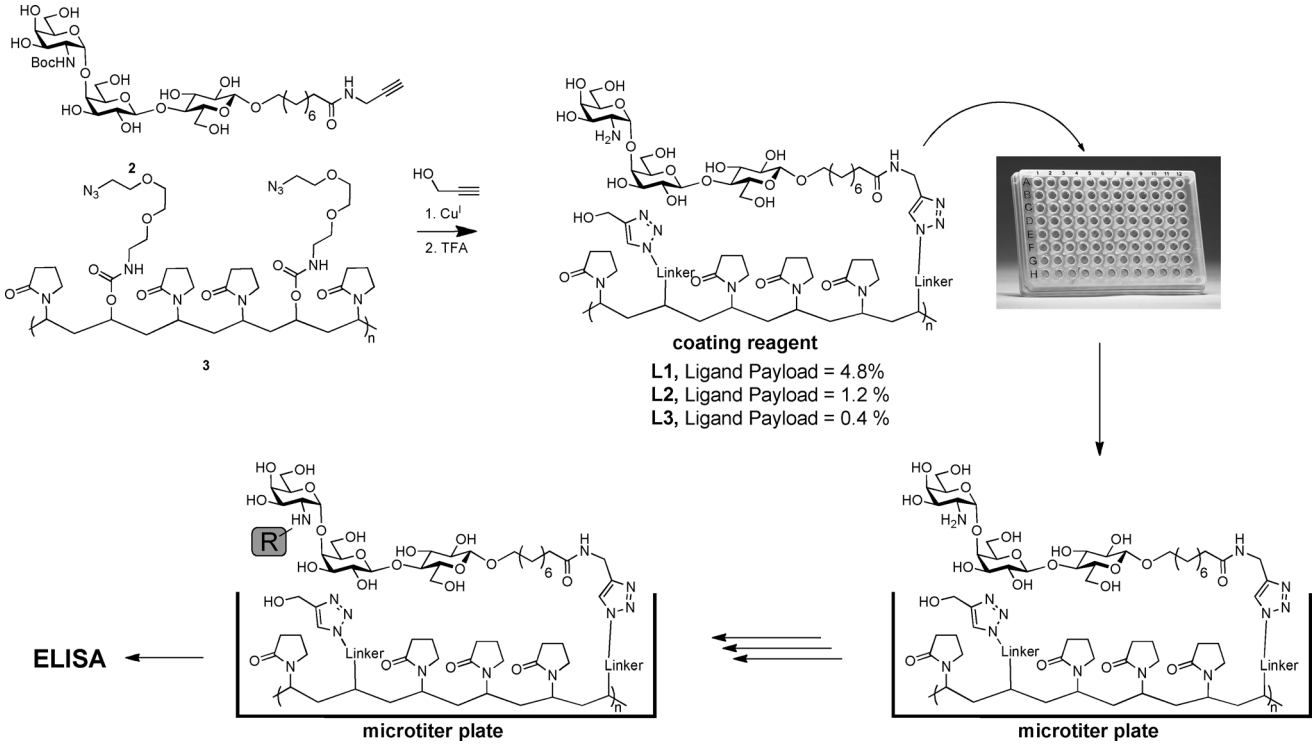
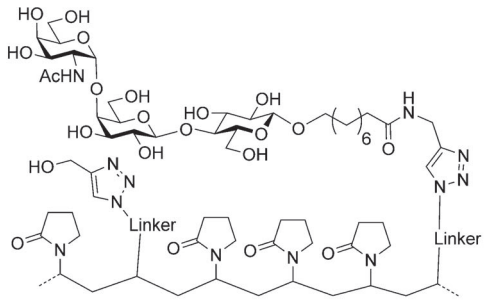


Figure 2. Variation of oligosaccharide ligand density on copovidone polymer using propargyl alcohol as a capping agent, deposition of this coating reagent onto ELISA plates, and in situ derivatization of the reactive amine group followed by ELISA evaluation of its binding affinity to Stx2a is schematically represented. The refined density variant ELISA approach identifies higher avidity polymers and hence improved monovalent ligands compared to the P^kNAc and P^k trisaccharides. For polymer **3** and the resulting conjugates, representative sections of the polymers are shown. Boc = *tert*-butoxycarbonyl, TFA = trifluoroacetic acid, ELISA = enzyme-linked immunosorbent assay.



L1Ac*, Payload = 4.4%
L1Ac, Payload = 4.8%
L2Ac, Payload = 1.2 %
L3Ac, Payload = 0.4 %

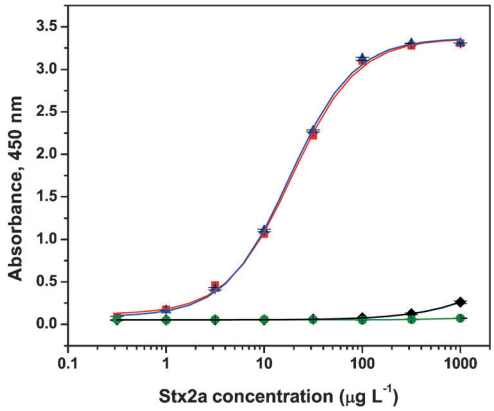


Figure 3. Binding of Stx2a to polystyrene microtiter plates coated with: **L1Ac*** (acetylated in bulk solution prior to coating; red ■), **L1Ac** (acetylated on the plate; blue ▲), **L2Ac** (acetylated on the plate; black ◆), **L3Ac** (acetylated on the plate; green ●). Assays were conducted in triplicate with error bars indicated.

a binding curve with complete saturation (Figure 3), thus confirming that 2″-*N*-acetyl-P^k-trisaccharide is a functional ligand for Stx2a. Stx2a showed identical binding to the plate coated with **L1Ac**, which was acetylated with acetic anhydride on the plate, and to an analogous polymer **L1Ac***, in which the ligand was converted to *N*-acetate prior to conjugation to the polymer and coating. After both negative and positive controls were established, we conducted a series of preliminary experiments to optimize the reaction conditions by using a variety of acylating reagents. We found that although some organic solvents (e.g., dichloromethane, dimethylformamide) are incompatible with the polystyrene plates, the acylation of the amino group can be achieved by activation of a carboxylic acid through using most of the common coupling reagents, such as EDC or HBTU–HOBt, and adding the mixture to the well in aqueous or methanolic solutions depending on the solubility of the reagent. Given that a large variety of commercial acyl chlorides are available, they can be conveniently utilized in this screening protocol through prior conversion into *N*-hydroxysuccinimide (NHS) esters.

Direct reaction of chloroformates and sulfonyl chlorides with amines is a common method for the generation of carbamates and sulfonamides, respectively. However, most of these reagents were found to be incompatible with standard polystyrene microtiter plates; they caused etching or discoloration of the wells, thereby rendering them unsuitable for ELISA. We found that, in addition to showing superior resistance to organic solvents, polypropylene microtiter plates support stable coating with polymers **L1**–**L3**, which permitted us to test a number of carbamate and sulfonamide derivatives.

The ligands modified with various acyl, carbamate, or sulfonamide groups that were tried in the first round of screening at high ligand density (**L1** polymers) showed a range of activities, mostly lower than the positive trisaccharide control P^{k}NAc. Comparison of the titration curves for the active ligands obtained by serial dilution of Stx2a did not permit clear discrimination of their activities. The lack of differentiation can be explained by the multivalency effect. Each of the five binding subunits in Stx1 has three distinct binding sites for its cognate ligand, the P^k-trisaccharide.[20] Two analogous binding sites, 1 and 2, were observed in the crystal structure of the Stx2a–P^{k}NAc complex. A small increase in each individual interaction leads to a large enhancement of avidity and quickly shifts the assay conditions to the signal saturation region. Multivalent interaction thus masks the activity differences of the active compounds. We expected that reducing the ligand density on the plate would reduce the number of ligands that could be simultaneously engaged by the multivalent receptor Stx2a, thereby extending the dynamic range of the assay.

The design of our experiment allows the ligand density to be uniformly and reproducibly tuned down by decreasing the payload of the pendant ligand on the polymer used for coating. When **L2** (1.2% loading versus 4.8% for **L1**) was used as a coating reagent, only weak binding of Stx2a to the plate was observed for acetate (Figures 3 and 4; black ◆) but now a number of ligands superior to acetate were discovered (Table S1 in the Supporting Information). Differentiation of the activities of most of the derivatives became possible at level 2 [for example, in Figure 4, compare L-AlaNAc (**5**; red ◆), with D-AlaNAc (**6**; red ◇)].

Amino acid derivatives performed better than other tested compounds at level 2, with only one derivative of a simple acid, 4-(thiophen-2-yl)butanoic acid (**4**), matching the activities of the amino acids. With the exception of L-lysine residues with both α and ε amino groups either free (**7**) or acetylated (**8**), all derivatives of L-Lys showed good activities at level 2.

However, the best ligand **L2-9** showed only weak activity when the ligand density was further reduced to that of level 3 (0.4% loading; compare Figures 3 and 4, blue ■). At this point, instead of continuing to screen more acylation reagents, we decided to elaborate the identified L-Lys hit as a new scaffold. To this end, we prepared an **L3** polymer bearing the trisaccharide N-acylated by L-Lys-N$_\alpha$Boc and treated the Lys ε-amino group with a large panel of acylating reagents (Table S1). Original screening was performed in triplicate with a high concentration of Stx2a, and Stx2a titration experiments were completed for each hit.

Unexpectedly, this round of screening did not yield any amino acids as good hits. Instead, some aromatic acids showed superior activities (Figure 5). The remaining α-amines were either protected with Boc, acetylated, or remained free. Of

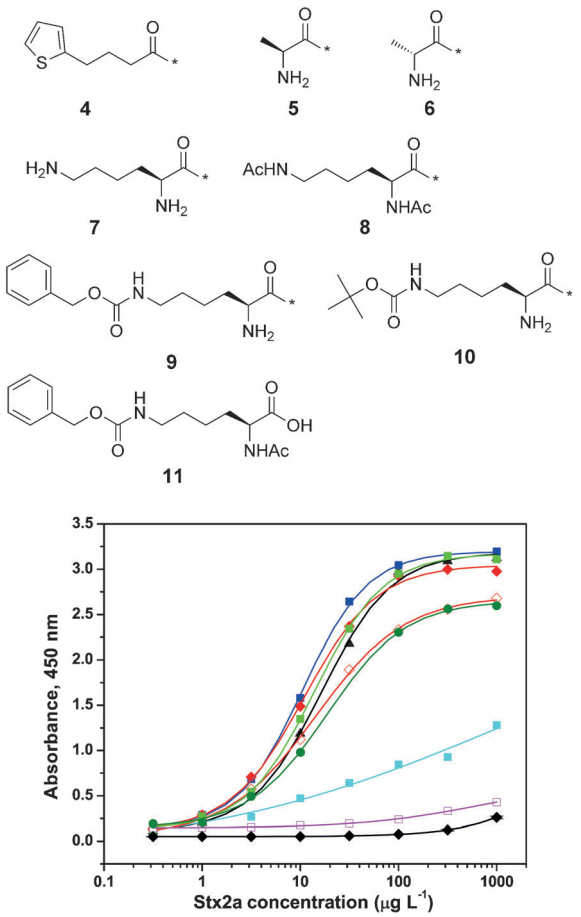


Figure 4. Level 2 screening. Binding of Stx2a to polystyrene microtiter plates coated with **L2** and modified with compounds **4**–**11** to furnish: **L2Ac** (black ◆), **L2-4** (black ▲), **L2-5** (red ◆), **L2-6** (red ◇), **L2-7** (violet □), **L2-8** (turquoise ■), **L2-9** (blue ■), **L2-10** (dark green ●), **L2-11** (light green ■).

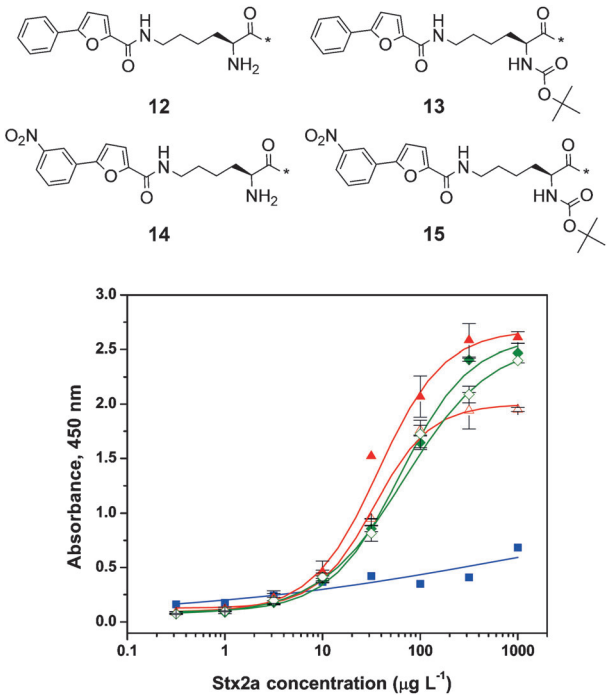


Figure 5. Level 3 screening. Binding of Stx2a to polystyrene microtiter plates coated with **L3Lys** and modified with compounds **12**–**15** to furnish: **L3-9** (blue ■), **L3-12** (red ▲), **L3-13** (red △), **L3-14** (green ◇), **L3-15** (green ◆).

Table 1: Properties of glycoconjugates.

Conjugate	Payload[a]	M_n[b]	Degree of incorporation	IC_{50} [nM][c]
L1	4.8	53 000	17.9	NT[d]
L2	1.2	41 000	4.4	NT
L3	0.4	38 000	1.6	NT
L3Lys	0.4	38 500	1.6	NT
L1Ac*	4.4	52 000	16.5	8.1
17	2.6	49 500	9.6	0.26

[a] Estimated by ^{1}H NMR spectroscopy. [b] Calculated based on M_n = 36 700 Da for unmodified polymer. [c] Based on M_n. [d] Not tested.

these three options, acetylation resulted in decreased activity while protection with Boc marginally increased Stx2a binding.

The activities of compound **16** and its poly(*N*-vinyl-2-pyrrolidone-*co*-vinyl alcohol) conjugate **17** as competitive inhibitors of Stx2a are shown in Figure 6. Ligand **16** is the first known monomeric inhibitor of Stx2a since the P^kNAc analogue failed as an inhibitor of Stx2a, even at millimolar concentrations. When attached to copovidone, an approximately 90 000-fold activity amplification per trisaccharide (ca. 17 000-fold by weight) was achieved. Despite a lower average number of pendant ligands per polymer chain, the polymeric analogue **17** is at least 30-fold more active than the P^kNAc-based polymer $\mathbf{L^1Ac}$* (Table 1).

Our approach to the on-plate generation and screening of a focused glycan library embodies several concepts separately reported by others.[21] Crucially, plate-adsorbed copovidone conjugates with variable ligand payloads address the issue of density variation in fine tuning multivalent interactions without masking ligand-affinity gains. Copovidone is an attractive choice as the polymeric construct because it nicely combines solubility properties with affinity for hydrophobic surfaces, which eliminates the need to introduce additional reactive groups or hydrophobic moieties solely for immobilization purposes. It is unlikely that the outcome of this work was crucially influenced by using a narrow-molecular-weight reversible addition–fragmentation chain-transfer (RAFT) polymer. For plate coating applications, any poly(NVP-*co*-VA), including commercial products such as Kollidon SR, could be employed after appropriate modification. Cost-efficient statistical carrier polymers suit this application without addressing the separate issue of optimal ligand presentation, which is better served by dendrimers[22] or precision polymers.[23]

Once prepared, only minute quantities of coating conjugate per plate are needed and the reproducibility of array manufacturing depends only on the quality of the commercial microtiter plates. This eliminates reaction conditions and quality of reagents as factors influencing the reproducibility of chemical immobilization methods. The copovidone conjugates support multiple cycles of a variety of on-plate

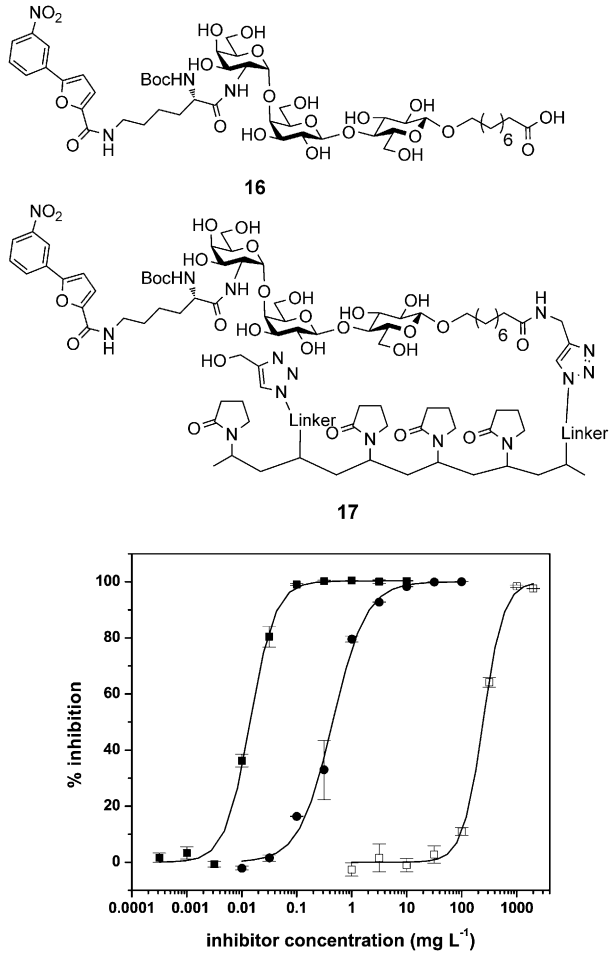


Figure 6. Inhibition of Stx2a binding to P^kNAc-coated plates. **L1Ac*** (■; IC_{50} 424 ng mL^{-1}, 8.1 nM), **17** (●; IC_{50} 13 ng mL^{-1}, 0.26 nM), **16** (□; IC_{50} 240 μg mL^{-1}, 0.22 mM).

chemical modifications and activity measurements, a property that permits the rapid in situ generation and evaluation of analogue libraries in a simple 96-well ELISA that does not require specialized equipment for activity readout. The best hit discovered at each preceding level of ligand density becomes a positive control reference for the next round of screening at a lower level of multivalency (Table S1).

Starting from a univalent trisaccharide scaffold that showed no measurable toxin activity, this approach generated the first sub-millimolar ligand for Shiga toxin type 2a and the most active monovalent ligand for any Shiga-like toxin. This approach should be applicable not just to carbohydrate-ligand discovery but to any system that employs multivalent interactions to boost low intrinsic affinity in order to generate a biological signal.

Received: October 29, 2013
Published online: January 22, 2014

Keywords: drug discovery · glycoarrays · haemolytic uremic syndrome · inhibitors · Shiga toxins

[1] a) P. I. Tarr, C. A. Gordon, W. L. Chandler, *Lancet* **2005**, *365*, 1073 – 1086; b) D. A. Rasko, D. R. Webster, J. W. Sahl, A. Bashir, N. Boisen, F. Scheutz, E. E. Paxinos, R. Sebra, C. S. Chin, D. Iliopoulos, A. Klammer, P. Peluso, L. Lee, A. O. Kislyuk, J. Bullard, A. Kasarskis, S. Wang, J. Eid, D. Rank, J. C. Redman, S. R. Steyert, J. Frimodt-Moller, C. Struve, A. M. Petersen, K. A. Krogfelt, J. P. Nataro, E. E. Schadt, M. K. Waldor, *N. Engl. J. Med.* **2011**, *365*, 709 – 717.

[2] a) J. C. Paton, A. W. Paton, *Clin. Microbiol. Rev.* **1998**, *11*, 450 – 479; b) P. M. Griffin, *Escherichia coli O157:H7 and other enterohemorrhagic Escherichia coli*, Raven, San Diego, **1995**; c) P. M. Griffin, R. V. Tauxe, *Epidemiol. Rev.* **1991**, *13*, 60 – 98.

[3] a) C. S. Wong, S. Jelacic, R. L. Habeeb, S. L. Watkins, P. I. Tarr, *N. Engl. J. Med.* **2000**, *342*, 1930 – 1936; b) C. M. McGannon, C. A. Fuller, A. A. Weiss, *Antimicrob. Agents Chemother.* **2010**, *54*, 3790 – 3798.

[4] a) A. E. Clatworthy, E. Pierson, D. T. Hung, *Nat. Chem. Biol.* **2007**, *3*, 541 – 548; b) L. Cegelski, G. R. Marshall, G. R. Eldridge, S. J. Hultgren, *Nat. Rev. Microbiol.* **2008**, *6*, 17 – 27.

[5] D. A. Rasko, V. Sperandio, *Nat. Rev. Drug Discovery* **2010**, *9*, 117 – 128.

[6] G. Schiavo, F. G. van der Goot, *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 530 – 537.

[7] L. Johannes, W. Romer, *Nat. Rev. Microbiol.* **2010**, *8*, 105 – 116.

[8] M. A. Karmali, M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, H. Lior, *J. Infect. Dis.* **1985**, *151*, 775 – 782.

[9] a) J. Lundquist, S. Debenham, E. Toone, *J. Org. Chem.* **2000**, *65*, 8245 – 8250; b) E. N. Kitova, P. I. Kitov, D. R. Bundle, J. S. Klassen, *Glycobiology* **2001**, *11*, 605 – 611.

[10] a) P. I. Kitov, J. M. Sadowska, G. Mulvey, G. D. Armstrong, H. Ling, N. S. Pannu, R. J. Read, D. R. Bundle, *Nature* **2000**, *403*, 669 – 672; b) G. L. Mulvey, P. Marcato, P. I. Kitov, J. Sadowska, D. R. Bundle, G. D. Armstrong, *J. Infect. Dis.* **2003**, *187*, 640 – 649; c) P. I. Kitov, G. L. Mulvey, T. P. Griener, T. Lipinski, D. Solomon, E. Paszkiewicz, J. M. Jacobson, J. M. Sadowska, M. Suzuki, K. Yamamura, G. D. Armstrong, D. R. Bundle, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 16837 – 16842.

[11] K. Nishikawa, K. Matsuoka, E. Kita, N. Okabe, M. Mizuguchi, K. Hino, S. Miyazawa, C. Yamasaki, J. Aoki, S. Takashima, Y. Yamakawa, M. Nishijima, D. Terunuma, H. Kuzuhara, Y. Natori, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 7669 – 7674.

[12] N. W. P. Rutjes, B. A. Binnington, C. R. Smith, M. D. Maloney, C. A. Lingwood, *Kidney Int.* **2002**, *62*, 832 – 845.

[13] R. L. Siegler, T. G. Obrig, T. J. Pysher, V. L. Tesh, N. D. Denkers, F. B. Taylor, *Pediatr. Nephrol.* **2003**, *18*, 92 – 96.

[14] a) M. R. Leslie, H. Parolis, L. A. S. Parolis, *Carbohydr. Res.* **2000**, *324-329*, 103 – 110; b) S. D. Gamage, C. M. McGannon, A. A. Weiss, *J. Bacteriol.* **2004**, *186*, 5506 – 5512.

[15] a) R. R. Kale, C. M. McGannon, C. Fuller-Schaefer, D. M. Hatch, M. J. Flagler, S. D. Gamage, A. A. Weiss, S. S. Iyer, *Angew. Chem.* **2008**, *120*, 1285 – 1288; *Angew. Chem. Int. Ed.* **2008**, *47*, 1265 – 1268; H. O. Yosief, S. S. Iyer, A. A. Weiss, *Infect. Immun.* **2013**, *81*, 2753 – 2760.

[16] J. M. Jacobson, J. Yin, P. I. Kitov, G. Mulvey, T. Griener, M. James, G. Armstrong, D. R. Bundle, *J. Biol. Chem.* **2013**, DOI:10.1074/jbc.M113.518886.

[17] P. I. Kitov, Y. Kotsuchibashi, E. Paszkiewicz, D. Wilhelm, R. Narain, D. R. Bundle, *Org. Lett.* **2013**, *15*, 5190 – 5193.

[18] Synthetic procedures, spectral data, conditions for plate treatment, and details for binding and inhibiton assays, are presented in the Supporting Information. In the present work, we used the previously described [17] copovidone sample. Briefly, a statistical copolymer (NVP/VA = 7.3:1, M_n = 36700 Da, PDI = 1.69) was obtained by RAFT polymerization of NVP and VA and characterized by size-exclusion chromatography and NMR spectroscopy. The degree of modification in all subsequent

derivatives of the original polymer was deduced from the NMR spectra.
[19] The sample of Stx2a was a generous gift from Prof. G. D. Armstrong. Stx2a was isolated and purified as described in G. Mulvey, R. Vanmaele, M. Mrazek, M. Gahill, G. D. Armstrong, *J. Microbiol. Methods* **1998**, *17*, 247 – 252.
[20] H. Ling, A. Boodhoo, B. Hazes, M. D. Cummings, G. D. Armstrong, J. L. Brunton, R. J. Read, *Biochemistry* **1998**, *37*, 1777 – 1788.
[21] a) K. Godula, C. R. Bertozzi, *J. Am. Chem. Soc.* **2012**, *134*, 15732 – 15742; b) C. D. Rillahan, E. Schwartz, C. Rademacher, R. McBride, J. Rangarajan, V. V. Fokin, J. C. Paulson, *ACS Chem. Biol.* **2013**, *8*, 1417 – 1422.
[22] S. L. Mangold, M. J. Cloninger, *Org. Biomol. Chem.* **2006**, *4*, 2458 – 2465.
[23] See, for example, D. Ponader, F. Wojcik, F. Beceren-Braun, J. Dernedde, L. Hartmann, *Biomacromolecules* **2012**, *13*, 1845 – 1852, and references therein.

