RATIONAL DESIGN OF SOLUBLE SUB−NANOMOLAR INHIBITORS FOR SHIGA TOXINS

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Introduction

Shiga−like toxins (SLTs) gain entry to epithelial cells in the gut by first binding to Gb_3 glycolipid. The intrinsic affinity of subunit B_5 for the P^k trisaccharide, α−D−Gal(1→4)−β−D−Gal(1→4)−β−D−Glc, is approximately 10^3 M^{-1}. By designing and synthesizing bridged P^k trisaccharide molecules we could enhance affinity 40 fold (ref. 1). To minimize the entropy cost of binding, tethers of appropriate length can be built between carbohydrate epitopes residing in different binding domains, so that resulting cluster could embrace the entire receptor surface and provide sufficient ligand copies to saturate all the important binding sites.

The crystal structure of the binding subunit B_5 (from SLT−1) in a complex with its natural receptor, P^k trisaccharide, reveals a doughnut−shaped pentameric protein that accommodates up to 15 units of trisaccharide in 3 distinct binding modes on the face opposite to the attachment site for the toxin subunit A (ref. 2; Fig. 1, left panel). The difference in crystallographic occupancies of three distinct carbohydrate binding domains (Fig. 1) suggests significantly higher affinities for the two peripheral binding sites 1 and 2 compared to those that are close to the core of the pentameric B_5 subunit (site 3). We decided to employ these sites for specific recognition, whereas, binding to the core sites would be hindered by construction elements of the carrier. Since the peripheral trisaccharides are closer to each other than their respective spacing from the centre of the pentameric complex, they can be tethered together before assembly of the dimers into a five−ray cluster (Fig. 1). We named this star−shaped cluster STARFISH. We report now the synthesis and biological evaluation of the multivalent pentameric dendrimer STARFISH built from a central penta−substituted glucose molecule.

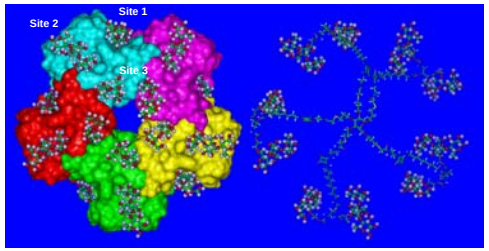


Figure 1. Structure−based design of multivalent inhibitors.

Versatility of the Approach

Shiga−like toxins Type 1 and Type 2 share 60% sequence homology and a common carbohydrate epitope, P^k trisaccharide. Since the crystal structure for SLT−2 is not yet available we used computer−assisted simulation to model the SLT−2/P^k interaction. Non−homologous amino acids were gradually substituted in the known SLT−1/ P^k crystal structure and after each step the energy of the resulting structure was minimized using the CVFF force field. Fig. 2 compares B subunits of SLT−1 and SLT−2. Three putative binding sites corresponding to sites 1−3 in the original SLT−1/P^k complex are formed primarily by conserved amino acids, which are shown in green. The common topology of multiple binding sites suggests that our approach may be applicable to different variants of Shiga toxin.

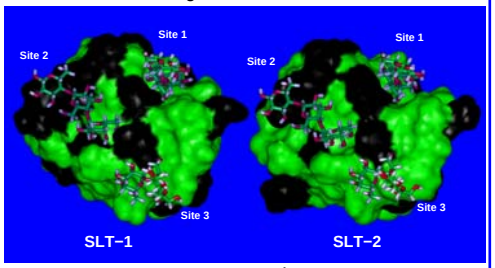
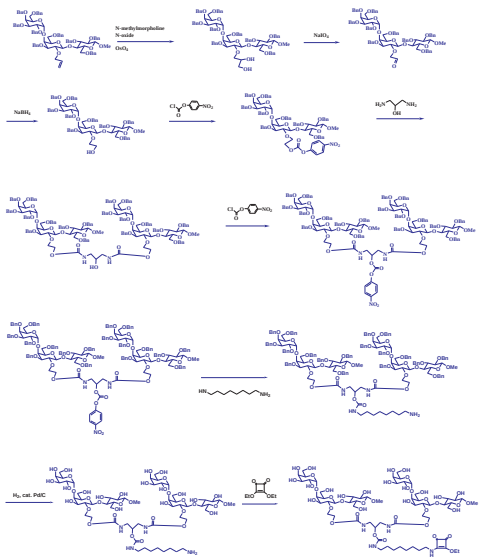


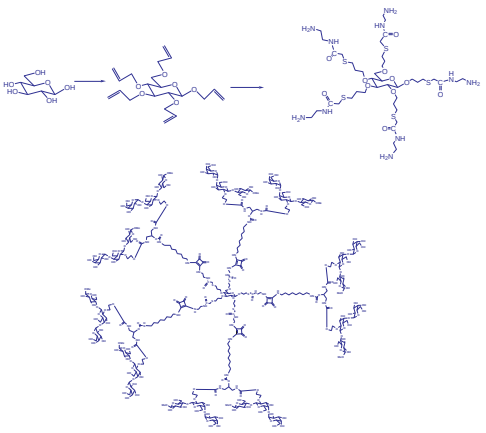
Figure 2. Comparison of SLT−1/P^k crystal structure and computer−assisted simulation of SLT−2/P^k complex.

Synthesis of P^k−STARFISH Molecule

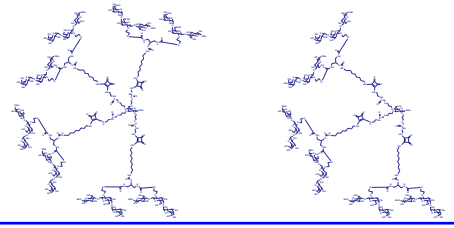
Assembly of P^k−trisaccharide dimer



Assembly of P^k−Starfish



Bridged P^k dimer with an amine−terminated linker, activated as a squaric acid derivative, was linked to a five−fold scaffold made from a functionalized glucose molecule to give a pentameric presentation of trisaccharide dimers. Tetra− and tri−arm analogues based on glucose core were obtained by a similar sequence of transformations.



Biological Evaluation

In order to assay synthetic P^k trisaccharide clusters 2 different competitive solid phase assays were developed. According to protocol A, a toxin was immobilized on the microtiter plate. An inhibitor in sequential dilutions was allowed to compete with biotin−labeled P^k/BSA conjugate for binding to the toxin. Amounts of bound biotin were detected by the standard biotin−streptavidine methodology.
In protocol B, a P^k−containing compound (commercially available glycolipid Gb_3 or synthetic P^k−glycoside) was immobilized on the plate, whereas, toxin and inhibitor were in solution. The amount of bound toxin was detected by an enzyme−linked antibody protocol.

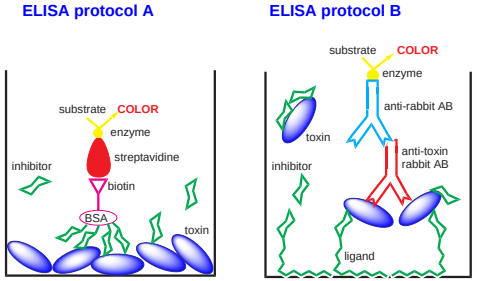
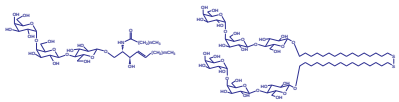


Figure 3. ELISA formats.

Ligands used in ELISA protocol B



Gb_3 P^k−trisaccharide disulfide (ref. 3)

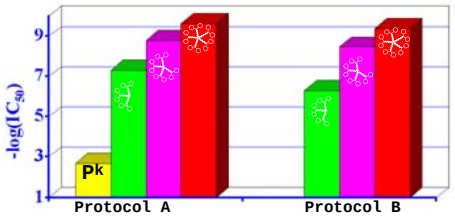


Figure 4. Inhibition assay. SLT−1 *vs.* multivalent P^k trisaccharide clusters.

As shown on Fig. 4, the inhibitory activities of multimeric P^k trisaccharide clusters against SLT−1 are greater than that of monovalent P^k glycoside, and exponentially increase with the number of arms, extending out from the central core. The inhibitory power of pentavalent STARFISH is 0.2 nM, i. e. 10^7 times higher that that of P^k glycoside and 10^4 times higher than for linear polyacrylamide−based P^k inhibitors (ref. 4).

The clinically−significant toxin SLT−2 binds the P^k trisaccharide with such weak affinity that assaying its binding is difficult. However, employing a novel glycolipid assay system, namely, protocol B, we could show that the multimeric inhibitors described above are also active against SLT−2 with an IC_{50} estimated value for pentamer of 1 nM .

Unusual Mode of Binding

Crystallographic studies of the adduct between the B_5 subunit of SLT−1 and P^k−STARFISH reveal a more complex picture than was originally thought. Instead of a 1:1 pentamer/STARFISH adduct, in which two P^k trisaccharides on one arm of the cluster would occupy two distinct binding sites of a B subunit (1 and 2), a sandwich−like 2:1 structure forms, in which STARFISH mediates face−to−face arrangement of pentameric protein. P^k trisaccharide occupies only sites 2 in both B_5 subunits. Since in this experiment the concentration of STARFISH was sufficient for 1:1 complex, the formation of the 2:1 sandwich was thermodynamically favoured, apparently due to higher affinity characteristic of the site 2.

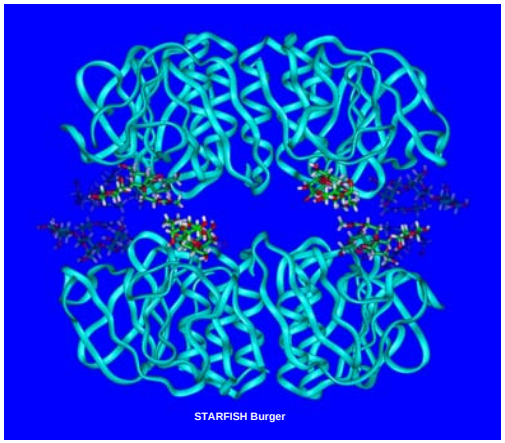


Figure 5. Crystal structure of 2:1 SLT−1/STARFISH adduct.

Conclusions

- High affinity inhibitors for the interaction of Verotoxins Type 1 and Type 2 with P^k trisaccharide have been designed based on the crystal structure of the P^k trisaccharide/SLT−1 complex.
- Two protocols for competitive inhibition assay of binding Shiga toxins to P^k trisaccharide have been developed.
- New crystal forms of SLT−1 complexed with pentameric P^k clusters have been observed.
- The binding motif seen in the bridged P^k STARFISH/SLT−1 complex suggests that STARFISH−type inhibitors can be used against other bacterial and viral toxins with pentameric structure, such as cholera toxin and heat−labile enterotoxin.

References

1. Kitov, P. I.; Bundle, D. R., unpublished results.
2. Ling, H.; Boodhoo, A.; Hazes, B.; Cummings, M. D.; Armstrong, G. D.; Brunton, J. L.; Reed, R. J., *Biochemistry*., 1998, 37, 1777−1788.
3. Kitov, P. I.; Railton, C.; Bundle, D. R., *Carbohydr. Res.*, 1998, 307, 361−369.
4. Kitov, P. I.; Bundle, D. R., Roy, R., unpublished results.