Inhibitors

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An Entropically Efficient Supramolecular Inhibition Strategy for Shiga Toxins**

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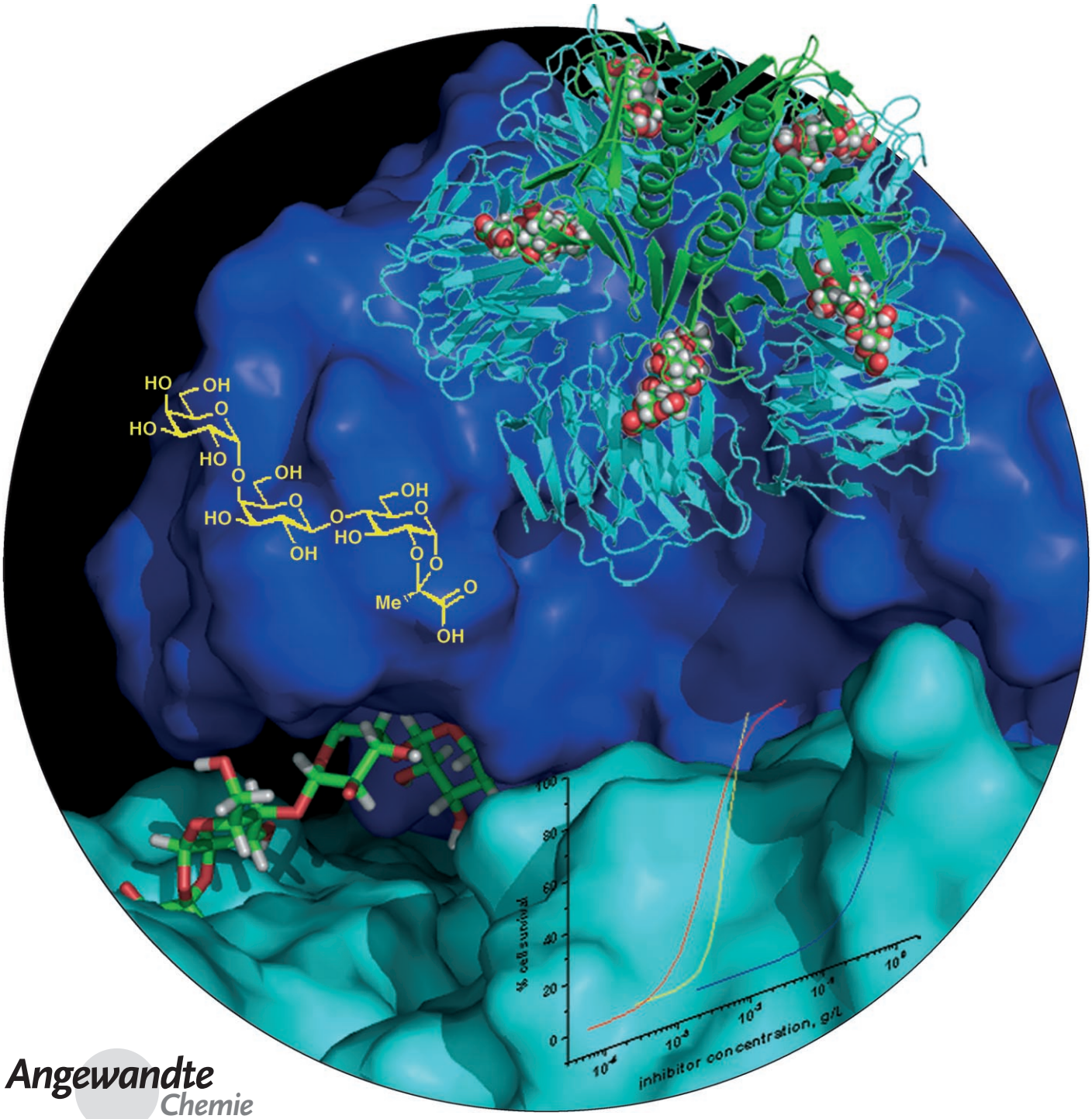
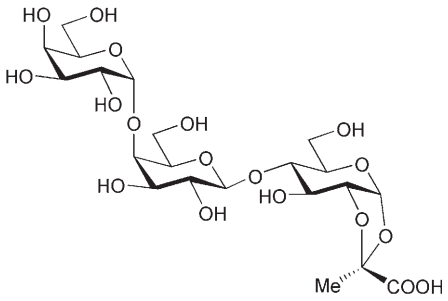


We describe the design, synthesis, and in vitro evaluation of a potent, low molecular weight (MW = 574) inhibitor for Shiga toxin that has activity in cell culture comparable to that of the most active multivalent inhibitors (MW = 8628). This exceptional activity is achieved through supramolecular protein aggregation, an emerging concept that offers exciting possibilities in drug design. Heterobifunctional ligands are a novel class of inhibitors that carry binding functionalities for the target receptor and an endogenous protein receptor, and induce formation of a three-component complex.[1–6] In the case of multivalent target receptors the affinity reinforcement by the supporting protein ("supramolecular effect") can enhance ligand–receptor interaction by several orders of magnitude without employing multivalent scaffolds.[1,2] A tether of appropriate length is needed to present the binding moieties to opposing receptors as well as to prevent clashes between proteins. The tether must be optimized to satisfy several mutually exclusive requirements. Short, rigid spacers can create steric constraints, whereas entropy costs are associated with long, flexible tethers.[7] Herein we show the first successful example of an "integrated" heterobivalent ligand conceived by rational design. Merging two binding moieties without any linker creates a compact, easily synthesized ligand that efficiently induces a supramolecular complex between two multimeric proteins, Shiga toxin type 1 (Stx1) or type 2 (Stx2) and serum amyloid P component (SAP), an endogenous protein. SAP is able to target bound ligands for clearance.[8]

Shiga toxins, the key virulence factors expressed by several pathogenic strains of *E. coli*, are radially symmetric homopentameric bacterial lectins. The five identical carbohydrate-binding B subunits are arranged in a pentagon-shaped multimer that carries a single enzymatic A subunit. Shiga toxin has low intrinsic affinity ($K_d \approx 1$ mM) for its native ligand, P^k-trisaccharide (αGal*p*-(1–4)-βGal*p*-(1–4)-βGlc*p*, the carbohydrate portion of glycosphingolipid Gb_3). The most successful approach to design of Shiga toxin antagonists to date has been the construction of multivalent molecules capable of simultaneously engaging most binding sites on the pentameric receptor.[9–11] Serum amyloid P component is an abundant serum protein that is a part of the innate immune system.[12] Like Shiga toxin's B_5 subunit, SAP is a pentameric protein with fivefold radial symmetry. SAP has one Ca^{2+}-dependent binding site per subunit and forms complexes with pyruvate ketals of polysaccharides as well as other ligands, such as D-proline.[12]

For molecular modeling we used two previously reported crystal structures: the complex between Stx1 and P^k-trisaccharide analogue (PDB entry 1BOS),[13] and the complex between SAP and the bivalent cyclic pyruvate of glycerol (PDB entry 2A3W).[7] Examination of the structures revealed that, when projected on a plane that is orthogonal to the fivefold axis of symmetry, the pyruvate moiety resides at a distance from the center of SAP that is the same as the distance of the glucose residue of P^k-trisaccharide from the center of Stx1, when this trisaccharide occupies the highest affinity binding site 2 of Stx1. Alignment of the two complexes in such a way that bound ligands overlap with their counterparts shows that a mutual arrangement of Stx and SAP can be found where the hydroxy groups at C1 and C2 of glucose in bound P^k-trisaccharide ligand are superimposed on the two oxygen atoms of the pyruvate's dioxane ring. This result suggested that a 1,2-*O*-carboxyethylidene derivative of glucose can mimic the pyruvate of glycerol, the ligand we designed to bind SAP.[7]

Further modeling studies led to the identification of structure **1**, which affords an excellent fit of both binding fragments with the respective binding sites without inducing



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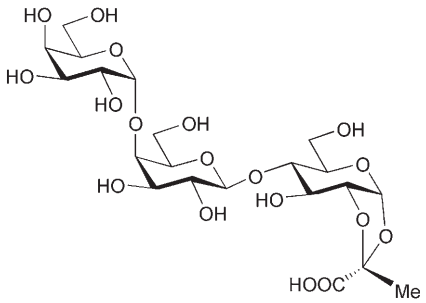

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any apparent unfavorable interactions between the two proteins as the heterobivalent ligand brings them into close face-to-face proximity (Figure 1). Most interactions between P^k-trisaccharide and Stx1 are preserved in ligand **1**, and the atoms of the fused cyclic pyruvate moiety of **1** occupy the same positions as a cyclic pyruvate found in a SAP–ligand complex: the carboxy group coordinates two Ca^{2+} ions and

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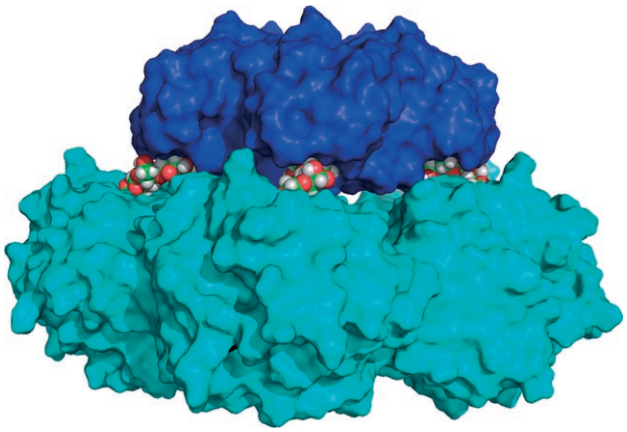


Figure 1. Molecular modeling of the face-to-face alignment between two aggregated proteins, Stx (top) and SAP (bottom), mediated by **1**. Two binding fragments of **1**, P^k-trisaccharide and cyclic pyruvate, fit into the binding sites of the corresponding proteins.

the methyl group fits into a small hydrophobic pocket formed by side chains of Lev62, Tyr64, and Tyr74.[7] It is essential for the stability of the supramolecular complex that the binding elements of the interceding ligand are spatially matched to the binding sites of the respective proteins. This molecular model predicts that the *S*-pyruvate isomer **1** should provide the proper presentation of the pyruvate moiety to SAP. Ligand **2** with the inverted configuration (*R* isomer) should not be able to engage face-to-face aligned Stx1 and SAP pentamers simultaneously.

To confirm the validity of this design we synthesized and evaluated the activities of compound **1** and its isomer **2**. The synthesis of **1** and **2** was accomplished by a chemoenzymatic pathway starting from D-lactose. Lactose was acetylated and converted into acetobromolactose, which was treated with KCN in the presence of Bu_4NBr[14] to give a mixture of the cyanoethylidene *S* and *R* isomers **3** and **4** (Scheme 1). The major *S* isomer (crystalline) and the minor *R* isomer (oil) were processed separately by standard methods. Deacetylation of each cyanoethylidene derivative **3** and **4** was accompanied by conversion of the cyano group into the corresponding methoxycarbonyl derivatives **5** and **6**. These products were enzymatically galactosylated by using a fusion enzyme consisting of UDP-Glc/Gal-isomerase and (1–4)-galactosyl transferase.[15] The methyl ester was hydrolyzed concurrently with glycosylation under the mild basic conditions (pH 8.2, 37 °C) to give the target compounds **1** and **2**. (Ester hydrolysis is attributed to lipases that contaminated the crude enzyme preparations.)

To compare the inhibitory potency of these designed ligands we used several solid-phase assay formats. The results are summarized in Table 1 (assay details given in the Supporting Information). SAP-dependent inhibition of Stx1 binding to P^k-trisaccharide-coated ELISA plates at constant SAP concentration was assayed in a competitive solid-phase assay (Table 1, entry A).[1] In assay B using the same P^k-coated ELISA plates the concentration of the inhibitor **1** was kept constant and the concentration of SAP was varied (Table 1, Figure 2). The affinity of the ligands for SAP was established by inhibition of SAP binding to D-proline-coated plates (Table 1, entry C), performed as previously described.[7] Direct solution phase binding of ligands to Stx1(B_5) was observed by nanoelectrospray FTICR mass spectrometry, and dissociation constants were deduced (Table 1, entry D).

Table 1: Binding data for inhibitors **1** and **2** in various assays.

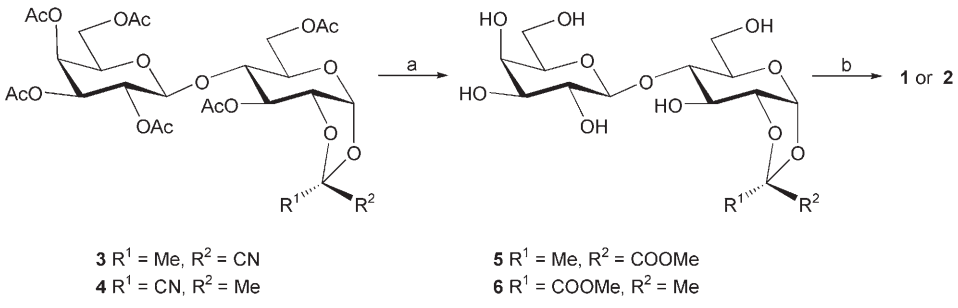
Assay	**1** [M]	**2** [M]
A	5.6×10^{-7}[a]	no activity
B	2.07×10^{-10}[b]	no activity
C	1.9×10^{-3}	1.2×10^{-3}
D	3.2×10^{-3}	6.0×10^{-3}

[a] IC_{50} with respect to **1** in the presence of 20 $\mu g\,mL^{-1}$ SAP. [b] IC_{50} with respect to SAP in the presence of 0.17 mM **1**.

The binding of isomers **1** and **2** to either Stx1 or SAP was very similar and approximately millimolar (Table 1, entries C and D). However, in the presence of SAP, isomer **1** showed around 4 orders of magnitude enhancement of Stx1 inhibition (Table 1, entry A). As predicted, isomer **2** was inactive. The SAP-mediated inhibition could be achieved at SAP concentrations as low as 0.1 $\mu g\,mL^{-1}$ (Figure 2).

Previously, we reported the design and activity of the first SAP-dependent inhibitor of Shiga toxin **7** (Figure 3).[1] The two binding fragments P^k-trisaccharide and cyclic pyruvate of glycerol are linked by a small but flexible linker, which contains five single bonds and may adopt a maximum of 3^5 (=243) staggered conformations. Therefore, upon ternary complex formation, the binding strength would be reduced approximately 100–1000-fold per ligand owing to loss of conformational entropy. Through design of an inhibitor that eliminates the linker between the binding moieties we expected to recover some of this loss. We observed an approximately 50-fold gain ($IC_{50} = 3\times10^{-5}$ M for **7**[1] versus $IC_{50} = 5.6\times10^{-7}$ M for **1**).

In considering this gain it is noteworthy that the pyruvate moiety in the five-membered dioxolane rings of ligands **1** and



3 R^1 = Me, R^2 = CN
4 R^1 = CN, R^2 = Me

5 R^1 = Me, R^2 = COOMe
6 R^1 = COOMe, R^2 = Me

Scheme 1. Synthesis of **1** and **2**. Reagents and conditions: a) NaOMe in MeOH then trifluoroacetic acid, 96%; b) UDP-glucose, α-(1,4)-GalT/UDP-4′-Gal-epimerase.

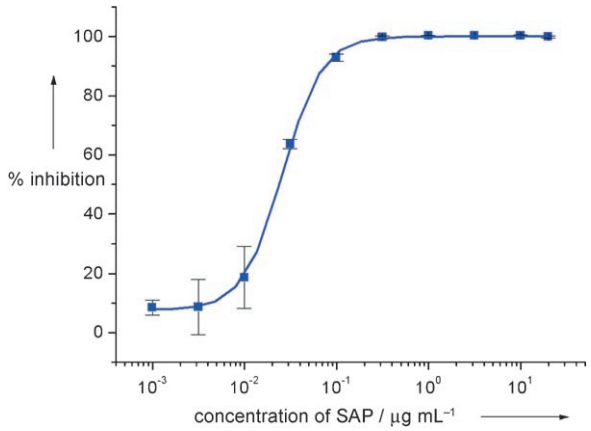


Figure 2. SAP-dependent inhibition of Stx1 (assay B). Titration curve for SAP in the presence of **1** (0.17 mM) and Ca^{2+} (1 mM). Error bars are given for triplicates.

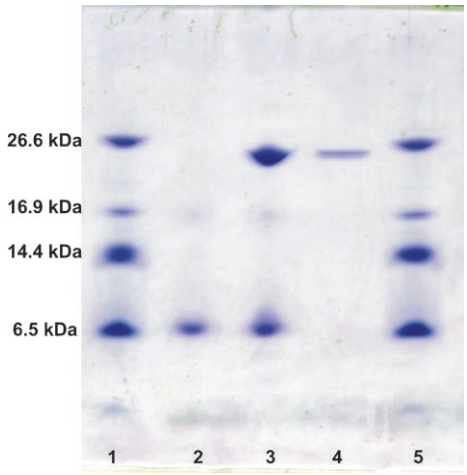


Figure 4. SDS-PAGE shows the presence of Stx1B and SAP in the peaks corresponding to complexes isolated by GPC. Line assignment: lines 1 and 5: mixture of reference proteins; line 2: StxB, line 3: complex SAP_5–$\mathbf{1}_5$–$Stx1B_5$, line 4: SAP. (SDS-PAGE for Sxt2 complex, see the Supporting Information.)

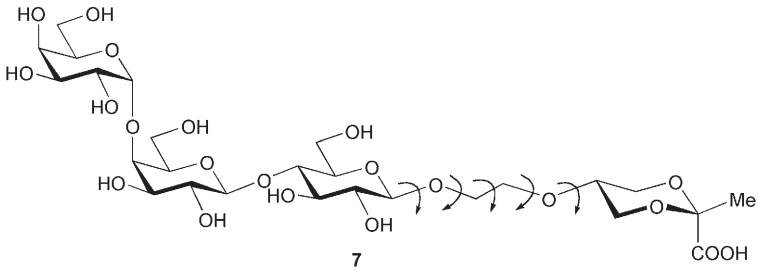


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Figure 3. Compound **7**, for which $IC_{50} = 3 \times 10^{-5}$ M in the presence of 20 μg mL^{-1} SAP.[1] Arrows mark single bonds, rotation around which changes the relative orientation of the two binding fragments.

2 has lower affinity for SAP (Table 1 entry C) than the six-membered dioxane ring of the cyclic pyruvate of glycerol ($IC_{50} = 5.3 \times 10^{-4}$ M),[7] present in **7**. Nevertheless, in the stabilization of the ternary complex, a lower affinity of individual ligands for SAP can be more than offset by the entropy savings achieved by elimination of the conformational freedom of the tether.

The ability of ligand **1** to induce a three-component complex between SAP and Shiga toxins was further confirmed by gel permeation chromatography (GPC). When SAP and Stx1B were mixed in the proportion 1:1 per subunit in the presence of **1** in Ca^{2+}-containing buffer, the toxin peak disappeared and a new peak appeared whose molecular weight, estimated by dynamic light scattering to be approximately 171 kDa, corresponds to that of the 1:1 complex SAP(pentamer)–$Stx1B_5$ (see the Supporting Information). The 1:1 composition of the isolated complex was further supported by SDS-PAGE (Figure 4.)

We also found that the B subunit of Shiga toxin type 2 (Stx2) shows the same behavior in a GPC experiment with SAP and **1**. The Stx2 is more closely associated with clinical disease.[16] This observation is consistent with our recently published mass spectrometry facilitated chemical mapping of Stx2 binding sites which suggested a dominant role for site 2 in both toxins.[17] This result is important proof of a uniform mode of action for **1** with respect to both toxins, since the known Ca^{2+}-independent interaction of SAP with Stx2[18–20] has prevented us from measuring the activity of ligand **1** with Stx2 in both solid-phase assays and in vivo. In the absence of the A subunit, Stx2B exists in solution as a mixture of oligomers, in which form it can be observed by GPC and FTICR mass spectrometry.[21] Remarkably, ligand-adaptor **1** induces supramolecular assembly of the pentameric form of Stx2B on SAP as a template.

To compare the activity of the heterobivalent ligand strategy to our previously reported decameric P^k-trisaccharide ligand (STARFISH), we tested the activity of these compounds in a Vero cell cytotoxicity assay. Although univalent with regard to the toxin specific ligand and 15-fold lower in molecular weight, the simple ligand **1** is as active as STARFISH (Figure 5). These STARFISH-type ligands induce face-to-face aggregation of two copies of Shiga toxin.[9] However, a supramolecular complex mediated by the heterobifunctional ligand **1** has a thermodynamic advantage, since the physiological concentration of SAP is 1000-fold higher than that required to bring about complex formation with Stx1. We imply in this case that the stability of the observed SAP_5–$\mathbf{1}_5$–$Stx1B_5$ complex is mediated by five heterobivalent ligands **1**. Although this appears to be the only reasonable thermodynamic rationale for the dramatic gain in avidity, unambiguous proof of the stoichiometry of the complex is lacking at this time.

Despite its exceptional in vitro activity in the cytotoxicity assay, compound **1** was found to be ineffective against in vivo administered Stx1 conducted in human SAP transgenic mice (see the Supporting Information),[22] whereas decameric ligands showed moderate efficacy by delaying the onset of symptoms.[10] The poor in vivo performance of **1** is attributed to its rapid clearance. We found that, after injection, the

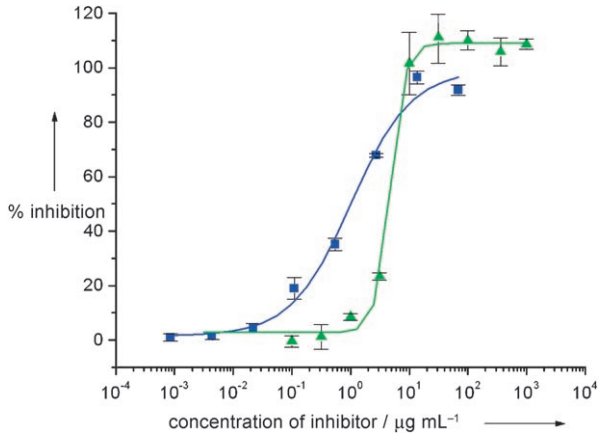

Figure 5. Cytotoxicity assay. Vero cells were incubated with synthetic inhibitors and a lethal dose of Stx-I. Blue squares: SAP-independent inhibition by decavalent inhibitor STARFISH (data from reference [9]), green triangles: SAP- and Ca^{2+}-dependent inhibition by **1**. Error bars are given for triplicates.

concentration of **1** in serum is rapidly decreased within the first hour and is virtually undetectable after 2 hours. We speculate that because of its higher mass (ca. 80 kDa) Shiga toxin persists in circulation longer than the inhibitor. The polar nature of **1** along with its low molecular weight facilitates fast excretion via the kidney, after which the animal is exposed to toxin that remains in circulation. Work is in progress to overcome the short half-life of ligand **1** in circulation by exploring different ways to retard excretion.

Heterobivalent ligands are able to mediate the high avidity association of two multimeric proteins in face-to-face aggregates despite the relatively weak intrinsic affinities of the component ligands for their respective protein. The concept is not limited to soluble receptors and may be applied to membrane-bound receptors. Preliminary data for an integrin–antibody pairing suggests that increased affinity for one receptor may be traded against a lower affinity for the second receptor. In the present work, judicious application of molecular modeling studies and rational design created a heterobivalent ligand that significantly reduces conformational entropy losses, thereby stabilizing multiprotein supramolecular assemblies. The successful implementation of this small-molecule strategy indicates that the supramolecular effect is a valuable tool for modulation of the inhibitory activity especially in the case of weak ligands for multivalent receptors. Therefore, finding the criteria for design of such inhibitor adaptors is an important goal. Supramolecular aggregation mediated by small readily synthesized molecules holds considerable promise for neutralization of multimeric microbial toxins and for targeting receptors on cell surfaces. Refinement of this approach will benefit from the selection of appropriate template proteins that possess effector functions to facilitate removal of the target protein or cell and formulation of heterobivalent ligands to increase their half-life in circulation.

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