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Designing a polyvalent inhibitor of anthrax toxin

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Screening peptide libraries is a proven strategy for identifying inhibitors of protein–ligand interactions. Compounds identified in these screens often bind to their targets with low affinities. When the target protein is present at a high density on the surface of cells or other biological surfaces, it is sometimes possible to increase the biological activity of a weakly binding ligand by presenting multiple copies of it on the same molecule. We isolated a peptide from a phage display library that binds weakly to the heptameric cell-binding subunit of anthrax toxin and prevents the interaction between cell-binding and enzymatic moieties. A molecule consisting of multiple copies of this nonnatural peptide, covalently linked to a flexible backbone, prevented assembly of the toxin complex *in vitro* and blocked toxin action in an animal model. This result demonstrates that protein–protein interactions can be inhibited by a synthetic, polymeric, polyvalent inhibitor *in vivo*.

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Anthrax toxin is produced by *Bacillus anthracis*, the causative agent of anthrax, and is responsible for the major symptoms of the disease[1]. Clinical anthrax is rare, but there is growing concern over the potential use of *B. anthracis* in biological warfare and terrorism. Although a vaccine against anthrax exists, various factors make mass vaccination impractical. The bacteria can be eradicated from the host by treatment with antibiotics, but because of the continuing action of the toxin, such therapy is of little value once symptoms have become evident. Thus, a specific inhibitor of the toxin's action might prove a valuable adjunct to antibiotic therapy. The toxin consists of a single receptor-binding moiety, termed protective antigen (PA), and two enzymatic moieties, termed edema factor (EF) and lethal factor (LF)[2]. After release from the bacteria as nontoxic monomers, these three proteins diffuse to the surface of mammalian cells and assemble into toxic, cell-bound complexes (Fig. 1). Cleavage of PA into two fragments by a cell-surface protease enables the fragment that remains bound to the cell, PA63, to heptamerize[3] and bind EF and LF with high affinity (K_d ~1 nM). After internalization by receptor-mediated endocytosis, the complexes are trafficked to the endosome. There, at low pH, the PA moiety inserts into the membrane and mediates translocation of EF and LF to the cytosol. EF is an adenylate cyclase that has an inhibitory effect on professional phagocytes, and LF is a protease[4] that acts specifically on macrophages, causing their death and the death of the host.

Here we report the designing and testing of a polyvalent inhibitor of anthrax toxin that binds to heptameric PA63 and blocks its interaction with EF and LF. First we identified from a phage-display library a dodecameric peptide that binds to PA63 and weakly inhibits its interaction with the enzymatic moieties of the toxin. We then covalently linked multiple copies of this peptide to a polyacrylamide backbone and demonstrated that the resulting polyvalent molecule strongly inhibited ligand binding by PA63 and toxin action in cell culture and in an animal intoxication model.

Results

Selection of peptides by phage display. One way to prevent the action of anthrax toxin would be to interfere with assembly of PA, LF, and EF into toxic complexes. To develop an inhibitor of this process, we first used phage display[5] to identify peptides that interfered with binding of EF and LF to PA63. A protocol was devised to select for members of a phage library that bind to PA63 and eliminate those that bind to the uncleaved PA molecule (Fig. 2A). This protocol enriched for phages that bind at or near the EF/LF binding site of PA63. We adsorbed PA63 onto a plastic surface and added a library of M13 phages displaying random 12-residue peptides fused to the N terminus of the pIII protein. After incubation, we washed the surface and then added intact PA to elute phages that bound to the whole protein. Finally soluble PA63 heptamer was added, and phages that adsorbed to it were recovered.

After three cycles of this protocol, we isolated phages displaying two different peptides: P1, HTSTYWWLDGAP, and P2, HQLPQYWWLSPG. ELISA showed that phages carrying either P1 or P2 bound to the PA63 heptamer, but not to intact PA (Fig. 2B). The binding of both phages was inhibited by LF_N, a 255-residue peptide corresponding to the N-terminal, PA63-binding domain of LF (and lacking its catalytic domain)[6]. The observation of inhibition of binding by LF_N supports the notion that phages carrying P1 and P2 bind PA63 at or near the EF/LF sites. A phage isolated in a separate screen (M. Mourez, unpublished data) and that carried an unrelated peptide (P3, EFYHGIWFYPWT) bound to intact PA as well as to PA63, and LF_N did not inhibit its binding (Fig. 2B). Phage carrying P3, but not ones carrying P1 or P2, also bound to a polypeptide corresponding to domains 3 and 4 of PA, a region believed not to participate in EF/LF binding (data not shown).

The P1 and P2 peptides share the hydrophobic sequence YWWL; this commonality suggests that this tetrapeptide may play a role in binding to PA63. The sequence YWWL is not present in EF, LF, or PA20. The side chains of three contiguous aromatic residues (Tyr22, Tyr23, and Phe24) of the PA20 moiety of native PA do, however, contact the hydrophobic surface of the PA63 moiety. We hypothesize that the YWWL sequence may bind to PA63 at this site, which is exposed to the solvent after removal of PA20 (ref. 3).

The P1 dodecapeptide (HTSTYWWLDGAP) was synthesized and found to disrupt the binding of radiolabeled LF_N to PA63 on Chinese hamster ovary (CHO) cells. A control peptide (FDLPFTMSTPTP) had no effect. The weak inhibitory activity of the P1 peptide (half-

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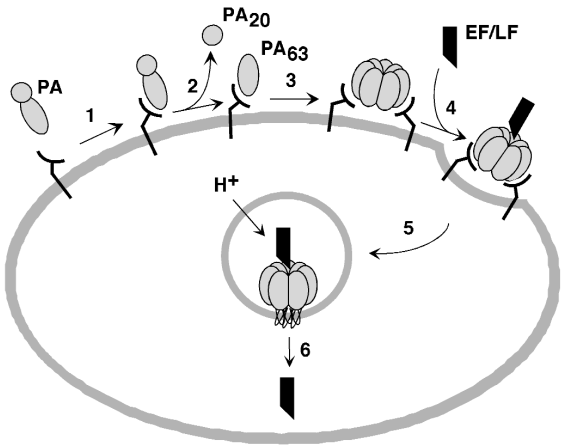

Figure 1. Assembly of anthrax toxin complexes and their entry into cells. (1) Binding of PA to its receptor. (2) Proteolytic activation of PA and dissociation of PA20. (3) Self-association of monomeric PA63 to form the heptameric prepore. (4) Binding of EF/LF to the prepore. (5) Endocytosis of the receptor–PA63–ligand complex. (6) pH-dependent insertion of PA63 and translocation of the ligand. The polyvalent inhibitor described in this report blocked step 4.

maximal inhibitory concentration or IC_{50} ~150 μM; see below) precluded its use as an inhibitor *in vivo*.

Synthesis of a polyvalent molecule. We[7–9] and others[10–13] have synthesized molecules presenting multiple copies of ligands and found that polyvalency can result in large enhancements in the efficiency of interaction of ligands with surfaces displaying multiple receptors. We synthesized a derivative of polyacrylamide that had multiple, covalently linked copies of the P1 peptide. This polymer (polyvalent inhibitor, or PVI) contained, on average, 22 peptide units and ~900 acrylamide monomers per molecule (one peptide per 40 acrylamide monomers). PVI bound to purified heptamer as evidenced by a mobility shift of the heptamer in native polyacrylamide gel electrophoresis (data not shown). PVI did not shift the mobility of whole PA, and the polyacrylamide backbone alone did not shift that of the heptamer.

Activity of the polyvalent inhibitor. PVI inhibited binding of radiolabeled LF_N to PA63 on CHO cells; the corresponding underivatized polyacrylamide did not (Fig. 3A). The IC_{50} of PVI, expressed in terms of the molar concentration of linked peptide, was 20 nM. This value represents an apparent 7,500-fold increase in inhibitory activity on a per-peptide basis, relative to the free peptide (IC_{50} of 150 μM). Increasing the peptide density on the polyacrylamide backbone (one peptide per 20 acrylamide monomers), or increasing the hydrophobicity of the inhibitor by further attaching ethanolamine, benzylamine, or tryptophan groups onto the peptide-containing polymeric backbone, did not lower the IC_{50} of PVI (data not shown).

To test the ability of PVI to inhibit the biological activity of anthrax toxin, we incubated CHO cells with PA and LF_NDTA, a fusion of the diphtheria toxin A chain (DTA) to the C terminus of LF_N. LF_NDTA binds to PA63 and enters cells by the same pathway as EF and LF. The DTA moiety catalyzes ADP-ribosylation of elongation factor-2 within the cytosol, and causes an inhibition of protein synthesis. This effect is easily measured[14]. PVI inhibited the toxicity of a mixture of PA and LF_NDTA with an IC_{50} of 6 nM, on a per-peptide basis (Fig. 3B). Neither the peptide nor underivatized polyacrylamide influenced toxicity.

We tested the efficacy of PVI in an animal model of intoxication using anthrax lethal toxin (Table 1). Fisher 344 rats are highly sensitive to this mixture of PA and LF (ref. 15), with death occurring within hours. We challenged rats with 10 times the minimal lethal dose (MLD) of PA and LF (1 × MLD = 50 pmol PA + 10 pmol LF). Inclusion of PVI (12 nmol peptide) in the mixture delayed symptoms, and a larger amount (75 nmol peptide) eliminated toxicity. The rats were also protected when PVI was injected 3–4 min after the challenge with PA and LF. Mixing both underivatized polyacrylamide backbone (125 μg) and monomeric peptide (75 nmol peptide) with the toxin had no effect on toxicity. There was no obvious toxicity associated with the injection of PVI during the week in which protected animals were monitored before being killed.

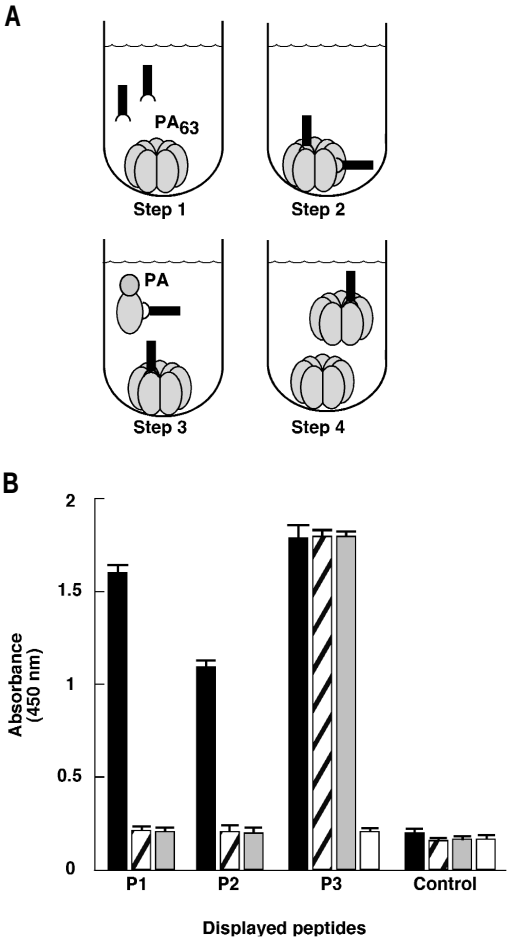

Figure 2. Selection of bacteriophages binding to PA63 heptamer at or near the EF/LF site. (A) Purified heptamer was coated on a plastic surface and a library of bacteriophages displaying 12-amino acid peptides was allowed to bind the heptamer (steps 1, 2). Purified intact PA was added to elute the phages that were bound to surfaces present both in intact PA and in the heptamer (step 3); the remaining phages were eluted with purified heptamer (step 4). (B) ELISA was done to assess the specificity of phages displaying different peptides (P1–3; see text for sequences), or the unselected PhD12 library as a negative control. The phages were allowed to bind to PA63 heptamer (black and striped bars) or intact PA (gray bars) adsorbed on the surface of a polystyrene plate. Each well was coated overnight with 1 μg protein in PBS. LF_N (10 μM) was added in some wells coated with heptamer (striped bars) to show that it could compete with phage binding. Phage displaying P3 did not bind to LF_N (white bars), demonstrating that it binds specifically to PA.

Discussion

The blockage of toxicity observed in our studies occurred by specific interaction of PVI with PA63 generated *in vivo* on host cells, and is not due to an interaction of PVI with native PA or LF in solution. This conclusion is supported by the finding that PVI affected the

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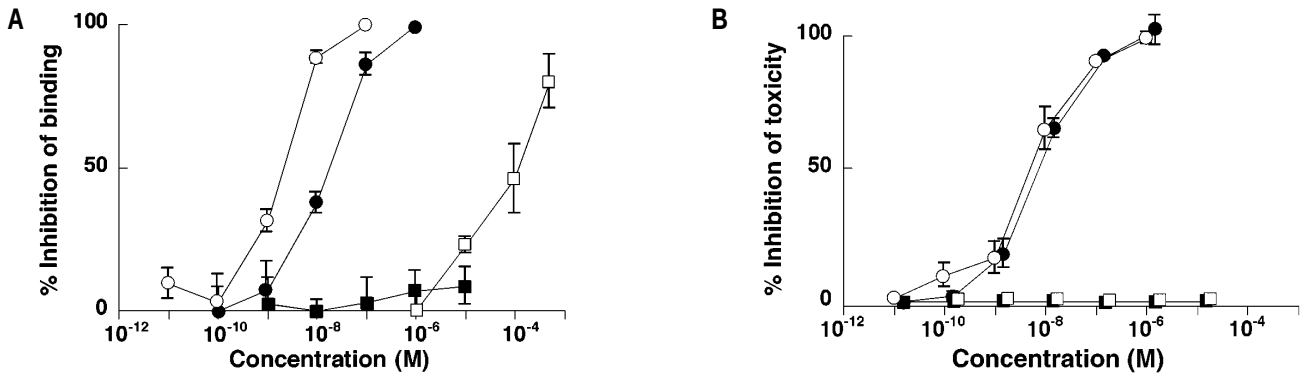

Figure 3. Inhibition of toxin action in cell culture. The effects of various amounts of LF_N (○), PVI (●), backbone (■) or monomeric peptide (□), were tested. The concentration of PVI is given as concentration of linked peptide, not the concentration of the whole PVI molecule. (A) Inhibition of toxin association. The association of toxin is measured as the binding of ^{35}S-labeled LF_N on CHO cells incubated with PA. (B) Inhibition of toxin cytotoxicity. The cytotoxicity is assayed by measuring the [^{3}H]leucine uptake in cells incubated with PA and LF_NDTA.

electrophoretic mobility only of PA63, and not of intact PA or LF (data not shown). As a further test, we incubated CHO cells with PA at 37°C and performed a western blot on cell extracts with anti-PA antibodies to quantify binding of PA to the surface of the cells and its conversion to heptameric PA63 (ref. 16). PVI had no effect on either parameter (data not shown). This observation indicates that the binding, proteolytic activation, and formation of the PA63 heptamer were unaltered by the inhibitor.

The efficacy of PVI in blocking the action of anthrax toxin *in vivo* suggests that it, or another inhibitor developed by a similar approach, could be a useful therapeutic ally against clinical anthrax. The approach used to develop PVI, involving the complementary technologies of combinatorial screening of small molecules and the generation of polyvalent ligands, may be generally useful in developing drugs and/or laboratory reagents. The use of a polymer with a flexible backbone avoids the necessity of understanding the spatial relationships among the binding sites on the target[7] and thus extends the applicability of our approach to targets in which these relationships are not known. We expect the approach to be most useful when the molecular target is oligomeric. It may thus be applicable, for example, to the development of inhibitors of oligomeric microbial structures or to strategies for increasing the therapeutic potency of antitumor peptides[17], growth factor receptor agonists[18], or molecules with related mechanisms of action.

Table 1. Inhibition of anthrax toxin action in a rat intoxication model

Inhibitor[a]	Amounts[b]			Outcome[c]
	PA	LF	Peptide	
None	0.5	0.1	0	Symptoms
Peptide + backbone	0.5	0.1	75	Symptoms
PVI	0.5	0.1	12	Delayed symptoms
PVI	0.5	0.1	75	No symptoms

[a]None: Purified protective antigen (PA) and lethal factor (LF) were mixed before injection with PBS; peptide + backbone: a mixture of monomeric HTSTYWWLDGAPK peptide and backbone; PVI: polyvalent inhibitor. In a separate experiment, PVI was administered 3–4 min after the toxin.
[b]The injected amounts of PA, LF, or peptide are given in nanomoles. For the PVI, the amount is given in nanomoles of peptide grafted on the polymeric backbones.
[c]After anesthesia, four Fisher 344 rats per group were injected intravenously in the dorsal vein of the penis[15], and the appearance of symptoms of intoxication was monitored. In the absence of active inhibitor, animals displayed symptoms ~1 h after injection. Delayed symptoms occurred after 2 h. Animals showing no symptoms were monitored for one week and then killed.

Experimental protocol

Phage-display selection and ELISA. Purified heptamer[19], 2 μg, was coated in Maxisorp tubes (Nunc, Roskilde, Denmark) in PBS overnight at 4°C. The tubes were blocked with PBS–2% BSA at 37°C for 2 h and washed with PBS. M13 bacteriophages (1.5×10^{11} plaque-forming units), present in a library displaying 12-amino acid peptides fused to the N terminus of the pIII protein (PhD12, New England Biolabs, Beverly, MA), were allowed to bind the heptamer in PBS–0.1% Tween 20 at room temperature for 60 min in round 1, 30 min in round 2, and 5 min in round 3. After binding, the wells were washed eight times. Purified intact PA (ref. 20; 15 μg in PBS) was added at room temperature for 1 h and then the remaining phages were eluted with 40 μg of heptamer in PBS at room temperature for 60 min in round 1 and overnight in rounds 2 and 3. The selection was repeated three times and the eluted phages amplified between rounds.

For ELISA, 1 μg of protein (PA63 heptamer, intact PA, or LF_N), purified as before[21], was coated in wells of a 96-well Maxisorp plate (Nunc) in PBS overnight at 4°C. The plate was blocked for 2 h at 37°C with PBS–2% BSA. Phages (10^8 plaque-forming units in PBS) were allowed to bind to the coated surface in the presence or absence of 10 μM LF_N. Bound phages were revealed using a monoclonal anti-M13 antibody coupled to horseradish peroxidase (Pharmacia, Uppsala, Sweden). The enzymatic activity was assayed by oxidation of 3,3′,5,5′-tetramethylbenzidine, measured by absorbance at 450 nm. ELISA were performed in duplicate and repeated twice. The results of a representative experiment are shown as mean ± s.e.m.

Peptide and polyvalent inhibitor synthesis. Peptides were synthesized, their N terminus acetylated, and their C terminus amidated by ADI Inc. (San Antonio, TX). All peptides were purified to >95% homogeneity, and characterized by HPLC and mass spectrometry. Poly (*N*-acryloyloxy succinimide) (PNAS) was prepared as described[22]. PNAS (20 mg, 118 μmol) was dissolved in 1.5 ml of *N*,*N*-dimethyl formamide (DMF). Triethylamine (50 μl, 360 μmol) and a solution of the peptide HTSTYWWLDGAPK (9.46 mg, 5.9 μmol) in DMF were added. We used a peptide with an additional lysine on the C terminus of the original P1 peptide to provide an easily accessible primary amino group (the ε-amine of lysine). The reaction mixture was stirred overnight, and then quenched by adding aqueous ammonium hydroxide (500 μl, 30% NH_3 by weight). The control polymer (polyacrylamide) was synthesized by the addition of aqueous ammonium hydroxide to a solution of the same sample of PNAS in DMF. The polymers were purified by exhaustive dialysis against distilled deionized water, followed by lyophilization. The size of the polyacrylamide backbone was determined by gel filtration chromatography after hydrolysis to polyacrylic acid (number-average molar mass, M_N = 65,000 Da; weight-average molar mass, M_W = 96,500 Da; polydispersity index = 1.48). The extent of coupling of the peptide to the polyacrylamide backbone was determined to be 2.5% by nuclear magnetic resonance (NMR), by comparing the integration of the aromatic peaks to the integration for the hydrogen α to the carbonyl group. The concentration of peptide in the polymer and of monomeric peptide was determined using the Edelhoch method[23].

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Cell binding of radioactively labeled LF_N. Confluent CHO cells in a 24-well plate were incubated for 1 h on ice in HAM's F12 medium buffered with 20 mM HEPES, pH 7.4, in the presence of 2×10^{-8} M PA cleaved by trypsin as described elsewhere[19]. LF_N was labeled with [^{35}S]methionine by *in vitro*–coupled transcription and translation, as described[24]. After one wash with cold PBS, radioactive LF_N was added for 1 h to the cells on ice in the presence of various amounts of LF_N, PVI, underivatized polyacrylamide, or monomeric peptide. The cells were then washed and lysed, and the radioactivity in the lysate was measured. The background of LF_N bound to cells in absence of PA was subtracted and was <5% of control. The inhibition of LF_N binding is expressed as the percentage of radioactivity of the control (radioactivity bound on cells incubated without inhibitor) that was not bound. The results are the mean ± s.e.m. of three independent experiments.

Cytotoxicity assay of LF_NDTA. Toxicity was assayed using LF_NDTA as described[14]. Briefly, confluent CHO cells in a 96-well plate were incubated with 10^{-9} M PA and 2×10^{-11} M LF_NDTA with various amounts of LF_N, PVI, backbone, or peptide. The cells were incubated for 4 h at 37°C, and then protein synthesis was assayed by monitoring [^{3}H]leucine incorporation in cellular proteins[14]. The amount of radioactivity incorporated in the absence of inhibitor was <2% of control. The inhibition of toxicity is expressed as the percentage of radioactivity of the control (radioactivity recovered from cells incubated without LF_NDTA). Each experiment was done in duplicate. The results are the mean ± s.e.m. of three independent experiments.

Rat intoxication. Purified PA (40 μg) and LF (ref. 21; 8 μg) diluted in PBS were mixed with PBS, a mixture of 125 μg of peptide, and 125 μg of polyacrylamide, 72 μg or 450 μg of PVI (the total volume injected per animal was 200 μl). Fisher 344 rats (250–300 g, Harlan, Indianapolis, IN) were injected intravenously in the dorsal vein of the penis[15] after anesthesia by intraperitoneal injection of ketamine and acepromazine. Four rats per group were injected with the different mixtures, and the appearance of symptoms of intoxication monitored. When the symptoms were obvious, the rats were killed to avoid unnecessary distress. In post-challenge protection experiments, four rats were injected with PA and LF diluted in PBS. After 3–4 min, a new syringe was used to inject at the same site PVI diluted in PBS.

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1. Dixon, T.C., Meselson, M., Guillemin, J. & Hanna, P.C. Anthrax. *N. Engl. J. Med.* **341**, 815–826 (1999).
2. Leppla, S.H. Anthrax toxins. In *Bacterial toxins and virulence factors in diseases. Handbook of natural toxins,* Vol. 8. (eds Moss, J., Iglewski, B., Vaughan M. & Tu, A.) 543–572 (Dekker, New York, NY; 1995).
3. Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H. & Liddington R.C. Crystal structure of the anthrax toxin protective antigen. *Nature* **385**, 833–838 (1997).
4. Duesbery, N.S. *et al.* Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science* **280**, 734–737 (1998).
5. Zwick, M.B., Shen, J. & Scott, J.K. Phage-displayed peptide libraries. *Curr. Opin. Biotechnol.* **9**, 427–435 (1998).
6. Arora, N. & Leppla, S.H. Residues 1–254 of anthrax toxin lethal factor are sufficient to cause cellular uptake of fused polypeptides. *J. Biol. Chem.* **268**, 3334–3341 (1993).
7. Mammen, M., Choi, S.-K. & Whitesides, G.M. Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew. Chem. Int. Edn. Engl.* **37**, 2754–2794 (1998).
8. Spaltenstein, A. & Whitesides, G.M. Polyacrylamides bearing pendant-sialoside groups strongly inhibit agglutination of erythrocytes by influenza virus. *J. Am. Chem. Soc.* **113**, 686–687 (1991).
9. Rao, J., Lahiri, J., Isaacs, L., Weiss, R.M. & Whitesides, G.M. A trivalent system from vancomycin. D-ala-D-Ala with higher affinity than avidin-biotin. *Science* **280**, 708–711 (1998).
10. Matrosovitch, M.N., Mochalova, L.U., Marinina, V.P., Byramova, N.E. & Bonvin, N.V. Synthetic polymeric sialoside inhibitors of influenza virus receptor-binding activity. *FEBS Lett.* **272**, 209–212 (1990).
11. Gordon, E.J., Sanders, W.J. & Kiessling, L.L. Synthetic ligands point to cell surface strategies. *Nature* **392**, 30–31 (1998).
12. Kramer, R.H. & Karpen, J.W. Spanning binding sites on allosteric proteins with polymer-linked ligand dimers. *Nature* **395**, 710–713 (1998).
13. Kitov, P.I. *et al.* Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. *Nature* **403**, 669–672 (2000).
14. Milne, J.C., Blanke, S.R., Hanna, P.C. & Collier, R.J. Protective antigen-binding domain of anthrax lethal factor mediates translocation of a heterologous protein fused to its amino- or carboxy-terminus. *Mol. Microbiol.* **15**, 661–666 (1995).
15. Ezzell, J.W., Ivins, B.E. & Leppla, S.H. Immunoelectrophoretic analysis, toxicity, and kinetics of *in vitro* production of the protective antigen and lethal factor components of *Bacillus anthracis* toxin. *Infect. Immun.* **45**, 761–767 (1984).
16. Milne, J.C., Furlong, D., Hanna, P.C., Wall, J.S. & Collier, R.J. Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *J. Biol. Chem.* **269**, 20607–20612 (1994).
17. Koivunen, E. *et al.* Tumor targeting with a selective gelatinase inhibitor. *Nat. Biotechnol.* **17**, 768–774 (1999).
18. Wrighton, N.C. *et al.* Small peptides as potent mimetics of the protein hormone erythropoietin. *Science* **273**, 458–463 (1996).
19. Miller, C.J., Elliott, J.L. & Collier, R.J. Anthrax protective antigen: prepore-to-pore conversion. *Biochemistry* **38**, 10432–10441 (1999).
20. Benson, E.L., Huynh, P.D., Finkelstein, A. & Collier, R.J. Identification of residues lining the anthrax protective antigen channel. *Biochemistry* **37**, 3941–3948 (1998).
21. Zhao, J., Milne, J.C. & Collier, R.J. Effect of anthrax toxin's lethal factor on ion channels formed by the protective antigen. *J. Biol. Chem.* **270**, 18626–18630 (1995).
22. Mammen, M., Dahmann, G. & Whitesides G.M. Effective inhibitors of hemagglutination by influenza virus synthesized from polymers having active ester groups. Insight into mechanism of inhibition. *J. Med. Chem.* **38**, 4179–4190 (1995).
23. Pace, C.N., Vajdos, F., Fee, L., Grimsley, G. & Gray, T. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4**, 2411–2423 (1995).
24. Wesche, J., Elliott, J.L., Falnes, P.O., Olsnes, S. & Collier, R.J. Characterization of membrane translocation by anthrax protective antigen. *Biochemistry* **37**, 15737–15746 (1998).