-Supporting Information-

Discovery of a TNF-α Antagonist Using Chondroitin Sulfate Microarrays

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Conjugation of CS Oligosaccharides to 1,2-(Bisaminooxy)ethane for Microarray Production

Ozonolysis of the anomeric allyl group and linkage of CS compounds **1-4**[1,2] to 1,2-(bisaminooxy)ethane[3] proceeded as follows: oligosaccharide (0.51 μmol) was dissolved in MeOH (500 μL) and cooled to –78 °C. O_3 was bubbled through the reaction until a blue color persisted (1 min). The reaction was then purged with N_2 until colorless, quenched with Ph_3P beads (3 mg), and gradually warmed to rt over 12 h. It was filtered and the product concentrated to afford the desired aldehyde as a white solid. The aldehyde (0.51 μmol) was then reacted for 14 h at rt with 1,2-(bisaminooxy)ethane hydrochloride (1.4 mg, 15 μmol) that had been dissolved in H_2O (100 μL) and pH adjusted to 5.0 with 1 M NaOH. The resulting oxime product was purified using a SepPak C18 column (500 mg, H_2O) and Sephadex G-10 (CS-E disaccharide, H_2O) or Sephadex G-25 (tetrasaccharides, H_2O) to afford a white solid in quantitative yield (0.51 μmol). **CS-A aminooxy:** ESI MS: *m/z*: calcd for $C_{32}H_{48}N_4Na_3O_{31}S_2$: 1117.1; found 1117.0. **CS-C aminooxy:** ESI MS: *m/z*: calcd for $C_{32}H_{48}N_4Na_3O_{31}S_2$: 1117.1; found 1117.0. **CS-E aminooxy:** ESI MS: *m/z*: calcd for $C_{32}H_{46}N_4Na_5O_{37}S_4$: 1321.0; found 1321.0. **CS-E di amminooxy:** ESI MS: *m/z*: calcd for $C_{18}H_{28}N_3Na_2O_{20}S_2$: 716.1; found 716.0. Mass spectra were obtained on a PerkinElmer/Sciex API 365 triple quadrupole/electrospray tandem mass spectrometer in the Protein/Peptide MicroAnalytical Laboratory at the California Institute of Technology.

The relative concentrations of the aminooxy oligosaccharides were calibrated to one another using the carbazole assay for uronic acid residues.[4] Briefly, the acid borate reagent (1.5 mL of 0.80 g sodium tetraborate, 16.6 mL H_2O, and 83.3 mL H_2SO_4) was added to 20-mL glass vials with Teflon caps. The

aminooxy oligosaccharides (50 μL of a 0.2 mg/mL stock in H_2O) were added and the solution placed in a boiling H_2O bath for 10 min. Following addition of the carbazole reagent (50 μL of 0.1% *w/v* carbazole in 100% EtOH), the solution was boiled for 15 min. The absorbance was read at 530 nm and compared to a D-glucuronolactone standard in H_2O.

Carbohydrate Microarrays

Solutions of the aminooxy oligosaccharides (in 300 mM NaH_2PO_4, pH 5.0, 10 μL/well in a 384-well plate) were arrayed on Hydrogel Aldehyde slides (NoAb Biodiscoveries) by using a Microgrid II arrayer (Biorobotics) to deliver sub-nanoliter volumes at rt and 50% humidity. Concentrations of carbohydrates ranged from 0 – 500 μM. The resulting arrays were incubated in a 70% humidity chamber at rt for 12 h and then stored in a low humidity, dust-free dessicator. The pH and reaction time were optimized to provide maximum immobilization of the compound. A pH screen with values between 4.0 and 9.0 (0.5 unit increments) showed that pH 5.0 yielded maximal binding to the slide and a time screen with values between 0 and 16 hours (4 h increments) showed that 12 h provided maximal immobilization. Non-specific attachment of CS oligosaccharides lacking the aminooxy linker (e.g., compounds **1-4**) was not observed. Prior to use, the arrays were outlined with a hydrophobic pen (Super Pap Pen, Research Products International) to create a boundary for the protein treatments and rinsed three times with H_2O. The slides were then blocked by treatment with $NaBH_4$ (125 mg) in 140 mM NaCl, 2.7 mM KCl, 5.4 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (phosphate buffered saline, PBS, 50 mL) at rt for 5 min with gentle rocking and washed five times for 3 min with PBS. For all incubations, the slides were placed in a covered pipette tip box. Human TNF-α (Peprotech), FGF-1 (R&D Systems; both reconstituted to 2 μM in 0.1% Triton X-100 in PBS), cell culture supernatant containing monoclonal anti-CS-A antibody, or cell culture supernatant containing monoclonal anti-CS-E antibody (both 1:1 in 0.1% Triton X-100 in PBS) were spotted onto the slides in 250 μL quantities, and incubated statically at rt for 2 h. The slides were then washed as previously described and incubated with the appropriate primary antibody [anti-TNF-α (Peprotech) or anti-FGF-1 (R&D Systems); 1:1000 in 0.1% Triton X-100 in PBS] for 2 h at rt with gentle rocking. Following the incubation, the slides were washed as previously described and treated in the dark at rt with a secondary IgG antibody conjugated to Cy3 (Amersham; 1:5000 in 0.1% Triton X-100 in PBS) at rt for 1 h with gentle

rocking. The slides were washed three times for 2 min with PBS, two times for 1 min with H_2O, and dried under a gentle stream of N_2. Microarrays were analyzed at 532 nm using a GenePix 5000a scanner, and fluorescence quantification was performed using GenePix 6.0 software after correction for local background. Each protein was analyzed in triplicate, and the data represent an average of at least five spots for a given carbohydrate concentration (Supporting Figure 1). All solutions used for the carbohydrate microarrays were sterile-filtered through a 0.2 μm syringe filter prior to use.

CS-A and CS-E Antibody Development

Balb/c mice were immunized with CS-A and CS-E tetrasaccharides conjugated to keyhole limpet hemocyanin (KLH), and sera and cell culture supernatants were tested for antibodies via ELISA and dot blot analysis. Ozonolysis of the anomeric allyl group of the tetrasaccharide (0.51 μmol) as previously described was followed by treatment with KLH (0.44 mg, 0.0063 μmol) and $NaCNBH_3$ (0.5 mg) in H_2O (pH'd with 5% K_2CO_3 to pH 9.5) for 2 d at rt. The product was then exhaustively dialyzed against 0.01 M Na_2HPO_4, 0.15 M NaCl, pH 7.4 at 4 °C and the protein concentration determined by BCA assay (Pierce). The epitope density was determined by comparing the conjugated proteins to the unconjugated proteins using the Habeeb assay.[5] In short, to the protein solution (10 μL) in PBS (40 μL) were added 0.1% trinitrobenzenesulfonic acid (50 μL) and 4% $NaHCO_3$, pH 9.5 (50 μL). The mixture was incubated at 40 °C for 2 h, quenched with 10% SDS (50 μL), 1 M HCl (25 μL), and H_2O (500 μL), and the absorbance at 363 nm was measured. The epitope densities were as follows: CS-A conjugate = 15 and CS-E conjugate = 14.

Three Balb/c female mice, 4-6 weeks old, were primed and boosted at 2-week intervals for a total of 5 intraperitoneal injections (5 μg per injection). CS-A- or CS-E-KLH conjugates were mixed with RIBI adjuvant (RIBI Immunochem) for the first two injections, and a final series of 3 boosts was performed without adjuvant. Bleeds were taken 1 week after each injection and monitored by dot blot analysis. The most responsive mouse was boosted and sacrificed after three days. Spleen cells were fused with HL-1 murine myeloma cells (Ventrex) using polyethylene glycol (PEG 1500, Boehringer-Mannheim) as described previously by Lebron *et al.*[6] Multiclonal and monoclonal cell lines were then screened via ELISA analysis.

Antibody ELISA Analysis

CS tetrasaccharides **1**-**3** were conjugated to bovine serum albumin (BSA) as follows. Ozonolysis of the anomeric allyl group of the CS-A, -C, and -E tetrasaccharides (0.51 μmol) as described above was followed by treatment of each compound with BSA (0.34 mg, 0.0051 μmol) and $NaCNBH_3$ (0.5 mg) in H_2O (pH 9.5 using K_2CO_3) for 2 d at rt. The CS-BSA conjugates were then exhaustively dialyzed against 0.01 M Na_2HPO_4, 0.15 M NaCl, pH 7.4 at 4 °C, and the protein concentrations were determined using the BCA assay (Pierce). The epitope densities were measured by comparing the conjugated proteins to the unconjugated proteins using the Habeeb assay. The epitope densities were as follows: CS-A conjugate = 14, CS-C conjugate = 16, CS-E conjugate = 14.

The BSA conjugates (1 μg/mL in 50 mM Na_2CO_3, pH 9.6) were added to a 384-well NUNC Maxisorp clear plate (25 μL per well), and the plate was sealed and incubated for 12 h at 4 °C. The wells were aspirated, washed four times with PBS containing 0.05% Tween-20 (PBST, 75 μL/wash), and blocked for 2 h at rt with 10% horse serum (Gibco) in PBS (75 μL). After the blocking step, the plate was washed four times with PBST, and the supernatants from the monoclonal anti-CS-A or CS-E antibody producing cultures (25 μL) were added to the wells and incubated at rt for 2 h. Following aspiration, the wells were washed four times with PBST and treated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Pierce; 1:10,000, 25 μL/well) in blocking buffer for 1 h at rt. The wells were again aspirated, washed four times with PBST, and then developed with ABTS liquid substrate solution (Sigma; 25 μL/well, solution at rt) for 30 min at rt. Color development was monitored on a Victor plate reader (PerkinElmer) at 405 nm. Only clones specific for the CS-A or CS-E tetrasaccharides and with absorbance values greater than 1.0 were kept for subsequent dot blot screening.

Dot Blot Analysis

Immunoblotting analysis was performed by spotting solutions of the BSA conjugates (relative epitope density adjusted, 1-100 ng, 1 μL/spot) in 10 mM Tris•HCl, 0.02% Nonidet P-40, pH 7.5 onto 0.45 μm nitrocellulose, allowing the spots to air dry, and fixing the blots with 40% MeOH, 10% AcOH, 50%

H_2O for 15 min at rt with gentle rocking. The dot blots were then blocked for 30 min in 5% non-fat milk containing 50 mM Tris•HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20 (TBST) followed by treatment with the monoclonal antibody producing cell culture supernatant in blocking buffer (1:1) for 2 h at rt with gentle rocking. The blots were then washed with TBST three times for 10 min and treated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Pierce; 1:10,000) in blocking buffer for 1 h at rt with gentle rocking. The dot blots were washed with TBST three times for 10 min and visualized by chemiluminescence (SuperSignal West Pico, Pierce). Consistent with the microarray data, highly selective binding of the antibodies to their respective sulfated antigens was observed, and weak binding of the CS-E antibody to the CS-C motif was noted at very high concentrations.

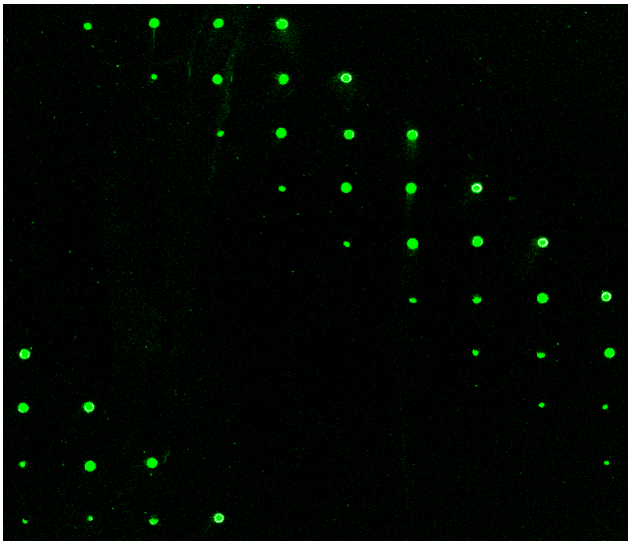
TNF-α ELISA Analysis

Human recombinant TNFR1 (Peprotech; 1 μg/mL in 50 mM Na_2CO_3, pH 9.6) was added to a 384-well NUNC Maxisorp clear plate (25 μL per well), and the plate was sealed and incubated for 12 h at 4 °C. The wells were aspirated, washed four times with PBS containing 0.05% Tween-20 (PBST, 75 μL/wash), and blocked for 2 h at rt with 1% BSA in PBS (75 μL). During this time, biotinylated TNF-α (PerkinElmer; 25 μL/well of a 1 nM solution in 0.05% Tween-20, 0.1% BSA in PBS) was pre-incubated with the indicated concentrations of polysaccharides enriched in the CS-A, CS-C, or CS-E motifs (Seikagaku; 0.1 – 100 μM in H_2O) or CS-E tetrasaccharide **3** (0.01 – 5 mM in H_2O). After the blocking step, the plate was washed four times with PBST, and the solutions of CS and TNF-α were added to the wells and incubated at rt for 2 h. Following aspiration, the wells were washed four times with PBST and treated with streptavidin-HRP (Pierce; 25 μL/well, 1:2000 in 0.05% Tween-20, 0.1% BSA in PBS) for 30 min at rt. The wells were again aspirated, washed four times with PBST, and then developed with ABTS liquid substrate solution (Sigma; 25 μL/well, solution at rt) for 30 min at rt. Color development was monitored on a Victor plate reader (PerkinElmer) at 405 nm. Each carbohydrate concentration was analyzed in triplicate, and the absorbance values were corrected for background in the absence of carbohydrate and normalized with respect to the absorbance value at the lowest carbohydrate concentration.

Caspase Assay

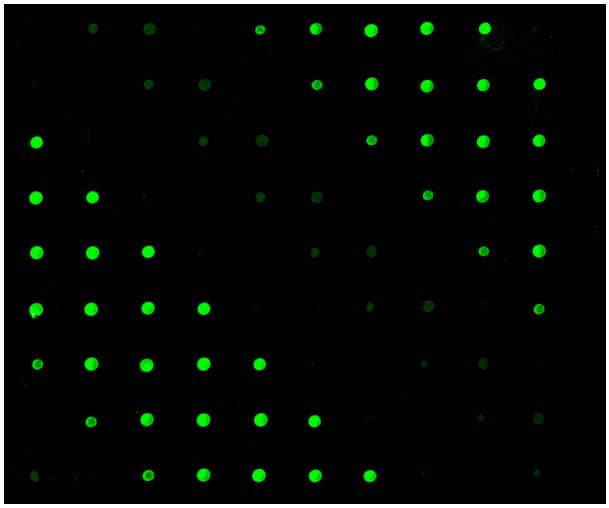
U937 cells (ATCC) sensitive to TNF-α-induced apoptosis[7] were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C and 5% CO_2/O_2. Cells were grown in every other well of a 384-well NUNC sterile, clear plate (25,000 cells/well, 25 μL/well) for 18 h. Solutions of the carbohydrates at the indicated concentrations (0.01 – 4000 μM in sterile PBS) were pre-incubated with TNF-α (1 μL/well of a 125 ng/mL solution in sterile PBS) at rt. After 2h, the solutions were added to the cells and incubated for 18 h. At this time, caspase 3/7 activity was analyzed as described by Shi *et al.*[8] using the Apo-One homogeneous caspase 3/7 assay kit (Promega) according to the manufacturer's protocol. Readings were taken every 30 min for 18 h at rt on a Victor plate reader. By this time, caspase 3/7 activity had reached a plateau, and the endpoints of the various treatments were used for data analysis. Endpoint fluorescence values for controls containing only cells were subtracted from the experimental endpoint values, and the resulting values were then normalized with respect to that of the lowest carbohydrate concentration. Each carbohydrate concentration was repeated in triplicate. The CS-E tetrasaccharide and polysaccharide had no effect on the extent of apoptosis in the absence of TNF-α.

A



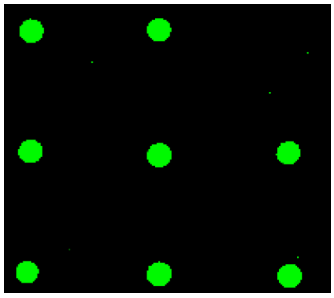
25E	5A	15A	25A	50A	70Di	200Di	0E	75E	12.5Di
12.5Di	22.5E	5A	15A	25A	50A	70Di	200Di	0E	7.5E
7.5 E	12.5Di	22.5E	5A	15A	25A	50A	70Di	200Di	0E
0E	7.5E	12.5Di	22.5E	5A	15A	25A	50A	70Di	200Di
200Di	0E	7.5E	12.5Di	22.5E	5A	15A	25A	50A	70Di
70Di	200Di	0E	7.5E	12.5Di	22.5E	5A	15A	25A	50A
50A	70Di	200Di	0E	7.5E	12.5Di	22.5E	5A	15A	25A
25A	50A	70Di	200Di	0E	7.5E	12.5Di	22.5E	5A	15A
15A	25A	50A	70Di	200Di	0E	7.5E	12.5Di	22.5E	5A
5A	15A	25A	50A	70Di	200Di	0E	7.5E	12.5Di	22.5E

B



90A	400A	2.5C	12.5E	17.5E	30E	60E	100E	Blank
Blank	90A	400A	2.5C	12.5E	17.5E	30E	60E	100E
40A	Blank	90A	400A	2.5C	12.5E	17.5E	30E	60E
100E	40A	Blank	90A	400A	2.5C	12.5E	17.5E	30E
60E	100E	40A	Blank	90A	400A	2.5C	12.5E	17.5E
30E	60E	100E	40A	Blank	90A	400A	2.5C	12.5E
17.5E	30E	60E	100E	40A	Blank	90A	400A	2.5C
12.5E	17.5E	30E	60E	100E	40A	Blank	90A	400A
2.5C	12.5E	17.5E	30E	60E	100E	40A	Blank	90A
400A	2.5C	12.5E	17.5E	30E	60E	100E	40A	Blank

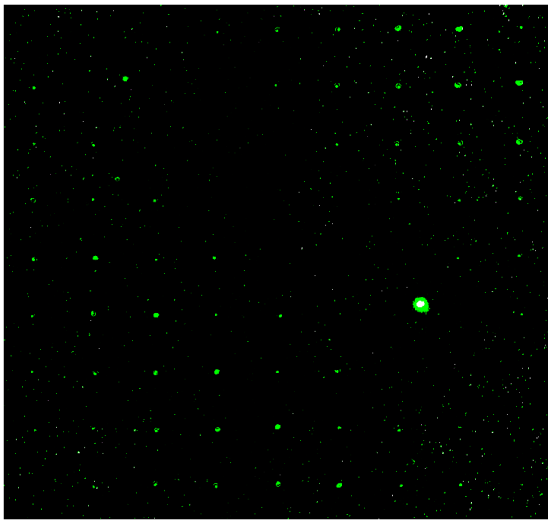
C



60E	100E	40A
30E	60E	100E
17.5E	30E	60E

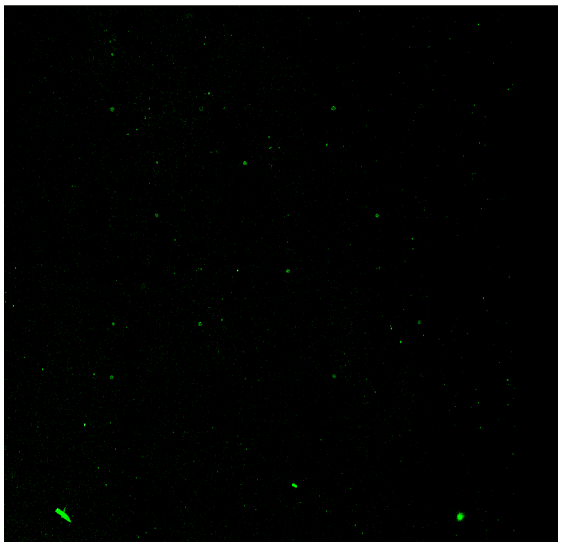
Supporting Figure 1. **CS microarrays for the analysis of glycosaminoglycan-protein interactions.** Each microarray contained 1500 spots. Representative portion of the microarrays, illustrating spot morphology and fluorescence intensity after incubation with (A) CS-A antibody and (B) CS-E antibody. (C) Higher magnification of a representative portion of the CS microarray after incubation with the CS-E antibody. The panels on the right indicate the corresponding oligosaccharides and their concentrations for each spot shown. Values are in μM. A = CS-A tetrasaccharide, C = CS-C tetrasaccharide, Di = CS-E disaccharide, E = CS-E tetrasaccharide.

A



60A	100A	500A	50C	10E	20E	35E	70E	25Di
50Di	60A	100A	500A	50C	10E	20E	35E	70E
25Di	50Di	60A	100A	500A	50C	10E	20E	35E
70E	25Di	50Di	60A	100A	500A	50C	10E	20E
35E	70E	25Di	50Di	60A	100A	500A	50C	10E
20E	35E	70E	25Di	50Di	60A	100A	500A	50C
10E	20E	35E	70E	25Di	50Di	60A	100A	500A
50C	10E	20E	35E	70E	25Di	50Di	60A	100A
500A	50C	10E	20E	35E	70E	25Di	50Di	60A

B



60A	100A	500A	50C	10E	20E	35E	70E	25Di
50Di	60A	100A	500A	50C	10E	20E	35E	70E
25Di	50Di	60A	100A	500A	50C	10E	20E	35E
70E	25Di	50Di	60A	100A	500A	50C	10E	20E
35E	70E	25Di	50Di	60A	100A	500A	50C	10E
20E	35E	70E	25Di	50Di	60A	100A	500A	50C
10E	20E	35E	70E	25Di	50Di	60A	100A	500A
50C	10E	20E	35E	70E	25Di	50Di	60A	100A
500A	50C	10E	20E	35E	70E	25Di	50Di	60A

Supporting Figure 2. **CS microarrays for the analysis of glycosaminoglycan-protein interactions.** Each microarray contained 1500 spots. Representative portion of the microarrays, illustrating spot morphology and fluorescence intensity after incubation with (A) TNF-α and (B) the negative control, FGF-1. The panels on the right indicate the corresponding oligosaccharides and their concentrations for each spot shown. Values are in μM. A = CS-A tetrasaccharide, C = CS-C tetrasaccharide, Di = CS-E disaccharide, E = CS-E tetrasaccharide.

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