Cationic Peptides and Peptidomimetics Bind Glycosaminoglycans as Potential Sema3A Pathway Inhibitors

Miriam Corredor, Roman Bonet, Alejandra Moure, Cecilia Domingo, Jordi Bujons, Ignacio Alfonso, Yolanda Pérez, and Ángel Messeguer

1Departamento de Química Biológica y Modelización Molecular and 2Servicio de Resonancia Magnética Nuclear, Instituto de Química Avanzada de Catalunya, IQAC-CSIC, Barcelona, Spain

ABSTRACT Semaphorin3A (Sema3A) is a vertebrate-secreted protein that was initially characterized as a repulsive-guidance cue. Semaphorins have crucial roles in several diseases; therefore, the development of Sema3A inhibitors is of therapeutic interest. Sema3A interacts with glycosaminoglycans (GAGs), presumably through its C-terminal basic region. We used different biophysical techniques (i.e., NMR, surface plasmon resonance, isothermal titration calorimetry, fluorescence, and UV-visible spectroscopy) to characterize the binding of two Sema3A C-terminus-derived basic peptides (FS2 and NFS3) to heparin and chondroitin sulfate A. We found that these peptides bind to both GAGs with affinities in the low-micromolar range. On the other hand, a peptoid named SICHI (semaphorin-induced chemorepulsion inhibitor), which is positively charged at physiological pH, was first identified by our group as being able to block Sema3A chemorepulsion and growth-cone collapse in axons at the extracellular level. To elucidate the direct target for the reported SICHI inhibitory effect in the Sema3A signaling pathway, we looked first to the protein-protein interaction between secreted Sema3A and the Nrp1 receptor. However, our results show that SICHI does not bind directly to the Sema3A sema domain or to Nrp1 extracellular domains. We evaluated a new, to our knowledge, hypothesis, according to which SICHI binds to GAGs, thereby perturbing the Sema3A-GAG interaction. By using the above-mentioned techniques, we observed that SICHI binds to GAGs and competes with Sema3A C-terminus-derived basic peptides for binding to GAGs. These data support the ability of SICHI to block the biologically relevant interaction between Sema3A and GAGs, thus revealing SICHI as a new, to our knowledge, class of inhibitors that target the GAG-protein interaction.

INTRODUCTION

Semaphorin3A (Sema3A) is a member of the class 3 secreted semaphorins. It was originally characterized as an axon guidance molecule that participates in regulating the process by which axon growth cones are directed to their proper targets during the formation of the nervous system. In the adult central nervous system (CNS), Sema3A acts as a canonical repulsive axon guidance molecule, inhibiting CNS regenerative axonal growth and sprouting. Thus, interfering with Sema3A signaling is an important therapeutic goal for achieving functional recovery after CNS injuries (1,2). Moreover, semaphorins also play a role in many processes outside the nervous system (3–5). Structurally, Sema3A is formed by an N-terminal, seven-bladed, β-propeller sema domain, followed by a cysteine-rich PSI (plexin, semaphorin, integrin) domain, an immunoglobulin (Ig) domain, and an Arg/Lys-rich basic region in the C-terminal end.

On the other hand, many recent studies have suggested a role for proteoglycans (PGs) in Sema3A and its Nr1 receptor function (6–11). These studies showed that full-length Sema3A adheres to the PG component of the extracellular matrix (ECM) at perineuronal nets or cortical neurons, and selectively binds to heparin and chondroitin sulfate-E (CS-E) glycosaminoglycan (GAG) units (9). It has been suggested that this interaction is mediated by the Sema3A C-terminal polybasic region (amino acids 716–771; Fig. 1 A) and that it can potentiate the Sema3A signaling pathway (6). Findings by de Wit et al. (6) indicated that a Sema3A fragment without the C-terminal part (comprising only the N-terminal semaphorin domain) is the predominant form in the extracellular medium, whereas the whole...
Sema3A (containing the polybasic region) binds mainly to CSPGs at the cell surface. Other studies reported that both the active full-length Sema3A (with collapsing activity) and the inactive Ig-basic C-terminal region (without the semaphorin domain) were able to bind dorsal root ganglion sensory axons in the dorsal columns, but a construct without the basic domain (comprising only Sema-PSI-Ig domains) exhibited 20-fold lower collapsing activity and weakly stained the dorsal columns (10,11). Moreover, it was shown that the polybasic C-terminal region is important for Sema3A biological activity through its binding to Nrp1, an interaction that is regulated by furin protease cleavage (13,14).

CSPGs are not only a structural component of the ECM, they also function as essential modulators of ligand-receptor interactions in key signaling pathways for many developmental processes and in the regeneration and plasticity of adult injured neurons. Several investigations have shown that CSPGs serve as axonal growth-inhibitory components in CNS tissue by direct interaction with specific protein receptors (e.g., LAR, RPTP, integrins, and NgR1/3) (12,15–17). Furthermore, other semaphorin family members besides the class 3 semaphorins and coreceptor Nrp1 interact with sulfated PGs. For example, Sema5A acts as both positive and negative guidance cues in neuronal outgrowth depending on its interaction with heparan sulfate (HS) or CS (Fig. 1B) (18). It is known that other secreted axon guidance molecules (e.g., Slits and Netrins) associate with GAGs (19,20). In addition, Sema3A receptor Nrp1 is also posttranscriptionally modified by GAGs (by CS at Ser612) in some nonneuronal cell types (21,22).

All of these findings support the notion that PGs could be an additional component of the Sema3A-PlxnA2-Nrp1 signaling system. PGs colocalize with Sema3A/Nrp1 at the cell surface, they have negative charges that could interact with the basic Sema3A tail, and their presence affects the Sema3A-Nrp1 protein-protein interaction. Noticeably, there is no evidence for a direct physical interaction between a specific sequence in the C-terminal region of Sema3A and GAGs. Herein, we describe the binding of two peptides (FS2 and (N)FS3; Fig. 1A) derived from the Sema3A C-terminal polybasic region to GAGs. We studied these interactions using a variety of biophysical techniques.

In addition, in a previous article (23) we identified a peptoid, termed SICHI (semaphorin-induced chemorepulsion inhibitor; cf. the structure in Fig. 1C), that blocks Sema3A chemorepulsion and growth-cone collapse in axons at submicromolar concentrations at the extracellular level (not directly affecting downstream signaling). The components of the protein-protein interacting signaling system—Sema3A, Neuropilin 1 (Nrp1), and Plexin A2 (PlxnA2) receptors (24)—were proposed as the main candidates for SICHI targeted inhibition, although this was not definitely proven. In an attempt to clarify the SICHI mode of action, we decided to confirm the direct target for the SICHI inhibitory effect in the Sema3A signaling pathway. We investigated not only the previously proposed protein targets (Sema3A and Nrp1) but also the possibility that SICHI could interact directly with GAGs. In addition to using different biophysical techniques, we employed molecular-dynamics (MD) simulations to characterize in silico the interaction between SICHI and GAGs.

**MATERIALS AND METHODS**

### Proteins, peptides, and GAGs

SICHI was prepared as described previously (25). Human Sema3A-65K (residues 26–555) and mouse Nrp1 a1a2b1b2 (residues 22–586) were cloned in the baculovirus vector pBAC3 (Merck-Millipore, Billerica, MA), which contains a His tag and a secretory signal in the N-terminus for expression of the proteins to the extracellular medium. The constructs were cotransfected in Sf9 cells using the BacMagic-3 kit (Merck-Millipore). After two rounds of amplification in Sf9 cells, recombinant viruses were used to infect Hi5 cells (ThermoFisher Scientific, Waltham, MA).
grown in suspension cultures at 28°C and 110 rpm. At 64 h postinfection, the supernatant was collected and proteins were purified using Ni2+ affinity and gel-filtration chromatography.

Synthetic peptides from the Sema3A C-terminal domain (FS2, FS3, and (NF)FS3; purity > 95%), without N- or C-terminal modification, were purchased from GenScript USA (Piscataway, NJ). Heparin is a degradation product derived from natural HS isolated from porcine lung, which typically consists of chains with a molecular mass in the range of 6–30 kDa. The structure of CS differs from that of HS/heparin by the presence of different disaccharide repeats and glycosidic linkages, the lack of N-sulfonation and a more even distribution of sulfation along the chain. Due to the limited commercial availability of CS-E, we decided to use high-molecular-weight (high-MW) heparin, dp14 heparin, and CS-A as GAG models (CS-A is mainly 4-sulfated, whereas CS-E is 4,6-disulfated at the GalNAc residues; Fig. 1). High-MW heparin sodium salt (from porcine intestinal mucosa, average molecular mass 12,000 Da), CS-A ammonium salt (from bovine trachea, average molecular mass 15,000 Da) GAGs, CDS (CS sodium salt from shark cartilage, a mixture comprised mainly of CS-A, CS-C, and CS-D), and hyaluronic acid (HA; from bovine vitreous humor, average molecular mass 300–800 kDa) were obtained from Sigma Aldrich (St. Louis, MO). Heparin dp14 oligosaccharide (average molecular mass ~4100 Da) was purchased from Iduron. All GAGs used in the binding assays were used without further purification.

Heparin attached covalently to 4% cross-linked agarose beads was purchased from Sigma-Aldrich (heparin-agarose, H0402). We checked the final heparin-agarose buffered suspension by adding 50% D2O and acquiring a 1H NMR spectrum (data not shown), and confirmed that no heparin had leached out into the supernatant solution.

NMR measurements

NMR experiments were acquired at 25°C on a 500 MHz spectrometer (Varian Inova; Agilent Technologies, Santa Clara, CA) equipped with an AutoX inverse double-resonance probe with a z-shielded pulsed-field gradient coil. Data were processed using the software programs VnmrJ3.2 (Agilent Technologies) and/or MNova (MestreLab Research, Santiago de Compostela, Spain). The samples were prepared in 5 mm Wilmad NMR 528-PP tubes in 10 mM phosphate buffer (for protein samples) or 10 mM Tris-d11 (for GAG samples) containing 150 mM NaCl dissolved in 90% H2O/10% D2O at pH 7.5. All spectra were obtained with a sweep width of 8 kHz and 16,384 acquired data points. The experiments were recorded using the standard saturation transfer difference (STD) or water-liquid observed via gradient spectroscopy (WaterLOGSY) pulse sequences in the Chempack pulse sequence library (STD_ES or WLOGSY_ES; Agilent Technologies), with excitation sculpting to suppress water resonances (26). For STD-NMR experiments, we used a 30 ms 3.1 kHz spin-lock pulse to reduce the background protein resonances. Saturation of the protein NMR signals was performed using a train of 40 selective 56 Hz Gaussian pulses with a duration of 50 ms, adding up to a total saturation time of 2 s. The on-resonance frequency was set to −4.0 ppm and off-resonance irradiation was applied at 80 ppm. STD-NMR spectra were acquired with a minimum of 1000 scans. For Water-LOGSY experiments, the first water-selective 180° pulse was 25 ms long. A weak rectangular pulsed field gradient was applied during the entire length of the mixing time (2). A short gradient recovery time of 0.5 ms was introduced at the end of the mixing time before the detection pulse. The spectra were acquired with 1200 transients and a spin-lock pulse of 50 ms at 3.7 kHz. To phase Water-LOGSY spectra correctly, it is necessary to use an internal reference compound. In our case, the reference is the residual ethanol absorbed/bonded in heparin-agarose resin (this interaction was previously confirmed with T1ρ filtered experiments), which appears as a positively phased signal. For the T1ρ relaxation filtered experiments, we used a proton pulse sequence with a spin-lock filter and excitation sculpting (PROTON_ES, Chempack Library), with 256 transients and a spin-lock pulse of 50 ms at 4 kHz.

Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed using a Biacore T-100 instrument (GE Healthcare, Little Chalfont, UK) in HBS-T buffer (10 mM HEPES, 150 mM NaCl, 0.05% Tween-20, pH 7.5) at 25°C. We immobilized the Sema3A-65K and Nrp1 (a1a2b1b2) proteins on a carboxymethyl dextran sensor chip CM5 at high densities (~6000 RU’s and 5500 RU’s, respectively) following standard amine coupling, and attaching the ligand (Sema3A or Nrp1 proteins) primary amine groups to the chip after activating the surface with 1-ethyl-3-(3-dimethyaminopropyl)-carboadiimide and N-hydroxysuccinimide. Using this procedure, the site of immobilization on the protein (and thus the orientation of the immobilized protein on the Biacore chips) is heterogeneous because the proteins contain several available amino groups. We checked that covalent attachment did not result in loss of protein activity (in our case, loss of protein-protein recognition by blockage of the PPI region). For binding studies, increasing concentrations of SICHI solution in running buffer were injected at a flow rate of 60 μl/min, with 60 and 120 s association and dissociation times respectively. An injection of MgCl2 was included at the end of each cycle to regenerate the sensor surface.

Methylene blue assay

UV absorbance was measured using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA). The experiments were performed with a 10 μM methylene blue (MB) solution in 5 mM Tris-HCl buffer at pH 7.5, to which increasing amounts of GAG (heparin, CSA, and HA) were added. We determined the binding constants and MB maximum binding number N per GAG molecule by fitting the experimental data to a linear regression equation published by Jiao et al. (27), which correlates the change in absorbance at 665 nm with the concentration of heparin (Eq. S1, Figs. S2–S4, and Supporting Materials and Methods in the Supporting Material). For the competition assays, solutions of MB and GAG (heparin or CSA) at fixed concentrations were titrated with increasing amounts of Sema3A peptides (FS2 or NF3S) and SICHI (Figs. S5–S8; see Supporting Materials and Methods for further details about the data analysis).

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was performed in a MicroCal VP-ITC instrument (GE Healthcare) in 5 mM Tris-HCl, pH 7.5, at 25°C. The cell (volume ~1.4 mL) and syringe (volume ~0.3 mL) contained 5 μM heparin and 1 mM SICHI/peptides, respectively. The experimental conditions were as follows: a first injection of 2 μL was followed by 30 injections of 10 μL, with a spacing of 600 s between injections and a stirring speed of 300 rpm. A blank titration was performed by injecting SICHI or the Sema3A-derived peptides into buffer in the same conditions. Raw data were analyzed with MicroCal Origin software (OriginLab, Northampton, MA), using a one-site model for the fittings.

The experiments with peptides FS2 and NFS3 showed an anomalous behavior at low peptide/GAG ratios, which we attributed to peptide aggregation. Attempts to use the smaller dp14 heparin led to reduced heat release without significantly diminishing this aggregation effect (data not shown). Therefore, since tighter binding allowed us to work at lower peptide concentrations and thereby minimize aggregation problems, we performed the ITC experiments reported here with crude high-MW heparin. Thus, the thermodynamic parameters obtained should be understood as averages among all the heparin species present in the samples. However, the influence of the heparin heterogeneity on the determined parameters was not limiting in our case, since the objective of this study was to compare the results for the peptides measured under the same conditions.
Molecular simulation methods

All molecular simulations were carried out with Schrödinger Suite 2014 through its graphical interface, Maestro (28). The program Macromodel (28), with its default force field OPLS 2005 (a modified version of the OPLS-AA force field) (29,30), and GB/SA water solvation conditions (31) were used for energy minimization. MD simulations were performed with the program Desmond 3.8 (32,33) using the OPLS 2005 force field. It was previously shown that this force field can provide reasonable results for the modeling of carbohydrates (34–37). Further details of the simulation protocols are given in Supporting Materials and Methods.

Fluorescence spectroscopy

Tryptophan fluorescence emission spectra were measured using a SpectraMax M5 spectrophotometer (Molecular Devices). A solution of FS2 (5 μM) in Tris buffer (5 mM, pH 7.5) was titrated with a solution of dp14 heparin in the same buffer. An excitation wavelength of 280 nm was used and spectra were recorded from 300 to 450 nm. For competition assays, a solution of FS2 (5 μM) and dp14 heparin (20 μM) was titrated with increasing concentrations of SICHI and the emission spectra were determined as described above. The experimental fluorescence change was analyzed using nonlinear fitting as described previously (38).

RESULTS AND DISCUSSION

Analysis of the interaction between GAGs and the Sema3A peptides FS2 and FS3

Sema3A is rich in basic amino acids at the C-terminus. In particular, in this region there are two clusters of Arg/Lys amino acids. We selected two short peptides, each of which includes one of the two basic clusters of the Sema3A C-terminal region (FS2 and FS3, marked in green on the Sema3A sequence in Fig. 1A) and also a furin protease site (-RDRKQR- or -KXRNRR-; furin cleavage liberates a C-terminal arginine that is necessary for binding to the b1 domain of Nrp) (13). FS3 contains a known consensus site, (X)BBXBX, for heparin binding (39), and FS2 contains the motif XBBXBBXBBX, which has not been described previously in the literature as a heparin-binding site.

NMR studies confirm the interaction between GAGs and peptides FS2 and FS3

We carried out NMR experiments to test the interaction between the positively charged Sema3A peptides FS2 and FS3 and GAGs. We decided to use a heparin agarose chromatographic resin to mimic to some extent the immobilized GAGs in a PG net, as well as to reduce the background resonances from glycan units. Fig. 2 shows an overall increase in the peak linewidths (i.e., intensity decrease) of peptide NMR resonances after the addition of heparin-agarose resin to a solution containing FS2 or FS3 peptides. We also assessed that the binding was specific to the GAG component of the resin, and not due to absorption into the agarose beads, by conducting a control NMR experiment using mannan agarose (a nonsulfated mannose polymer) instead of heparin (sulfated GAG) agarose (Fig. S1A). In another type of NMR experiment commonly used for ligand-receptor interaction studies, WaterLOGSY, positively phased signals in the presence of a high-MW receptor (in our case, heparin-agarose) originate from the bound ligand, whereas negatively phased signals come from small molecules that do not interact with the receptor (40). The WaterLOGSY NMR spectrum of the FS3 peptide in the presence of heparin agarose showed positively phased peptide resonances, supporting a peptide-GAG interaction (Fig. S1B). The NMR qualitative binding studies were performed with peptide FS3, which does not include the previous residue in the protein sequence (asparagine). After confirming by NMR the binding of FS3 peptide to GAGs, we decided to incorporate the asparagine at the N-terminus (hereafter referred to as...
peptide NFS3) because the known heparin binding consensus sequence XBBXBX includes a nonbasic amino acid at the N-terminal end. We then used peptide NFS3 for additional quantitative biophysical studies employing ITC and UV-visible (UV-Vis) MB assays.

Investigation of peptide binding to GAGs by spectrophotometry using a cationic dye

We used the cationic dye MB, which is widely known as a heparin sensor (27,41), in a UV-Vis spectroscopic competition assay to characterize the binding of FS2/NFS3 peptides to GAGs. To determine the binding constants between MB and GAGs, we used the method published by Jiao et al. (27). The binding of MB to GAGs led to the phenomenon of metachromasia, characterized by a decreased absorbance at 665 nm (Fig. 3 A). Although other dyes that are able to work in the presence of salt could have been used (42), MB was considered useful for comparison purposes because of its easy accessibility and well-supported performance. The use of a high salt concentration (i.e., 150 mM NaCl) in the buffer was avoided because the increased ionic strength would have interfered with the interaction between MB and heparin due to a competitive effect. Under these conditions, the titration of FS2/NFS3 peptides into the preformed [MB-GAG]-complex solution increased the absorbance intensity to that observed for the free MB (Fig. 3 B). Thus, the ability of Sema3A cationic peptides to displace MB from its corresponding complex with GAGs was proved.

Analyzing data from this kind of assay is challenging for several reasons. GAGs have multiple binding sites along the polymer chain. Moreover, commercial GAG samples are polydisperse materials (43). Thus, it is difficult to extract a binding constant from these assays, for a simple comparison of the relative binding performances, we calculated the charge excess values (CE50, +: - charge ratio, number of peptide positive charges divided by the number of negative charges present on the GAG) at 50% dye displacement, as proposed by Bromfield and Smith (44). This measure reflects how much cationic charge is required to displace 50% of MB from its complex with GAGs (a lower number indicates greater efficiency and better binding).

This MB-displacement assay was performed for heparin (it was assumed that the disaccharide repeating unit has four negative charges homogeneously distributed along the heparin chain) and CS-A (three negative charges, with a different disposition of charge along the chain compared with heparin). From the titration curves, the charge excess during an interaction with heparin was estimated for FS2 and NFS3 peptides (CE50 = 0.46 and 0.57, respectively) (Table S1). These CE50 values indicate that less than one positive charge on the peptides is used for each GAG negative charge. On the other hand, during titration of the CS-A/MB complex, the peptide NFS3 required 50% less charge (CE50 = 0.36) than the FS2 peptide (CE50 = 0.75) to displace CS-A (Table S2), indicating that NFS3 uses its positive charge more efficiently than FS2.

Characterization of the peptide-heparin interaction using ITC

Peptide-heparin binding was also characterized by ITC (for FS2 and NFS3, see Fig. 4, A and B, respectively), a biophysical technique that is commonly used to study cationic peptide-GAG interactions quantitatively (45). Fig. 4 shows the calorimetric titration plot obtained for FS2/NFS3 peptides with heparin at 25°C. The ITC titration curve showed a two-phase exothermic process (46). The first part of the curve was attributed to an aggregation process, which we confirmed by studying samples via dynamic light scattering using the same peptide/GAG ratio (data not shown). This process was probably due to the formation of clusters of one peptide molecule and several heparin chains. After some points in the titration, the expected sigmoidal decrease in heat release for the peptide-heparin interaction was observed until it dropped to zero when the binding was complete. The complexity of ITC curves does not allow for a concise data analysis. For these experiments and for comparison purposes, only the sigmoidal part of the isotherms was fitted to a 1:1 model. Binding stoichiometries (n) and

![Image](https://example.com/image.png)

FIGURE 3 (A) UV-Vis absorption band at 665 nm of MB (9.2 μM) sensor before (blue) and after binding to heparin (5.2 μM, black), and after peptide (70 μM, green) or SICHI (334 μM, red) competition. (B) Relative increase of MB absorbance at 665 nm versus the charge ratio after the addition of FS2 (green) and NFS3 (blue) peptides or SICHI (red) to the preformed MB/heparin complex. To see this figure in color, go online.
dissociation constants \((K_D)\) were determined using an equation that has been widely employed for the binding of cationic peptides to heparin (47). This model assumes that the polyelectrolyte molecule (heparin) can bind several peptide molecules in \(n\) independent and equivalent sites, all of which have the same \(K_D\) for the ligand peptide. Fitting of the ITC data using this model gave similar values for peptides FS2 \((K_D = 1.8 \pm 0.1\ \mu M\) and \(n = 18.6 \pm 0.3\)) and NFS3 \((K_D = 3.2 \pm 0.6\ \mu M\) and \(n = 15.9 \pm 0.4\)). The slightly lower \(K_D\) values and higher number of heparin-bound peptide molecules observed for FS2 correlate with the larger number of basic residues relative to NFS3 (seven R/K residues, net charge +6 at neutral pH for FS2 versus five R/K residues and net charge +5 for NFS3).

Characterization of the interaction between FS2 and dp14 heparin by fluorescence spectroscopy

For intrinsic tryptophan fluorescence-based affinity measurements, low-MW dp14 heparin was used to avoid turbidity effects (due to interfering aggregates when using high-MW heparin). The fluorescence emission spectrum of a solution of FS2 peptide (5 \(\mu M\) in Tris buffer (5 mM, pH 7.5) exhibited a maximum at 350 nm (Fig. 4 C). Tryptophan’s fluorescence depends on the peptide environment, which changes due to the binding of the highly electronegative GAGs. Fluorescence data acquired on FS2 bound to dp14 revealed increased intensity at the emission maximum (~7-fold at saturation; Fig. 4 C), confirming FS2 peptide binding to dp14 heparin. Shifting of the tryptophan wavelength emission maximum was not observed, suggesting that the average environment around the tryptophan residues was similar in polarity/hydrophobicity after heparin addition.

Using the experimental data from the average of three independent measurements, we performed nonlinear fitting of the titration values to Eq. S5 and determined a \(K_D = 1.3 \pm 0.2\ \mu M\) (Fig. S9; Supporting Materials and Methods). The assay was shown to be fairly reproducible but very sensitive to small experimental changes. Therefore, the apparent \(K_D\) value obtained in this way must be taken as a rough estimate of the true value. It is worth noting that both ITC and fluorescence spectroscopy measurements gave low-micromolar \(K_D\) values for the peptide FS2-GAG (heparin or dp14 heparin) interaction.

![ITC binding isotherm](image)

**FIGURE 4** (A) ITC binding isotherm of an in-cell 5 \(\mu M\) heparin injected with 1 mM FS2 peptide. Thermodynamic parameters from the fit: \(K_D = 1.8 \pm 0.1\ \mu M\), \(\Delta H = \pm 0.1\ \mu M\), \(\Delta S = \pm 39 \pm 5\ \mu M\), and \(n = 18.6 \pm 0.3\). (B) ITC binding isotherm of an in-cell 5 \(\mu M\) heparin injected with 1 mM NFS3 peptide. Thermodynamic parameters from the fit: \(K_D = 3.2 \pm 0.6\ \mu M\), \(\Delta H = \pm 21 \pm 1\ \mu M\), \(\Delta S = 11 \pm 2\ \mu M\), and \(n = 15.9 \pm 0.4\). (C) Changes in FS2 peptide tryptophan intrinsic fluorescence emission spectra in the presence of increasing concentrations of dp14 heparin. Peptide (5 \(\mu M\)) was incubated with increasing amounts of dp14 heparin (0-50 \(\mu M\), bottom line to top line). After excitation at 280 nm, the spectra were recorded from 300 to 450 nm. To see this figure in color, go online.
All of these measurements confirm that the peptide sequences corresponding to the basic Sema3A C-terminal region (725-VWKDRKQRRQR736 and 755-NKKGRNRR760) interact with GAG structures. The $K_D$ values obtained by ITC for FS2/NFS3 peptides/high-MW heparin and by fluorescence spectroscopy for FS2 peptide/heparin dp14 are in the low-micromolar range, on the order of those reported for known biologically relevant heparin-peptide interactions (48,49).

Similarly to Sema3A, orthodenticle homeobox protein 2 (Otx-2) binds to CS-E motifs in perineuronal nets (50). Beurdeley et al. (50) used ITC to study the interaction of an Otx-2 peptide (RKQRERTTTFRQAQL) with different GAG variants, and did not observe binding to CS-A (4-sulfated CS) or a higher affinity for heparin ($K_D = 193 \pm 20$ nM) than for CS-E. FS2 peptide has a basic amino acid sequence (BBXBBXBXX) similar to that of Otx-2 peptide (BBXBBXBX), but it binds to both CS-A and heparin (as shown by NMR and MB assays). The experimental conditions used in our study and that of Beurdeley et al. are not equivalent, which could explain the differences in specificity. Because the binding between cationic peptides or proteins with GAGs involves an important ionic contribution, measurements of $K_D$ are highly dependent on the experimental conditions, in particular the ionic strength. In this study, due to the characteristics of MB, we performed our measurements in the absence of sodium chloride, whereas Beurdeley et al. obtained their measurements in physiological salt conditions. Also, note that in other published cellular assays, different observations were made regarding the specificity of Sema3A binding to CSPGs (6,7,9).

The case of SICHI: does it have a protein target or is there an alternative mode of inhibition?

The antecedents of Sema3A cascade inhibitors are limited because only two small-molecule inhibitors of the Sema3A pathway have been found: SM-216289 (Xanthofulvin) (1) and SICHI. SICHI, a peptoid that was previously synthesized by our group and specifically blocks Sema3A biological functions in vitro, was proposed to be an inhibitor that is able to disrupt the Sema3A pathway, perturbing protein-protein interactions and then the formation of higher-order receptor signaling complexes. However, whether this compound exerts its action by a direct interaction with Sema3A or Nrp1 was not proven. In vitro assays showed that SICHI dramatically decreased the binding of Sema3A to both Nrp1/PlxnA2 complexes and Nrp1 alone (23). Sema3A is a cell-secreted protein and its interaction with Nrp1 occurs outside the cells. The x-ray structure of the ternary Sema3A-Nrp1-PlxnA2 complex published by Janssen and co-workers (24) showed that Sema3A interacts directly with Nrp1 but only contacts PlxnA2 through Nrp1 intervention. Moreover, as summarized in the Introduction, several studies have revealed the importance of CSPGs for Sema3A/Nrp1 biological activity. Furthermore, our results with Sema3A peptides confirmed the binding of the C-terminal basic region to GAGs. Therefore, we sought to determine the SICHI point of intervention (Sema3A-Nrp1 PPI, GAGs, or GAG-protein interaction; Fig. 5). Such knowledge is crucial for developing improved SICHI-like molecules in response to the growing interest in therapeutic approaches to promote axonal regeneration.

**Analysis of the interaction of SICHI with Sema3A and Nrp1 by NMR and SPR**

First, we evaluated the direct interaction of SICHI with the two potential protein targets (Sema3A and Nrp1) using STD-NMR. STD-NMR is a reliable way to screen compounds for binding to proteins; however, since detection by this method is limited to affinities in the range of $10^{-8}$ to $10^{-3}$ M, we decided to use SPR as a complementary methodology. To check that covalent protein attachment at the surface of the chip did not result in loss of protein activity (by blockade of the protein-protein interaction surface), we measured the specific interaction between

**FIGURE 5** Schematic illustration of the mechanism of action proposed for SICHI through the direct inhibition of a biologically relevant protein-GAG interaction. Structural features of GAGs, such as the length, flexibility, and structural diversity along the chain, make them scaffold molecules for promoting protein-protein interactions. It was previously suggested that in the system studied here, CS/HSPGs act as scaffolds to aid the approach of Sema3A (a secreted protein) to its partner receptor proteins Nrp1 and PlxnA2. By binding simultaneously to Sema3A and Nrp1, CS/HSPGs increase the chance of the protein partners getting close and interacting. The scheme shows the proposed inhibition mechanism of the Sema3A pathway by hindering the colocalization of secreted Sema3A with its receptor proteins (Nrp1-PlxnA2) due to SICHI binding to HS/CS sites, blocking them for protein binding. Single letter labels indicate SICHI intervention sites: (a) GAGs, (b) Sema3A-GAGs, and (c) Sema3A and CS-modified Nrp1. To see this figure in color, go online.
chip-immobilized Nrp1 and Sema3A, and determined their affinity ($K_D = 19 \mu M$, data not shown), which was in good agreement with a previously reported value ($K_D = 46 \mu M$) (24). The reverse experiment with chip-immobilized Sema3A also showed an interaction with the Nrp1 construct (data not shown). Thus, using the combination of NMR and SPR, we found that SICHI marginally binds (i.e., in the millimolar range) to the Sema3A domain and the Nrp1 extracellular domains (a1a2b1b2) (Fig. S10), excluding both proteins as prime candidates for SICHI action. Moreover, these results were incompatible with the activity observed in the previous in vitro cellular studies.

As mentioned above, Sema3A binding to the cell surface is important for its chemorepulsive activity. We believe that this is connected to the role played by PGs and GAGs as a “rack” for hanging different proteins (in this case, Sema3A) at the right site (near Nrp1 and PlxnA2 receptor proteins), disposition, and moment, resulting in an increased concentration at the local level, the creation of gradients, and/or protection against degradation. Sema3A binding (helped by the Nrp1 bridging to stabilize the heterotrimeric assembly and/or by GAGs) dimerizes and possibly clusters the plexin intracellular region, leading to signaling. Therefore, we propose the novel, to our knowledge, hypothesis that SICHI could compete with Sema3A for the interaction with GAGs, inhibiting/modulating Sema3A pathway by hindering the colocalization of secreted Sema3A with its receptor proteins (Nrp1-PlxnA2) (Fig. 5). In this work, as a second approximation, we tested this hypothesis by examining whether a small molecule like SICHI, positively charged at physiological pH, is able to bind to GAGs and disrupt the interaction between the Sema3A C-terminus-derived peptides and GAGs (heparin, heparin dp14, or CS-A).

**Analysis of the interaction of SICHI with GAGs by NMR, MB assay, and ITC**

Initially, since SICHI contains four amino groups, we determined their $pK_a$ values by means of potentiometric titrations (Fig. S11; Table S3). The results showed that the triprotinated form of SICHI was the main species at neutral pH. We also tested different buffer conditions for NMR studies of SICHI in solution (Fig. S12), and obtained the best signal/noise ratio with Tris buffer. Next, we evaluated the binding of SICHI to GAGs by using different biophysical techniques. Using the MB method under the same conditions employed for the FS2/NFS3 peptide-GAG interaction assays, we observed that titration of SICHI into the preformed [MB-GAG] solution increased the absorbance intensity until it reached that of the free MB (Fig. 3 A). As was the case with the peptides, this MB-displacement assay was performed with both high-MW heparin and CS-A. In all cases, SICHI was able to displace MB from the corresponding complex with the GAG (Fig. 3 B), exhibiting more efficient displacement capability with CS-A/MB ($CE_{50} = 0.67$) than with heparin/MB ($CE_{50} = 1.71$) (Tables S1 and S2). When we compare the charge efficiencies of SICHI and peptides as heparin binders, we find that the FS2 and NFS3 peptides ($CE_{50} = 0.46$ and 0.57, respectively) surpass SICHI ($CE_{50} = 1.71$) (Table S2). These results support the notion that SICHI is an efficient binder for GAGs. Further, they suggest that the interaction is not due only to simple electrostatic contacts and some structural effects play an important role.

For a further characterization of the interaction, we used ITC and NMR. The ITC studies showed a $K_D$ between SICHI and heparin in the micromolar range ($K_D = 17 \pm 2 \mu M$, $n = 15.3 \pm 0.5$) (Fig. 6 B), which is similar to reported values for other peptoid-GAG interactions (51).

![FIGURE 6 SICHI peptoid binding to GAGs. (A) From bottom to top: NMR spectrum of 1 mM SICHI alone, with 0.09 mM CS-A (15 kDa, 4-sulfated CS), and with 0.3 mM heparin (12 kDa, 2,6-disulfated). All spectra were acquired in 10 mM Tris/150 mM NaCl, pH 7.5 in 90% H$_2$O/10% D$_2$O. (B) ITC binding isotherm (at 25°C) for the interaction of SICHI (1 mM, syringe) with heparin (5 mM, cell). The peaks were integrated and the total heat per injection (peak area) is plotted as a function of molar ratio in the bottom figure. Thermodynamic parameters from the fit: $K_D = 17 \pm 2 \mu M$, $n = 15.3 \pm 0.5$, $\Delta H = -5.2 \pm 0.2$ kJ/mol, and $7\Delta S = 22.0 \pm 0.5$ kJ/mol. To see this figure in color, go online.](https://example.com/figure6.png)
Interestingly, the binding process was enthalpically and entropically favored ($\Delta H = -5.2 \pm 0.2$ kJ/mol and $\Delta S = 22.0 \pm 0.5$ kJ/mol), as is commonly observed for electrostatic and polar interactions in water. Using 1D $^1$H spectra to study the interaction of peptoids with GAGs by NMR spectroscopy is a challenging task due to the flexible and polymeric nature of the anionic GAGs, and also to the presence of different SICHI conformations in solution (52). For the peptoid-GAG NMR binding experiments, we used ligand-based NMR screening methods such as $^1$H NMR with a $T_1$ spin-lock filter and WaterLOGSY pulse sequences (26). Interestingly, in the presence of sulfated GAGs (heparin, heparin agarose, CS-A, or CDS), we observed chemical-shift changes and signal broadening of SICHI resonances in the relaxation-filtered experiments (Figs. 6A and S13A), demonstrating the interaction between SICHI and GAGs and that this binding occurred in the intermediate-exchange regime. However, the same NMR experiments showed that SICHI did not bind to the less-charged glycan HA (Fig. S13C). Further evidence that SICHI binds to sulfated GAGs was the sign change of the resonances observed in the WaterLOGSY spectra of SICHI in the presence of heparin agarose (Fig. S13B).

Considering the small size of SICHI and that its design was not optimized for GAG binding (we selected SICHI after screening a combinatorial peptoid library (23), its micromolar affinity for binding to GAGs is promising. In the past few years, there has been increasing interest in developing cationic drugs that are able to bind GAGs and produce diverse biological effects. A large variety of molecules have been synthesized for this purpose, including peptoids (51), spectrophotometric/fluorescent sensors (53), small organic molecules (54,55), and self-assembling heparin ligands (56,57). In a previous work (51), several peptoids were synthesized with the objective to neutralize heparin anticoagulant activity. The published results showed that peptoids with three monomer units and one positive charge (from a monomer unit with a lysine analog side chain) bound to heparin with $K_D$ values in the range of 160–220 $\mu$M (determined by ITC, measured in 50 mM phosphate buffer, pH 7.4). Our highly charged SICHI peptoid has a $K_D$ of ~17 $\mu$M (ITC, measured in 5 mM Tris buffer, pH 7.5).

**MD simulations to characterize the binding of SICHI to heparin**

To gain further insight into the interaction of SICHI with heparin, we carried out 100 ns MD simulations on systems containing one to five molecules of SICHI and one molecule of a heparin octamer (dp8) model. Using a threshold of 3Å as an arbitrary criterion to decide whether a SICHI molecule is in close contact with heparin, the simulations showed that the number of SICHI molecules with at least one atom within that threshold increased approximately linearly with the number of SICHI molecules included in the simulation (Fig. S14). A similar trend was also observed when the number of SICHI atoms within the threshold was counted (Fig. S14). Thus, averaging over the equilibrated second half of the simulation time (i.e., 50–100 ns), the simulations with five SICHI molecules show that ~4.6 molecules and 59 atoms are at a close distance from heparin (Fig. 7). Moreover, looking at different snapshots of the simulation (Fig. S15), it is clear that five molecules of SICHI can be simultaneously complexed to the dp8 heparin model (Fig. 7A). As expected, molecules that display the larger number of contacts show that these are polar in nature; that is, hydrogen bonds between the polar charged and noncharged groups of SICHI and those of heparin, as well as electrostatic interactions between charged groups of both molecules, were observed (Fig. 7B).

We carried out similar MD simulations with systems containing one to five molecules of MB and one molecule of the dp8 heparin model, to ascertain whether they exhibited similar trends. In this case, the number of molecules in close contact also increased with the number of molecules included in the simulation (Fig. S16). However, for the simulation with five molecules of MB, the average number of bound molecules was only ~2.7, although at some points the five MB molecules were within the chosen threshold (Fig. 7D). Taking into account that MB is smaller in size than SICHI, this would suggest a lower affinity for the former, which seems reasonable considering that MB holds only one positive charge that is delocalized through its aromatic system, and that because of its structure, it cannot establish as many polar interactions with heparin as does SICHI. This is clear when one looks at the structures of the complexes from the simulation (Fig. S17).

To assess the competence of SICHI and MB to bind to the heparin model, we carried out further simulations that included five molecules of SICHI and five of MB, in addition to the molecule of heparin. To improve sampling and check the reproducibility, we repeated this simulation three times, in each case starting from a situation where the ligands (SICHI and MB) and the receptor (heparin) molecules were disposed in different random arrangements inside the simulation box. Fig. 7, E and F, show the results of one of these simulations. Looking at SICHI, it is apparent that the presence of MB molecules does not change much from what was observed in the simulations where SICHI was the only ligand (compare Fig. 7E and 7C). In contrast, for MB there is indeed a large change in the MD profiles, which indicates that a lower number of molecules are in close contact with heparin when SICHI is also present in the simulation. The other two simulations carried out for this system showed essentially the same results (Fig. S18), which indicates that SICHI can compete with and displace MB from binding to heparin.
This is in good qualitative agreement with what we observed experimentally.

**SICHI targets and modulates the Sema3A peptide-GAG interaction**

Finally, after confirming with different experimental techniques that both Sema3A peptides and SICHI bind GAGs with affinities (expressed as $K_D$ or CE 50) in the same range, we evaluated whether SICHI is able to compete with Sema3A peptides (as representatives of the C-terminal polybasic region) for binding to GAGs. For this purpose, we performed relaxation-filtered $^1$H/WaterLOGSY NMR experiments and intrinsic tryptophan fluorescence spectroscopy. By NMR, after monitoring the perturbation of asparagine and lysine multiplets at 2.65 and 2.90 ppm, respectively, we found that the addition of increasing amounts of SICHI resulted in partial displacement of peptide FS3 (Fig. 8A). For the longer FS2 peptide, we checked the region between 1.50 and 1.70 ppm of the $^1$H spin-lock filtered spectra, free of SICHI resonances, to evaluate the competition effect (Fig. 8B). In addition, we found that SICHI and peptide FS3 were able to bind simultaneously to heparin, as we observed in the WaterLOGSY NMR spectra (Fig. 8C) of both ligands in the presence of the heparin resin. To confirm these results, was studied the displacement of FS2 from the [FS2-dp14] complex with SICHI by measuring the FS2 fluorescence spectra. A partial recovery of the initial tryptophan fluorescence after titration with SICHI was observed (Fig. 8D).

Both the NMR and fluorescence spectroscopy experiments showed a dose-dependent SICHI competition effect. Also, the acquisition of all NMR spectra in the presence of salt (150 mM NaCl) showed that SICHI could compete with cationic peptides for binding to GAGs in physiological salt conditions. By comparing the $K_D$ values obtained by ITC for the interaction between peptides or SICHI with heparin, we observed that the affinity of FS2 and NFS3 peptides to heparin is 5- to 10-fold stronger than the affinity observed for SICHI. These results are in agreement with the partial displacement of peptides from heparin exerted by SICHI, and observed by NMR and fluorescence spectroscopy. Protein interactions are governed by several different forces, such as compartmentalization and electrostatic and hydrophobic effects. Colocalization increases the effective concentration of biomolecules and, together with other mechanisms, can lead to the formation of interacting domains. Our data support the hypothesis that the interaction between Sema3A and GAGs observed at the cellular level...
and described in the literature takes place through the involvement of the C-terminal polybasic region. Given the importance of the Sema3A-GAG interaction in the Sema3A signaling pathway, this knowledge is valuable for developing future therapeutic strategies for Sema3A modulation.

CONCLUSION

Taken together, our results suggest that SICHI, a small peptoid molecule, could interfere with the interaction between the Sema3A C-terminal region and GAGs by displacing the Sema3A C-terminal domain from the GAGs or by binding directly to them. This interference could explain the in vitro inhibition of Sema3A chemorepulsive activity exhibited by SICHI in growth-cone-collapse assays (23).

Thus, we propose a new, to our knowledge, mechanism for inhibiting the Sema3A pathway by targeting the GAG-Sema3A interaction, and provide information that can be exploited in future studies for the development of protein-GAG interaction inhibitors of therapeutic interest. We deem that our model of activity should be the starting point for further optimization of the structural and pharmacological characteristics of SICHI. Work along this line is in progress in our laboratory.

SUPPORTING MATERIAL

Supporting Materials and Methods, eighteen figures, three tables, and three movies are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(16)00145-4.

AUTHOR CONTRIBUTIONS

M.C.: fluorescence, UV, chemical synthesis, and experiments; R.B.: ITC, SPR, protein recombinant expression, and experiments; C.D. and A.
Corredor et al.

Moure: chemical synthesis and experiments; Y.P.: NMR and experiments; J.B.: MD calculations. All authors contributed to designing the experiments and analyzing the results, participated in writing the manuscript, and read and accepted the manuscript.

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SUPPORTING CITATIONS


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