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Small molecule inhibitors of protein interaction with glycosaminoglycans (SMIGs), a novel class of bioactive agents with anti-inflammatory properties☆

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ABSTRACT

Background: Small molecule inhibitors of biologically important protein–glycosaminoglycan (GAG) interactions have yet to be identified.
Methods: Compound libraries were screened in an assay of L-selectin–IgG binding to heparin (a species of heparan sulfate [HS-GAG]). Hits were validated, IC-50s established and direct binding of hits to HS-GAGs was investigated by incubating compounds alone with heparin. Selectivity of inhibitors was assessed in 11 different protein-GAG binding assays. Anti-inflammatory activity of selected compounds was evaluated in animal models.
Results: Screening identified a number of structurally-diverse planar aromatic cationic amines. Scaffolds similar to known GAG binders, chloroquine and tilorone, were also identified. Inhibitors displayed activity also against bovine kidney heparan sulfate. Direct binding of compounds to GAGs was verified by incubating compounds with heparin alone. Selectivity of inhibitors was demonstrated in a panel of 11 heparin binding proteins, including selectins, chemokines (IL-8, IP-10), Beta Amyloid and cytokines (VEGF, IL-6). A number of selected lead compounds showed dose-dependent efficacy in peritonitis, paw edema and delayed type hypersensitivity.
Conclusions: A new class of compounds, SMIGs, inhibits protein–GAG interaction by direct binding to GAGs. Although their IC-50s were in the low micro-molar range, SMIGs binding to HS-GAGs appeared to be stable in physiological conditions, indicating high avidity binding. SMIGs may interfere with major checkpoints for inflammatory and autoimmune events.
General significance: SMIGs are a class of structurally-diverse planar aromatic cationic amines that have an unusual mode of action — inhibiting protein–GAG interactions via direct and stable binding to GAGs. SMIGs may have therapeutic potential in inflammatory and autoimmune disorders.

1. Introduction

Many proteins involved in the inflammatory response, such as selectins, chemokines and cytokines, are known to interact with glycosaminoglycans (GAGs) via their heparin-binding domains [1]. GAGs are linear polysaccharides synthesized by a variety of different cell types and localized on the cell-surface and in the extra cellular matrix. Important protein families, including extracellular matrix proteins (e.g., collagen), growth factors and their receptors, cytokines, chemokines, amyloid peptides, viral envelope proteins, enzymes and cell adhesion molecules, possess heparin-binding domains that are responsible for tight binding to sulfated GAGs, especially heparan sulfate glycosaminoglycans (HS-GAGs) [2–5]. HS-GAGs consist of repeating disaccharide units of D-glucuronic/L-iduronic acid and N-acetyl- or N-sulfo-D-glucosamine, which have variable sulfation patterns, and display high molecular diversity [2,3]. Heparin, which is particularly abundant in mast cells, is an example of a highly sulfated HS-GAG [2,3,6]. In tissues, GAGs are usually covalently linked to a protein core, forming proteoglycans. Over the last decade understanding of the significance of regulatory protein interactions with HS-GAGs has evolved considerably [1–5]. Heparins and GAG-related molecules have provided new clues and

Abbreviations: GAG, glycosaminoglycan; SMIG or SMIGs, small molecule inhibitors of protein interaction with glycosaminoglycans; HS-GAG, heparan sulfate; DTH, delayed-type hypersensitivity; VEGF, vascular endothelial growth factor

☆ Dedication: We wish to dedicate this manuscript to the memory of Professor Vivian I. Teichberg (The Weizmann Institute of Science), an inspirational scientist, mentor, and colleague.

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explanations of the process of inflammation and suggested new therapeutic approaches to human disease [1,7–10]. The interactions between the proteins and GAGs play important roles in the cell–matrix and cell–cell adhesion, processes known to be involved in certain diseases and inflammatory disorders. The modulation of these protein–GAG interactions may have therapeutic implications. Synthetic HS-GAGs, or molecular mimics of HS-GAG sequences [11,12], may provide new approaches for combating viral infections, cancer, and Alzheimer's disease. In inflammation and autoimmune diseases, use of non-coagulant heparins [13] provided early indication for the role of HS-GAGs in these diseases. Also, mammalian endoglycosidase heparanase that digests HS-GAGs [14], has been implicated in inflammation [15].

One class of carbohydrate-binding cell adhesion molecules is the selectins [16]. The selectins are responsible for leukocyte capture from the blood stream mediating their intermittent attachment and consequent "rolling" along the endothelial cell surface. This capture initiates a cascade of secondary, tighter cell-adhesive events to take place. Selectins mediate their adhesive functions via lectin domains that bind to carbohydrate ligands [16,17]. There is evidence that GAGs, and in particular HS-GAGs, are one class of carbohydrate receptors with which the selectins interact [18–21]. Consistent with this observation, heparin, non-coagulant heparins, HS-GAG and heparin-derived oligosaccharides blocked L-selectin-dependent adhesion directly and short sulfated heparin-derived tetra-saccharides reduced binding of neutrophils to COS cells expressing P-selectin [18]. Heparin's anti-inflammatory effects required glucosamine 6-O-sulfation and were mediated by blockade of L- and P-selectins [20]. The role of selectin–ligand interactions in the inflammatory response has been demonstrated in various animal models, prompting considerable attention from the pharmaceutical industry [22]. Drugs in development included monoclonal antibodies, carbohydrate-based mimetics and Bimosiamose — a small molecule [22,23], however, none of these molecules target the interactions of L- and P- selectins with sulfated GAGs.

The heparin binding sites of proteins frequently contain clusters of basic amino acids intertwined with non-basic residues. The key amino acids comprising the heparin binding domain, however, are quite diverse [24,25]. Basic residues contributing to heparin binding can be close in space but remote from each other in amino acid sequence. A heparin binding domain, therefore, often constitutes a discontinuous binding domain [26] that is difficult to predict from DNA sequence. The superfamily of heparin-binding domain proteins has several hundred members including chemokines, cytokines, cell adhesion molecules, enzymes, growth factors, viral envelope proteins, etc. An important example of a heparin-binding protein is fibroblast growth factor (FGF) [27]. FGF interacts with a HS-GAG sequence for full biological activation of the FGF receptor. Crystal structure analysis of FGF with its receptor showed two separate HS binding sites in FGF [28]. The heparin binding sites of chemokines are required for chemokine gradient formation, and inhibition of chemokine–GAG interaction has been described as a novel and promising anti-inflammatory therapeutic strategy [29–32].

In spite of the emerging significance of the heparin-binding domains of peptides and proteins, there is a lack of suitable tools required to dissect such interactions at the molecular level. Studies have been carried out with mutant chemokine peptides that retain their ability to bind GAGs [30–33] and some of these peptides are being developed as anti-inflammatory therapeutics [33]. Other approaches to inhibit GAG interactions with heparin-binding proteins included small molecule anionic compounds that act as GAG mimetics and have also limited selectivity [34]. Methylene blue, chloroquine and some cationic acridines and phenothiazines have long been known to bind to GAGs [35], however, these compounds have not been used to analyze protein–GAG interactions, perhaps due to their pleiotropic properties. There are few if any well-characterized small molecule inhibitors of GAG–protein binding [36] and overall, there is a lack of small molecule compounds that would have sufficient binding affinity and selectivity to be useful as molecular tools for dissecting the functional significance of heparin-binding domains of various biologically important proteins that interact with GAGs.

In this paper we describe the identification of a number of small molecule inhibitors of heparin-binding domain protein interactions with GAGs via screening of chemical compound libraries that do not include GAG mimetics. The initial focus of the compound screening was on inhibitors of selectins, chemokines and cytokines. The screening and subsequent work revealed a broad class of novel bioactive compounds that directly bind to HS-GAGs and are able to inhibit HS-GAG interactions with a number of biologically important proteins. The report describes the chemical structures of these small molecule inhibitors and their characterization in molecular interaction assays in vitro, as well as the efficacy of selected compounds in animal models of inflammation.

2. Experimental procedures

2.1. Materials

Recombinant human L-selectin/IgG (Cat. No.728-LS); recombinant human P-selectin/IgG (Cat. No.137-PS); Recombinant human VEGF (Cat. No. 293-VE); anti-human VEGF (1:2000; Cat. No. BAF293) were all purchased from Research and Development Systems. CMV envelope glycoprotein B (gB) was from Research Diagnostics, Inc., Cat. No. RDI-RCMVAG-B. Bovine Kidney heparan sulfate (Cat. No. H7640); porcine intestinal mucosa heparin (Grade 1-A; Cat. No. H-3393); anti-beta-amyloid monoclonal antibody; anti-human IgG peroxidase conjugate (A8667); Tween 20 (P-1379); porcine intestinal mucosa heparin conjugated to bovine serum albumin (Heparin-BSA; Cat. No. H0403), Diclofenac (Cat. No. D6899) and fucoidan (Cat. No. F5631) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Chondroitin sulfate from shark cartilage and dermatan sulfate from hog skin were purchased from Seikagaku Ltd. (Japan). Chromogen tetramethyl benzidine (TMB) was from Dako (Cat. No. S1599); anti-human L-selectin monoclonal antibody (DREG-55) was from Bender MedSystems (Burlingame, CA; Cat. No. BMS121); goat anti-mouse IgG (H&L) peroxidase conjugated antibody was from Chemicon International, Inc. (Cat. No. AP124P); and 96-well polystyrene ELISA plates were from Sigma-Aldrich Corporation (NUNC Cat. No. 442404). Compounds were purchased from suppliers of chemical compound libraries ChemDiv Labs (San Diego, CA), Chemibridge Inc. (San Diego, CA), Tripos Inc. (St. Louis, MO), Life Chemicals Ltd. (Kiev, Ukraine) and Enamine Ltd. (Kiev, Ukraine). All of the purchased compounds and analogs listed in this publication met standard criteria for purity (>95%) and structures were confirmed by NMR and mass spectrometry, provided by vendors. In addition, all hit compounds' structures were independently verified by NMR and mass spectrometry at Bar-Ilan University (Ramat Gan, Israel). Re-synthesis of several key compounds was also performed (e.g., RX-111, RX-211).

2.2. Assay for L- and P-selectin binding to GAGs and screening of compound libraries

An in vitro assay [37) was used to assess the ability of test compounds to inhibit the interactions of an L-selectin-IgG chimera [21, 38) with HS-GAGs. The assay was suitable for determining the concentration required for 50% inhibition (IC-50) for each specific compound. In most assays, the GAG used was heparin. Thus, Heparin-BSA at 5 mg/ml in phosphate-buffered saline (PBS; pH 6.5) was added to a 96-well polystyrene ELISA plate (0.1 ml per well) and incubated overnight at 4 °C. Following the incubation, the plate was washed thoroughly, by immersion, with deionized water and PBS (pH 6.5). The ELISA plate was then blocked with 3% BSA for 1 h at room temperature. Following blocking, the plate was washed with deionized water, and then with PBS containing Tween 20 (0.05%). Recombinant human L-selectin–IgG dissolved in PBS supplemented with BSA (0.1%) and calcium chloride (1 mM) was

added to the ELISA plate (100 μl per well) and incubated for 60 min at room temperature with shaking. Following incubation, the plate was washed with deionized water and three times with PBS containing Tween 20. Anti-human IgG peroxidase conjugate (1:5000) diluted in PBS supplemented with BSA (0.1%) and calcium chloride (1 mM) was added to the ELISA plate and incubated for 30 min at room temperature with shaking. The plate was then washed with deionized water and three times with PBS containing Tween 20. The peroxidase substrate TMB was added to the ELISA plate and incubated at room temperature. After 15 min, ELISA stop solution (hydrochloric acid 1 N, sulfuric acid 3 N) was added to stop the peroxidase catalyzed colorimetric reaction. The optical density (OD) of the samples was measured at 450 nm using an ELISA plate reader (Dynatech MR5000). All assays were done in 96-well plates in triplicates. Data were analyzed with Graphpad Prism software and IC-50 values were established. Hill coefficients were also determined using GraphPad Prism. The P-selectin assay was carried out in a similar fashion, except that recombinant human P-selectin/IgG was used. Compounds were dissolved in DMSO, diluted in PBS and added to the wells at various concentrations in the range of 0.01 to 300 μM. Compounds were dissolved in DMSO at 10 mM and further diluted prior to assay. DMSO concentration in the screening well was up to 2%. Individual compounds at a final concentration of 30 μM were co-incubated with L-selectin/IgG on plates containing immobilized heparin. After washing, bound L-selectin was detected with antibody conjugated to horseradish peroxidase. Following color development, the % inhibition compared to control (no compound) for every compound was determined. Compounds that inhibited at least 30% of the signal were scored as hits. None of the compounds listed in this publication interfered with the colorimetric detection assay for horseradish peroxidase.

2.3. Inhibition of human L-selectin binding to immobilized heparin by soluble heparin

Plates were prepared as described above. Recombinant human L-selectin-IgG dissolved in PBS (pH 6.5; supplemented with BSA (0.1%) and $CaCl_2$ (1 mM)) was incubated with soluble Heparin at different concentrations (0–3000 ng/ml), in separate tubes, each concentration in triplicate, for 1 h at RT.

2.4. Inhibition of human L-selectin binding to heparin by anti-L-selectin monoclonal antibody

Recombinant human L-selectin-IgG dissolved in PBS (pH 6.5; supplemented with BSA (0.1%) and calcium chloride (1 mM)) was incubated separately either with (1) anti-human L-selectin monoclonal antibody (DREG-55); or with (2) anti-beta-amyloid monoclonal antibody. L-selectin/IgG (10 ng/ml) was incubated with a range of antibody concentrations (5–160 ng/ml) in a final volume of 100 μl; each concentration in triplicate for 1 h at room temperature.

2.5. Assays for protein interactions with GAGs from different tissue sources

Bovine kidney HS-GAG, shark cartilage chondroitin sulfate, hog skin dermatan sulfate, and low molecular weight heparins were commercially available. HS-GAG was conjugated to BSA to prepare a synthetic HS-GAG–BSA complex in which the HS-GAG was coupled via its reducing aldehyde terminus to the protein using sodium cyanoborohydride [37]. Other GAGs were coupled to BSA in a similar fashion. The binding assay was similar to the assay described above. Following the incubation, the plate was washed, incubated with an antibody specific to L-selectin–IgG and finally TMB was added to the ELISA plate. After 15 min, ELISA stop solution was added and the optical density of the samples was measured at 450 nm using an ELISA plate reader. All assays were done in 96-well plates in triplicates.

2.6. Assays for binding of cytokines, chemokines and other proteins to heparin and compound screening

These assays were in principle similar to L-selectin–IgG assay described above [37]. An example is given for VEGF below and similar assays were developed for other chemokines, cytokines, etc. Recombinant human VEGF dissolved in PBS supplemented with BSA (0.1%) was added to the ELISA plate. The test compounds were dissolved in DMSO, diluted in PBS and added to the wells at various concentrations in the range of 0.01 to 200 μM (final volume 100 μl per well) and incubated for 60 min at room temperature with shaking. Following incubation, the plate was washed with deionized water and PBS (pH 7.3) containing Tween 20. Anti-human VEGF (1:2000) diluted in PBS supplemented with BSA (1%) was added to the ELISA plate (100 μl per well) and incubated for 60 min at room temperature with shaking. Following incubation, the plate was washed with deionized water and then with PBS (pH 7.3) containing Tween 20. streptavidin-HRP (1:200; Research and Development Systems Cat. No. DY998) diluted in PBS supplemented with BSA (1%) was added to the ELISA plate (100 μl per well) and incubated for 20 min at room temperature with shaking. All assays were done in 96-well plates in triplicates.

2.7. "Pre-incubation" and "Co-incubation" assays to demonstrate direct interaction of inhibitor compounds with heparin and other HS-GAGs

In order to demonstrate that the inhibitor compounds bind directly to heparin or to other HS-GAGs, individual compounds were incubated with immobilized heparin in the absence of L-selectin–IgG. 96 well ELISA plates were coated with heparin–BSA, then blocked with BSA as described above. In "pre-incubation assay", inhibitor compounds were incubated in the ELISA plate for 90 min, and then plates were washed extensively with incubation buffer. After washing, L-selectin–IgG was added to these wells. At the same time, in separate control wells, in "Co-incubation assay", L-selectin–IgG was co-incubated with inhibitor compounds for 90 min and then was added to wells coated with heparin–BSA. Following the incubation, L-selectin–IgG bound to the plate was quantified by antibody conjugated to horse radish peroxidase followed by optical density measurement, as described above. A similar assay was used also for VEGF. All assays were done in 96-well plates in triplicates.

2.8. A model of leukocyte and neutrophil infiltration into mouse peritoneum

BALB/c mice (Velaz, Prague; 6 weeks old, ~20 g in weight, 15 mice/group) received intraperitoneal injection of an inhibitor compound in 0.2 ml DMSO/Tween/sterile saline 1 h before administration of thioglycollate (Sigma-Aldrich Corporation). Control groups received vehicle and sham controls received no thioglycollate. Mice were injected intraperitoneally with 1 ml of 3% thioglycollate broth [39]. Mice were sacrificed after 3 h, and the peritoneal cavities were lavaged with 5 ml of ice-cold saline containing 2 mM EDTA to prevent clotting. After red blood cell lysis, leukocytes were counted in a hemocytometer. Neutrophils were counted after staining with Türck's reagent (Merck, Darmstadt, Germany). Under these conditions, thioglycollate administration induced approximately 3-fold increase in neutrophil accumulation in the peritoneal cavity. Data was expressed as mean ± SEM, and statistical analysis was performed by Student *t*-test. A value of $p < 0.05$ was taken to denote statistical significance. Fucoidan (50 mg/kg) was used as a positive control and inhibited neutrophil infiltration by 30%.

2.9. Delayed-type hypersensitivity (DTH)

ICR mice (Velaz, Prague, Czech Republic; 8 weeks old; 15 animals per group) were sensitized by topical application of a 2% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazoline-5-one; Sigma-Aldrich Corporation, St Louis, MO) solution in acetone/olive oil (4:1 vol/vol) to

shaved abdomen (50 μl) and to each paw (5 μl) [40]. Five days after sensitization, right ears were challenged by topical application of 10 μl of a 1% oxazolone solution, whereas left ears were treated with vehicle alone. Compounds were administered 1 h prior to challenge. The extent of inflammation was measured 24 h after challenge, using the mouse ear-swelling test. Animals were numbered (tail marking) and weighed and the thickness of both ears was recorded with a constant-loading dial micrometer (Mitutoyo, Tokyo). Data were expressed as mean ± SEM, and the unpaired Student *t*-test was used for statistical analyses. Dexamethasone at 10 mg/kg (i.p.) was used as positive control and it inhibited ear swelling to 46% of control values.

2.10. Carrageenan-induced paw edema

Acute edema was induced in the left hind paw of BALB/c mice (12 mice/group) by injecting 0.02 ml of freshly prepared solution of 2% carrageenan (Sigma-Aldrich Corporation) after 60 min of test compound administration [41]. The right paw received 0.02 ml of saline, which served as a control. Carrageenan was injected under the plantar region of right hind paw and the paw thickness was measured at 2, 4 and 24 h after carrageenan challenge using a Mitutoyo engineer's micrometer. Data were calculated as the difference between right and left pad thicknesses and expressed as mean ± SEM, and statistical analysis was performed by Student *t*-test. Indomethacin, diclofenac and dexamethasone were used as positive controls. It is important to note that in our paw edema studies, paw swelling was measured as a difference in paw thickness rather than difference in paw volume; thus the reported efficacy of compounds is likely an underestimate.

All animal experiments were reviewed and approved by Ethical Committee for animal experimentation of the Institute of Animal Physiology, approved by State Veterinary and Food Administration of the Slovak Republic, and were performed in accordance with Slovak legislation based on EC Directive 86/609/EEC on the protection of animals used for experimental and other scientific purpose.

3. Results

3.1. Compound screening in assays for L-selectin–IgG and P-selectin–IgG binding to HS-GAGs

In order to identify compounds that inhibit protein-GAG interaction we employed ELISA molecular interaction assays in 96 well plates. We used heparin, a highly sulfated HS-GAG, readily available and in sufficient quantity, as a source of HS-GAGs [2,20,37]. A 96-well plate assay was employed to detect the binding of an L-selectin–IgG chimera [21,38] to immobilized heparin. The immobilization of heparin was achieved by coating 96-well plates with a heparin–BSA conjugate. The amount of bound L-selectin–IgG to immobilized heparin was determined by an ELISA using an anti-IgG peroxidase conjugate, followed by quantitative color development of second antibody-conjugated horseradish peroxidase. Control plates were coated with BSA instead of heparin–BSA. The background absorbance signal was very low and did not noticeably interfere with binding assays and/or inhibition of binding by compounds. L-selectin–IgG binding to heparin–BSA was optimal in buffers containing calcium (data not shown). Fig. 1A shows the saturation curve of the L-selectin–IgG binding to heparin. The L-selectin ELISA assay was validated in two independent ways as follows. As shown in Fig. 1B, soluble heparin inhibited L-selectin–IgG binding to immobilized heparin. A mAb directed against the carbohydrate-binding domain of L-selectin (Dregg-55) [42] inhibited L-selectin–IgG binding to heparin (Fig. 1C), while a non-specific antibody such as a monoclonal anti-beta amyloid antibody did not inhibit the binding, thus providing further confirmation of the specificity of binding.

The L-selectin assay was used to screen a collection of about 2800 diverse compounds on 96-well plates. For this purpose, the compounds at a final concentration of 30 μM, were co-incubated with L-selectin–IgG

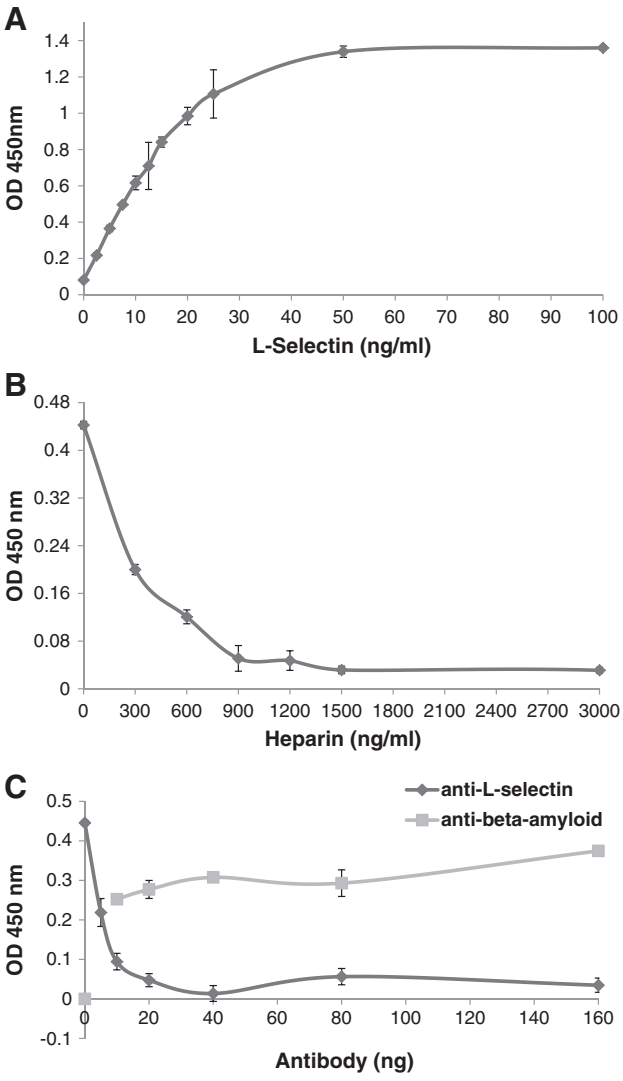

Fig. 1. Characterization of L-selectin–IgG chimera binding to immobilized heparin. Assays were carried out on 96-well plates i–n triplicates with immobilized heparin as described in Experimental procedures section. A: Saturation curve using increasing concentrations of L-selectin–IgG. B: Inhibition of L-selectin–IgG binding to immobilized heparin by soluble heparin. C: Inhibition of L-selectin–IgG binding to heparin by anti-L-selectin antibody DREGG-55 and anti-beta-amyloid antibodies (used as a negative control).

on plates containing immobilized heparin–BSA. Following color development, the % inhibition for every compound was determined. Positive and negative controls were included on every plate. Compounds which inhibited at least 30% of the signal were scored as hits. A total of 85 hits were identified. Compounds which inhibited more than 50% of signal were selected for further analysis — 37 compounds. Dose–response compound inhibition curves were generated for these 37 hits and 16 compounds were found to have IC-50 values ≤15 μM. An example of an IC-50 curve is shown in Fig. 2A and IC-50s for selected compounds (inhibitors of L and P selectin) are given in Table 1, As indicated in Table 1, a wide range of scaffolds has been identified. Examples of chemical structures are shown in Fig. 3. In addition, the compound screening also identified compounds that were very similar to tilorone, chloroquine and quinacrine. The chemical structures of three such compounds, RX-101, RX-0023 and RX-0025, are shown in Fig. 4.

In order to further investigate the properties of inhibitor compounds listed in Table 1, we acquired analogs of selected chemical series (RX-101, RX-105, RX-107, RX-108, RX-110 and RX-114). About sixty thieno[2 [3],c]pyridines related to RX-105 (2-[[4-[(ethylbutylamino)

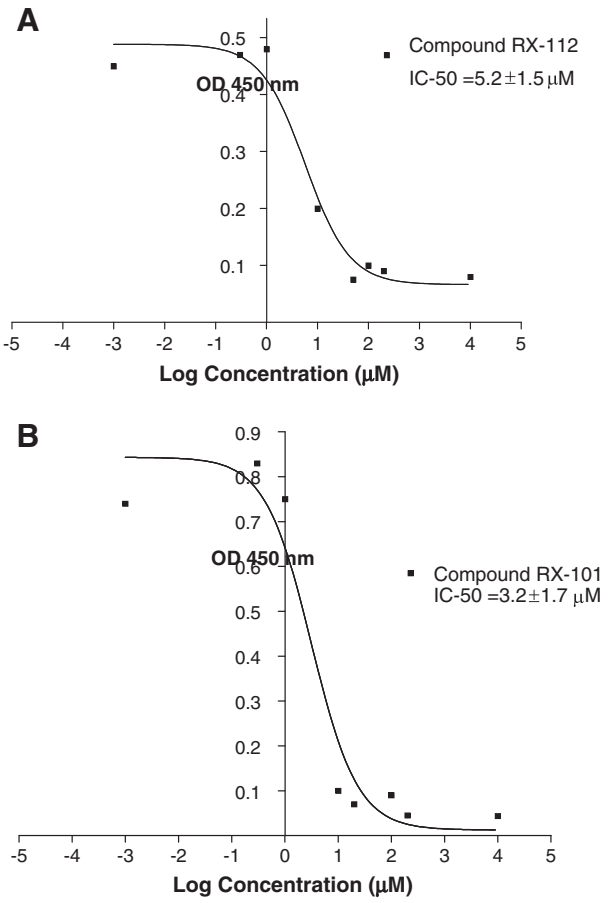

Fig. 2. Examples of small molecule compound's inhibition curves of L-selectin and P-selectin binding to HS-GAGs. A: Inhibition of L-selectin–IgG chimera binding to immobilized heparin by increasing concentrations of compound RX-112 (for structure, see Fig. 3, legend). B: Inhibition of P-selectin–IgG chimera binding to immobilized heparin by compound RX-101. The assays are described in Experimental procedures section. Curves were generated with GraphPad Prism software. All dose–response curves were done twice and a representative graph is shown.

sulfonyl]benzoyl]amino]-3-(benzothiazol-2-yl)-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine) were tested in L-selectin–heparin assays and many of these analogs had inhibitory activity (>30% inhibition) at 30 μM concentration (data not shown). RX-111 (2-[[4-[(diethylamino) sulfonyl]benzoyl]amino]-3-(benzothiazol-2-yl)-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine (Fig. 3, see legend) appeared to be one of the most potent inhibitors with IC-50 = 1 μM. Among other series, about 45 analogs of RX-110 were tested with several compounds having inhibitory activity comparable to RX-110 (data not shown).

We also developed a 96-well ELISA assay for P-selectin interaction with heparin. This assay is very similar to the L-selectin assay using the P-selectin–IgG chimera. The P-selectin ELISA assay was validated in two independent experiments: (i) increasing P-selectin–IgG concentrations gave corresponding dose–response curves; and (ii) P-selectin–IgG binding was inhibited in the presence of soluble heparin (data not shown). The P-selectin assay was used to screen the compound collection on 96-well plates. This screening resulted in the identification of several hits with IC-50 values in the low micro-molar range, however, the hits overall had higher IC-50s than in L-selectin screen. Some hits were identical to those obtained in the L-selectin screen, however, many hits were unique to each of the two selectins. An example of an inhibition curve is shown in Fig. 2B and IC-50 values for some compounds are shown in Table 1.

Table 1
Selected compounds that inhibit binding of selectins to heparin.

Compound no.	L-selectin[a] IC-50 [μM]	P-selectin[a,b] IC-50 [μM]	Heparin binding[c]	Chemical scaffold
RX-101	15.0 ± 2.6	3.2 ± 0.4	+	Iminofluorene
RX-102	2.4 ± 0.4	n.a.	n.d.	Benzothiazole
RX-103	3.9 ± 0.5	9.0 ± 1.7	−	Quinazoline
RX-104	5.6 ± 0.8	n.a.	−	Benzothiophen
RX-105	13.0 ± 2.0	n.a.	+	Thieno[2,3c]pyridine
RX-106	1.6 ± 0.3	n.a.	−	2,6-Dioxo-purin
RX-107	7.0 ± 0.9	n.d.	−	Quinazoline–carboxamide
RX-108	2.4 ± 0.3	25 ± 5%[b]	+	Coumarinoid
RX-109	1.6 ± 0.3	61 ± 11%[b]	−	Thiazolidinone
RX-110	0.7 ± 0.2	n.a.	+	Pyrido[1,2-a]pyrimidin thiazolidinylidene
RX-114	11.0 ± 1.8	13.0 ± 1.9	+	Imidazobenzimidazole
RX-0222	14.0 ± 2.0	n.a.	+	Benzo[de]isoquinoline 1,3-dione
RX-0023	1.6 ± 0.3	36 ± 6.1	+	Quinazoline
RX-0025	2.6 ± 0.3	7.6 ± 1.0	+	Benzo[g]quinazolin

[a] IC-50s of compound inhibition of L-selectin–IgG and P-selectin–IgG binding to heparin are shown.
[b] In some cases IC-50 in P-selectin assay was not measured and % inhibition at 30 μM concentration is shown instead.
[c] Direct binding of SMIGs to heparin is indicated by a plus sign; no evidence for direct binding by a minus sign. These data were obtained as % inhibition in the assay of L-selectin–IgG binding to immobilized heparin at 30 μM compound concentration, by comparing binding inhibition in the pre-incubation assay vs. co-incubation assay (see Results section for description). Abbreviations are: n.a. — no significant activity; n.d. — not determined; + active; − no significant activity. For simplicity, the standard deviation is not shown; the coefficient of variation did not exceed 25% for any IC-50 value shown. All assays were done on 96-well plates in triplicates and IC-50 inhibition curves were done at least twice.

3.2. Mechanism of action of inhibitor compounds

In order to investigate in more detail how compounds inhibit L-selectin binding to heparin and other HS-GAGs, we measured the inhibitory action of several compounds in standard assay as described above, the "Co-Incubation" assay, and in parallel performed "Pre-Incubation" assay in which individual compounds (30 μM final conc.) were incubated with immobilized heparin–BSA on 96-well plates in the absence of L-selectin–IgG. Initially we screened about 40 compounds, 50% of which bound stably to heparin even after multiple washes of plates with buffer and deionized water. Some of the inhibitor compounds that bound directly to heparin are listed in Table 1 and in Table 2 (as "Pre-Incubation" versus "Co-Incubation"). As shown in Table 2, inhibition ranged between 40 and 70% and "Pre-Incubation" assay values were comparable to "Co-Incubation" assay values (i.e., control values, represented by standard assay with co-incubation of compounds and L-selectin–IgG). Negative control plates were coated with BSA only (without heparin) and did not demonstrate any noticeable L-selectin–IgG binding activity and/or compound inhibition activity (data not shown). Subsequently, similar data were obtained with other heparin-binding domain proteins such as VEGF, P-selectin and CMV viral envelope protein. Fig. 5 shows concentration–response curves for RX-111 inhibition of VEGF binding to heparin. "Co-Incubation" vs. "Pre-Incubation" curves were very similar, demonstrating direct and tight binding of RX-111 to heparin. Similar data for RX-111 ("Pre-Incubation" versus "Co-Incubation") were obtained in L-selectin–IgG assays (data not shown).

The compounds, based on their direct action with GAGs and their inhibitory activity of protein–GAG interactions, were named small molecule inhibitors of protein interaction with glycosaminoglycans (SMIGs).

3.3. Selectivity of inhibition with different heparin-binding domain proteins

We set up a panel of protein–heparin binding assays, replacing L selectin with other heparin-binding proteins, to establish the potential of SMIGs to inhibit these interactions with selected SMIGs. The panel

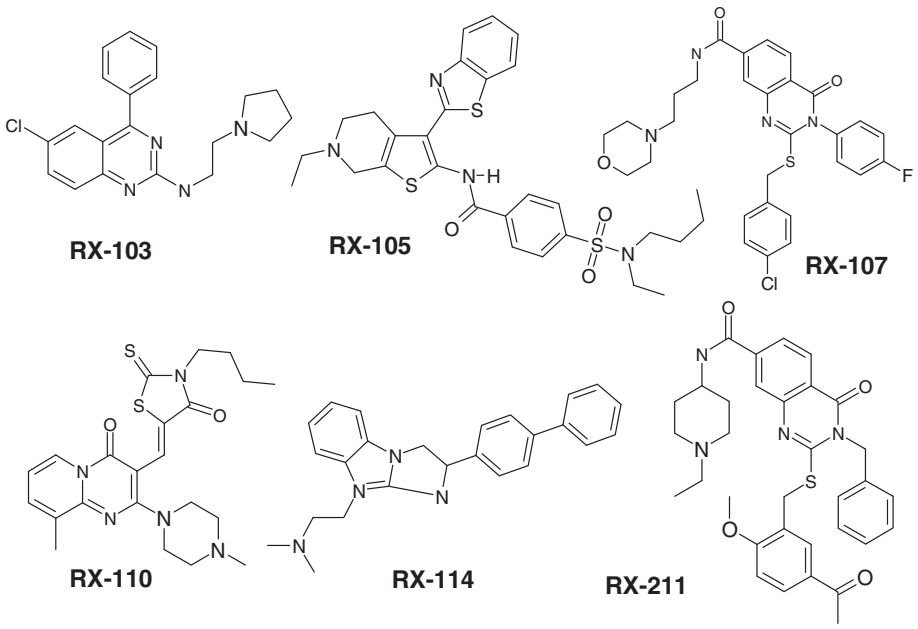

Fig. 3. Chemical structures of selected compounds. Structures of five compounds that inhibited L-selectin/IgG binding to heparin and had significant anti-inflammatory activity are shown. For more details see also Table 1. RX-112 is identical to RX-107 except that the chloride is replaced by a methyl group. RX-111 is identical to RX-105 except that the butyl group is replaced by ethyl.

contained 11 proteins containing heparin-binding domains and the results of this screen are shown in Table 3.

3.4. Range of action of SMIGS towards different GAGs

In order to investigate inhibitory properties of SMIGs towards different GAG species (commercially available), binding assays were developed for shark cartilage chondroitin sulfate, hog skin dermatan sulfate and bovine kidney heparan sulfate binding to L-selectin–IgG, chemokines and cytokines. As shown in Fig. 6, compounds that inhibited binding to heparin also inhibited binding to immobilized bovine kidney HS-GAGs (no attempt was made to perform full dose–response curves due to limited availability of bovine kidney HS-GAGs). Table 4 shows the results of IP-10 binding to shark cartilage chondroitin sulfate and the inhibition of this binding by several different inhibitor compounds. Overall there was a relative lack of inhibitory activity towards chondroitin sulfate and dermatan sulfate, with predominant activity of SMIGs being towards HS-GAGs.

3.5. Anti-inflammatory activity of SMIGs

The ability of SMIG compounds to reduce leukocyte migration to sites of acute inflammation was evaluated. As reviewed in the Introduction section, inhibitors of L-selectin–HS-GAG interaction might prevent leukocyte rolling on endothelium and cell migration towards inflammatory sites. We have therefore selected ten L-selectin–HS-GAG inhibitor compounds representing different chemical series, and evaluated these compounds in a thioglycollate-induced model of peritonitis in BALB/c mice. In the first series of experiments, 10 compounds were tested in thioglycollate-induced peritonitis and as many as 5 compounds were found to have statistically significant inhibitory activity in this model, as shown in Table 5. Compound RX-105 was studied in more detail at three doses, 2 mg/kg, 10 mg/kg and 50 mg/kg. As shown in Fig. 7, Compound RX-105 was a potent inhibitor of neutrophil infiltration. Infiltration was reduced by 76% at 50 mg/kg, by 46% at 10 mg/kg and by 25% at 2 mg/kg. The efficacy of the RX-105 thienopyridine chemical series was confirmed with their analogs (data not shown). Subsequent studies

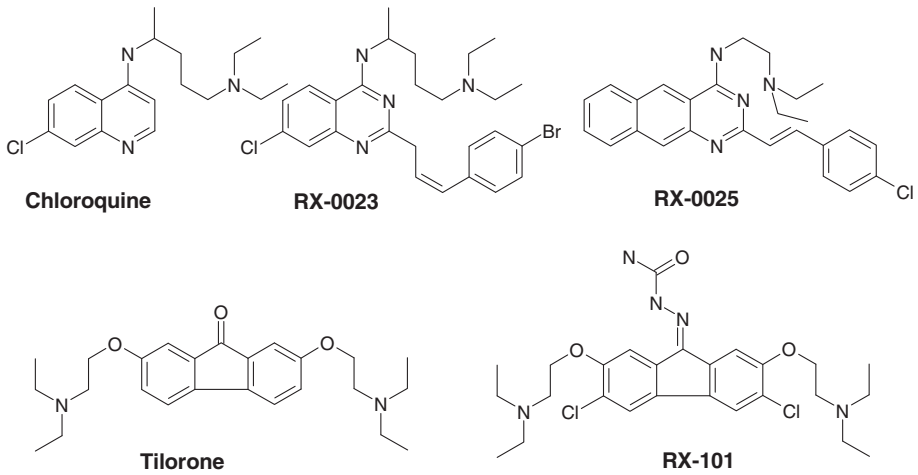

Fig. 4. Selected hit compounds that are structurally similar to chloroquine and tilorone. Chemical structures of RX-0023 and RX-0025 are similar to chloroquine, whereas RX-101 is similar to tilorone. These compounds bind directly to heparin (Table 1).

Table 2
Demonstration of direct binding of L-selectin inhibitor compounds and VEGF inhibitor compounds to heparin.

Compound no.	L-selectin pre-incubation % inhibition	L-selectin co-incubation % inhibition	VEGF pre-incubation % inhibition	VEGF co-incubation % inhibition
RX-101	70.6 ± 14.1	77.4 ± 16.9		
RX-0025	68.6 ± 12.6	75.2 ± 13.7		
RX-0222	58.5 ± 11.7	61.2 ± 12.5		
RX-108	48.2 ± 10.2	65.9 ± 12.0		
RX-110	40.4 ± 10.7	44.4 ± 10.3	81.0 ± 17.0	79.0 ± 19.9
RX-111	57.5 ± 11.0	68.0 ± 12.1	73.0 ± 16.8	72.0 ± 19.0

For assay description, see Experimental procedures and Results sections. Test compounds were used at 30 μM. All assays were done on 96-well plates in triplicates and each experiment was done at least twice.

with the closely related RX-111 compound demonstrated activity in paw edema after per oral and i.v. administration (data not shown).

Selected compounds were tested in another model of acute inflammation, paw edema. Edema was induced in the left hind paw of Balb/c mice by injecting a solution of 2% carrageenan. Test compounds were injected 1 h before carrageenan. As shown in Table 5, several test compounds significantly reduced carrageenan induced paw edema. Compound RX-105 was selected for more detailed analysis and it inhibited paw edema dose dependently (Fig. 8). Subsequent studies with closely related analog RX-111 (see Fig. 3 legend for structure) confirmed the anti-inflammatory effect of this chemical series on paw edema. In order to compare the efficacy of different anti-inflammatory compounds, we routinely tested the performance of dexamethasone, a steroid compound, and Diclofenac, a non-steroidal anti-inflammatory compound. Throughout these studies, dexamethasone (3 mg/kg i.p.) inhibited paw edema to about 44% of control. Diclofenac, at 10 mg/kg (p.o.), gave 23–36% inhibition in several studies.

The anti-inflammatory activity of compounds has been further evaluated in mice using the DTH animal model that is associated with infiltration by macrophages and T-cells, and is predictive of therapeutic potential in autoimmune indications such as rheumatoid arthritis and multiple sclerosis. As shown in Table 5, DTH ear swelling was efficiently inhibited by administration by two lead compounds, RX-105 and RX-112.

4. Discussion

This report describes a first and systematic approach to identify small molecule compounds that inhibit interactions between heparin-binding domain containing proteins and GAGs. A wide range of structurally diverse inhibitor compounds that interact with GAGs have been identified and named small molecule inhibitors of protein interaction with glycosaminoglycans ("SMIGs"). SMIGs are not related to small molecule GAG mimetics such as sulphonic or sulfate-containing anionic small molecule compounds (28) and have a different mechanism of action.

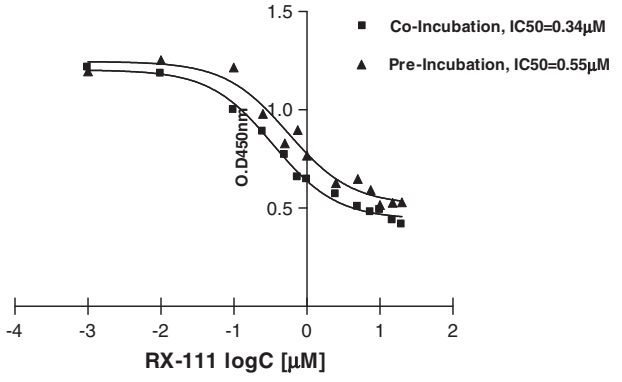

Fig. 5. "Co-incubation" vs. "Pre-incubation" curves. Example of a SMIG compound that binds directly to heparin. RX-111 (Fig. 3, legend) inhibited VEGF binding to heparin in both pre-incubation and co-incubation assays with a similar inhibition dose–inhibition curve.

Table 3
Comparison of inhibitory activity of selected SMIGs towards different heparin-binding proteins.

	RX-101	RX-110	RX-111 (RX-105 series)	RX-211 (RX-107 series)
L-selectin	15.0 ± 2.6	0.7 ± 0.2	1.0 ± 0.2	5.5 ± 0.7
P-selectin	3.2 ± 0.4	–	–	–
MCP-1	nd	–	–	–
MCP-3	112.0 ± 24.2	–	–	12.0 ± 2.1
RANTES	25.0 ± 4.5	–	–	7.0 ± 1.4
IP-10	nd	–	5.0 ± 0.8	–
IL-8	nd	–	–	19.0 ± 3.6
VEGF	3.2 ± 0.6	2.3 ± 0.4	1.0 ± 0.2	3.4 ± 0.7
IFNγ	nd	–	–	36.0 ± 7.0
IL-6	nd	–	–	2.5 ± 0.4
Aβ	–	1.5 ± 0.2	75.0 ± 14.7	–
Fibronectin	nd	–	–	17.0 ± 3.3
CMV-gB*	90 ± 14%	90 ± 10%	–	nd

The table shows inhibitory characteristics of four compounds (RX-101, RX-110, RX-111 and RX-211) in various protein–heparin binding assays. All values (except for CMV-gB) are given as IC-50 in μM. For simplicity, standard deviation is not shown. The coefficient of variation did not exceed 25% for any IC-50 value shown. For CMV-gB, only % inhibition at 30 μM was measured. Compounds at the top of the table in the bracket indicate origin of the chemical series, i.e., RX-111 is a close analog of RX-105 and RX-211 is a close analog of RX-107 (Fig. 3). nd — not done. When a compound had no inhibitory activity in a particular assay, a dash is shown. All assays were done on 96-well plates in triplicates and IC-50 inhibition curves were done at least twice.

The L-selectin–IgG fusion protein [21,38], a bivalent L-selectin chimera, reflective of in vivo acting L-selectin, was used. Heparin, a highly sulfated analog of HS-GAG, was used as a surrogate for HS-GAGs [2,20,37]. An antibody directed against L-selectin was used to validate the specificity of the L-selectin–IgG–heparin binding assay. The Dreg-55 antibody, known to interact with the carbohydrate-binding domain of L-selectin, inhibits neutrophil accumulation in vitro and inflammation in vivo [42]. Screening for L-selectin–heparin inhibitors identified a number of hits with diverse chemical structures. Concentration–response studies determined that the IC-50 for inhibition was in the range of 0.1–70 μM for the active compounds. The most potent inhibitors had IC-50 in the 0.1–1 μM range and a number of compounds had

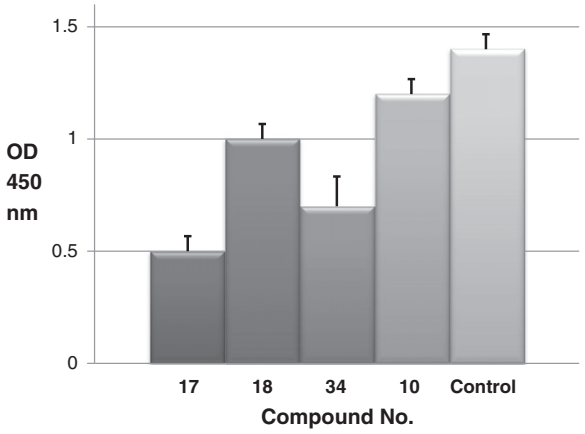

Fig. 6. Inhibition of L-selectin–IgG binding to immobilized bovine kidney heparan sulfate by different inhibitor compounds. Compounds shown are RX-110 (compound 10) and three members of thieno[2,3c]pyridines related to RX-105: 2-[[(4-(3,4- dihydro-2(1H)-isoquinolinyl)sulfonyl] benzoyl]amino]-6-(1-methylethyl)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxamide (compound 17); 2-[[4-[[ethyl(phenylmethyl)amino]sulfonyl] benzoyl]amino]-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxamide (Compound 18) and 2-[[4-(3,4-dihydro-1(2H)-quinolinyl)sulfonyl]benzoyl]amino]-4,5,6,7-tetrahydro-5,5,7,7-tetramethyl thieno[2,3-c]pyridine-3-carboxamide (compound 34). The experiments were done in triplicate wells and coefficient of variation did not exceed 30%.

Table 4
Compounds that inhibit IP-10 binding to chondroitin sulfate.

Compound	Inhibition of IP-10 binding to chondroitin sulfate[a] (control = 100%)
RX-102	–
RX-105	11 ± 3%
RX-106	–
RX-109	54 ± 8%
RX-110	57 ± 9%
RX-111	22 ± 4%
RX-211	75 ± 18%

[a] Percent inhibition at 30 μM compound concentration is shown. When a compound had no inhibitory activity in a particular assay, a dash is shown. All assays were done on 96-well plates in triplicates and each experiment was done at least twice.

IC-50 in the 10–50 μM range. In order to confirm that compounds bind to GAGs other than heparin, we examined the inhibitory activity of these compounds using different commercially available GAGs purified from mammalian tissues. These studies confirmed that compounds with inhibitory activity in heparin–protein assays also had inhibitory activity in bovine kidney HS-GAG–protein assays (Fig. 6 and data not shown). There were considerably less compounds with inhibitory activity against chondroitin sulfate and very little evidence for inhibitory compounds against dermatan sulfate. Compounds active in the IP-10/chondroitin sulfate assay are shown in Table 4. These studies were limited to a few commercially available sources of GAGs and should be expanded in future studies to include additional sources of tissue and cell sources of mammalian GAGs. Based on our current data, the compounds are predominantly targeting interactions of HS-GAGs.

We next examined the mechanism of inhibition by hit compounds. In principle, compounds that inhibit interaction between L-selectin and heparin can function in one of two ways (i) bind to L-selectin and prevent its interaction with heparin; or (ii) bind to heparin and prevent its binding to L-selectin. We considered a third possibility of joint binding of inhibitor compound to both L-selectin and heparin, but except for rare causes, found no evidence for this mechanism of action. Since many inhibitor compounds are cationic amines, we first considered the possibility that the compounds interact with heparin, which has anionic character. One notable empirical observation was that, in spite of their moderate IC-50 values in the micro-molar range, inhibition appeared to be persistent, even after short incubation times, under the assay conditions (data not shown). In a series of key experiments (described as "Co-Incubation" assay vs. "Pre-Incubation" assay in the Results section), inhibitor compounds were applied to 96-well plates containing only immobilized heparin and these plates were subsequently washed extensively with buffer and water. The standard L-selectin binding assay was subsequently performed with addition of L-selectin–IgG, washing, addition of second antibody, washing, etc. Inhibitor compounds remained bound to immobilized heparin as evinced by their inhibitory action against L-selectin binding. These experiments (shown in Table 2) indicated that inhibitor compounds bind to heparin strongly and persistently. Controls, such as plates coated with BSA only did not show any L-selectin–IgG binding activity and/or compound inhibition

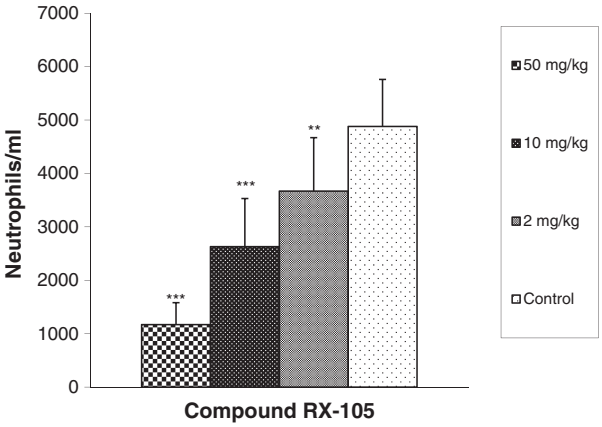

Fig. 7. Dose-dependent inhibition of neutrophil infiltration by RX-105 in mouse peritonitis animal model. For experimental details, see Experimental procedures section.

activity. Initially we screened about 40 compounds in this manner and about 50% of these compounds bound to heparin. Some inhibitor compounds did not show activity in this assay (e.g., RX-103, RX-107), possibly due to faster off-rate kinetics which prevented their detection under the assay conditions. Further evidence for direct binding of SMIGs to heparin came from concentration–response studies. IC-50 values for inhibitory compounds was nearly identical in "Pre-Incubation" vs. "Co-Incubation" assays (Table 2). Subsequently, direct binding of SMIGs to heparin was verified with a second heparin-binding protein, VEGF (Table 2). Although we have not investigated the kinetics of compound binding to heparin in detail, empirical observations indicated fast association rate and a slow dissociation rate. The curve of RX-110 and other related compounds often gave Hill coefficients in the range of −2 to −5, raising the possibility of cooperativity in binding. High salt concentration appeared to inhibit SMIGs binding to heparin (data not shown), indicating that charge interactions, such as between cationic amines and sulfate groups of HS-GAGs, may have a significant role in the interaction (however, ionic charge interactions alone would not be expected to account for the stable binding of SMIGs to GAG, see below). The evidence for direct binding of SMIGs to GAGs was verified by the results of compound library screening, whereas compounds RX-0023, RX-0025 and RX-101, which are highly similar to chloroquine and tilorone (see below), were identified as binders of GAGs (Fig. 4 and Table 1).

Whereas certain small molecule compounds have been known to bind to GAGs, the biological significance of this interaction has not been determined. Among compounds known to bind to GAGs is Methylene Blue, a compound with pleiotropic biochemical and therapeutic properties [43], as well as other cationic phenothiazines such as chlorpromazine, a well-known anti-psychotic drug that has anti-prion amyloidosis properties (35). Certain acridines such as acridine orange, tacrine and quinacrine are also cationic compounds known to bind GAGs [35]. Binding of tilorone and chloroquine to GAGs has been described in the past by physico-chemical methods such as UV spectroscopy and circular dichroism [35,44,45]. However, the biological significance

Table 5
Anti-inflammatory activity of selected compounds in peritonitis, paw edema and delayed-type hypersensitivity.[a]

Chemical series	Dose, i.p.	Peritonitis	Dose, i.p.	Paw edema	Dose	DTH
RX-101	5 mg/kg	1560 ± 610 (54%; $p < 0.001$)				
RX-103	1 mg/kg	2800 ± 1040 (67%; $p < 0.001$)				
RX-105	10 mg/kg	2550 ± 650 (69%;$p < 0.001$)	10 mg/kg	0.844 ± 0.167 (70%; $p < 0.001$)	3 mg/kg i.v.	0.111 ± 0.029 (56%; $p < 0.001$)
RX-112[b]	10 mg/kg	1360 ± 660 (47%; $p < 0.001$)	5 mg/kg	0.64 ± 0.196 (65%; $p < 0.001$)	5 mg/kg i.m.	0.235 ± 0.066 (77%; $p < 0.05$)
RX-108	5 mg/kg	2270 ± 1130 (79%; NS)	5 mg/kg	0.91 ± 0.274 (80%; $p < 0.05$)		
RX-110	10 mg/kg	2140 ± 1140 (74%; $p < 0.05$)	5 mg/kg	0.745 ± 0.305 (66%; $p < 0.01$)		NA

[a] Data are expressed as mean ± SD. Percent of control values (100%) are given in parentheses, peritonitis results are shown as % inhibition of neutrophil infiltration, paw edema data are shown as % inhibition of paw swelling and DTH data are shown as percent inhibition of ear swelling.
[b] RX-112 is a close analog of RX-107 (see Fig. 3 legend for structure). RX-107 was used in peritonitis. Test compounds were administered at doses indicated, each group consisting of 12–15 animals. The statistical significance as determined by Student's *t*-test is given. NS, not significant; NA, not active.

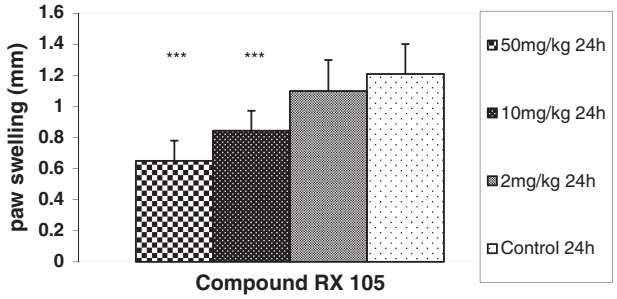

Fig. 8. Dose-dependent inhibition of paw swelling by RX-105 in paw edema animal model. Data show reduction in paw swelling 24 h after induction of edema with carageenan. Less pronounced, albeit statistically significant effects were observed also after 4 h interval with the two higher concentrations of RX-105. Subsequent studies with closely related analog RX-111 gave similar data and confirmed the anti-inflammatory effect this chemical series on paw edema.

of the binding of these compounds to GAGs on the cell surface has not been understood and until now the functional significance of heparin binding domains has not been explored as a strategy for small molecule drug discovery. Among small molecules reported to bind GAGs is bis-2-methyl-4-amino-quinolyl-6-carbamide, also known as surfen [36], which is a weakly di-cationic aminoquinoline compound. Surfen blocked FGF2-binding and signaling that depended on cell surface heparan sulfate and prevented both FGF2- and VEGF-mediated sprouting of endothelial cells in Matrigel. Surfen also blocked heparan sulfate-mediated cell adhesion to the Hep-II domain of fibronectin and prevented infection by HSV-1 that depended on glycoprotein D interaction with heparan sulfate [36]. Another small molecule reported to interact with GAGs is adhesamine, a dumbbell-shaped dicationic compound that supports cell adhesion [46].

Previous studies did not predict the very wide range of chemotypes that are capable of binding directly and tightly to GAGs and exerting biological activity. Examples of the many chemotypes identified in the course of this work are shown in Figs. 3 and 4. A few of them are related to acridines, but the majority represent novel scaffolds and chemical series. The interaction between SMIGs and HS-GAGs appears to be due to a combination of at least two factors: (i) charge interactions between sulfate groups of HS-GAG and the protonated nitrogen of SMIGs; and (ii) the planar aromatic character of the compounds. The cooperative binding of compounds to GAG suggests that multiple compounds interact with a single GAG chain. In this respect, binding of SMIGs to GAGs may resemble binding of intercalators to DNA, which also involves charge interactions and planar aromatic rings. It is of interest that both quinacrine and methylene blue are DNA intercalators [47]. Future work should be focused on a much more detailed structural analysis of these SMIG–HS-GAG interactions by biophysical techniques [6,48].

The therapeutic potential of the "hit" compounds identified in the screening assays has been explored by testing in animal models of inflammation. We used thioglycollate-induced peritonitis, because L-selectin inhibitors are known to be active in this model of neutrophil infiltration [39]. A number of compounds displayed statistically significant anti-inflammatory activity in this animal model. Subsequent work with analogs of 3 lead compound series confirmed their anti-inflammatory activity. These are the first reported small molecule compounds, which are capable of modulating the interaction between L-selectin and HS-GAGs and have anti-inflammatory activity. A detailed study of RX-105 demonstrated dose-dependent inhibition of leukocyte infiltration into mouse peritoneum (Fig. 7). A closely related analog, RX-111 (Fig. 3), had potent anti-inflammatory effects in paw edema, DTH, Experimental Allergic Encephalomyelitis and an animal model of Trinitrobenzenesulfonic acid-induced colitis (data not shown; manuscript in preparation). Results of paw edema for compounds such as RX-107 and RX-110 are listed in Table 4 and Fig. 8. Inhibitors of neutrophil infiltration may be useful as therapeutics for acute inflammatory diseases such as ischemia–reperfusion injury [39], whereas compounds active in DTH may have therapeutic potential in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis [49]. For SMIGs to be candidate anti-inflammatory/autoimmune disease therapeutics, it is important to assess their selectivity for different chemokines and cytokines containing heparin-binding domains. Although initial data (comparing L-selectin to P-selectin) pointed to rather good selectivity of these inhibitors, further work indicated that other proteins containing heparin-binding domains may also be inhibited. As a first step towards a more detailed evaluation of selectivity, we set up a panel of HS-GAG-protein binding assays (that were similar in experimental design to L-selectin-heparin binding assay) and measured inhibitory activity of selected SMIGs. The panel contained 11 proteins containing heparin-binding domains, divided into six classes: 2 selectins, 4 chemokines, 3 cytokines, amyloid beta, fibronectin and a cytomegalovirus envelope protein gB. These proteins, except for fibronectin and cytomegalovirus envelope protein gB, are known to possess a single heparin-binding domain. The result of this screen showed that some compounds had a narrow selectivity profile, inhibiting only one or two proteins, whereas others were promiscuous having a broad spectrum (Table 3).

Overall, there was a relatively high hit rate of SMIGs with anti-inflammatory activity (Table 5 and data not shown). This raises the possibility that SMIG binding to HS-GAGs and/or SMIG inhibition of protein binding to GAGs may represent a major checkpoint for events associated with the inflammatory response. Consistent with this notion, GAG binders chloroquine and quinacrine are potent well-known anti-inflammatory drugs. It is hoped that SMIGs with superior therapeutic properties will be identified.

In view of our results, it may be reasonable to consider modulation of GAG–protein binding as a mechanism of action for chloroquine. Of note, scaffolds related to chloroquine, such as compounds RX-0023 and RX-0025, inhibited binding of both selectins to heparin (Table 1). Although not the focus of this work, we have found that chloroquine itself did not significantly inhibit any of the proteins in Table 3 (data not shown). Given the large size of the heparin-binding domain superfamily this is not particularly surprising. Chloroquine is an anti-malarial drug that is used for the treatment of lupus and rheumatoid arthritis (and also has anti-viral properties) and despite substantial research efforts, its molecular mechanism of action has not been convincingly established [50]. Conceivably, some of the observed biochemical and biological activities of chloroquine may be due to its binding to GAGs and inhibition of protein–GAG interactions.

The mechanism responsible for the anti-inflammatory properties of the SMIGs described (Tables 1 and 2) remains to be determined on a case by case basis. Selectins and HS-GAGs have been implicated independently and jointly (via L-selectin–HS-GAG binding) in the inflammation process. The SMIGs described here inhibited L-selectin (and/or P-selectin) binding to HS-GAGs. It is conceivable that the same SMIGs inhibit a additional protein–HS-GAG interactions such as those of pro-inflammatory chemokines and cytokines. In order to develop SMIGs as therapeutics, it is also necessary to consider their cytotoxicity and possible interference with the coagulation cascade. SMIGs might be also useful for neutralization of the anti-coagulant activity of heparin, although preliminary investigation with compounds reported herein did not find any potent compounds (data not shown).

In conclusion, we describe a novel, structurally-diverse class of compounds that apparently have potent biological activity owing to their direct interaction with GAGs, specifically HS-GAGs. The compounds, termed small molecule inhibitors of protein interaction with glycosaminoglycans (SMIGs), inhibit binding of heparin-binding domain proteins to GAGs. A number of SMIGs have demonstrable anti-inflammatory properties. SMIGs represent a novel class of compounds, that given an acceptable toxicological profile, may be therapeutic in inflammatory and autoimmune disorders as well as in other human diseases, where GAGs are known to have key biological roles.

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References

[1] E.M. Muñoz, R.J. Linhardt, Heparin-binding domains in vascular biology, Arterioscler. Thromb. Vasc. Biol. 24 (9) (2004) 1549–1557.
[2] J.T. Gallagher, Heparan sulphate: a heparin in miniature, Handb. Exp. Pharmacol. 207 (2012) 347–360.
[3] R. Sasisekharan, G. Venkataraman, Heparin and heparan sulfate: biosynthesis, structure and function, Curr. Opin. Chem. Biol. 4 (2000) 626–631.
[4] U. Lindahl, J.P. Li, Interactions between heparan sulfate and proteins-design and functional implications, Int. Rev. Cell Mol. Biol. 276 (2009) 105–159.
[5] S. Sarrazin, W.C. Lamanna, J.D. Esko, Heparan sulfate proteoglycans, Cold Spring Harb. Perspect. Biol. 3 (7) (2011).
[6] B. Mulloy, Structure and physicochemical characterization of heparin, Handb. Exp. Pharmacol. 207 (2012) 77–98.
[7] N.S. Gandhi, R.L. Mancera, Heparin/heparan sulphate-based drugs, Drug Discov. Today 15 (23–24) (2010) 1058–1069.
[8] U. Lindahl, L. Kjellén, Pathophysiology of heparan sulphate: many diseases, few drugs, J. Intern. Med. 273 (6) (2013) 555–571.
[9] K.R. Taylor, R.L. Gallo, Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation, FASEB J. 20 (1) (2006) 9–22.
[10] J.P. Li, I. Vlodavsky, Heparin, heparan sulfate and heparanase in inflammatory reactions, Thromb. Haemost. 102 (5) (2009) 823–828.
[11] M. Irony-Tur-Sinai, I. Vlodavsky, S.A. Ben-Sasson, F. Pinto, C. Sicsic, T. Brenner, A synthetic heparin-mimicking polyanionic compound inhibits central nervous system inflammation, J. Neurol. Sci. 206 (1) (2003) 49–57.
[12] B. Casu, A. Naggi, G. Torri, Heparin-derived heparan sulfate mimics to modulate heparan sulfate-protein interaction in inflammation and cancer, Matrix Biol. 29 (6) (2010) 442–452.
[13] O. Lider, E. Baharav, Y.A. Mekori, T. Miller, Y. Naparstek, I. Vlodavsky, I.R. Cohen, Suppression of experimental autoimmune diseases and prolongation of allograft survival by treatment of animals with low doses of heparins, J. Clin. Invest. 83 (3) (1989) 752–756.
[14] I. Vlodavsky, Y. Friedmann, M. Elkin, H. Aingorn, R. Atzmon, R. Ishai-Michaeli, M. Bitan, O. Pappo, T. Peretz, I. Michal, L. Spector, I. Pecker, Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis, Nat. Med. 5 (7) (1999) 793–802.
[15] C.R. Parish, The role of heparan sulphate in inflammation, Nat. Rev. Immunol. 6 (9) (2006) 633–643.
[16] L. Lasky, Selectin–carbohydrate interactions and the initiation of the inflammatory response, Annu. Rev. Biochem. 64 (1995) 113–139.
[17] H. Kawashima, Roles of sulfated glycans in lymphocyte homing, Biol. Pharm. Bull. 29 (12) (2006) 2343–2349.
[18] R.M. Nelson, O. Cecconi, W.G. Roberts, A. Aruffo, R.J. Linhardt, M.P. Bevilacqua, Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation, Blood 82 (11) (1993) 3253–3258.
[19] L. Borsig, L. Wang, M.C. Cavalcante, L. Cardilo-Reis, P.L. Ferreira, P.A. Mourão, J.D. Esko, M.S. Pavão, Selectin blocking activity of a fucosylated chondroitin sulfate glycosaminoglycan from sea cucumber. Effect on tumor metastasis and neutrophil recruitment, J. Biol. Chem. 282 (20) (2007) 14984–14991.
[20] L. Wang, J.R. Brown, A. Varki, J.D. Esko, Heparin's anti-inflammatory effects require glucosamine 6-O-sulfation and are mediated by blockade of L- and P-selectins, J. Clin. Invest. 110 (1) (2002) 127–136.
[21] K.E. Norgard-Sumnicht, N.M. Varki, A. Varki, Calcium-dependent heparin-like ligands for L-selectin in nonlymphoid endothelial cells, Science 261 (5120) (1993) 480–483.
[22] S.J. Romano, Selectin antagonists: therapeutic potential in asthma and COPD, Treat. Respir. Med. 4 (2) (2005) 85–94.
[23] R. Kranich, A.S. Busemann, D. Bock, S. Schroeter-Maas, D. Beyer, B. Heinemann, M. Meyer, K. Schierhorn, R. Zahlten, G. Wolff, E.M. Aydt, Rational design of novel, potent small molecule pan-selectin antagonists, J. Med. Chem. 50 (6) (2007) 1101–1115.
[24] S. Ye, Y. Luo, W. Lu, R.B. Jones, R.J. Linhardt, I. Capila, T. Toida, M. Kan, H. Pelletier, W.L. McKeehan, Structural basis for interaction of FGF-1, FGF-2, and FGF-7 with different heparan sulfate motifs, Biochemistry 40 (48) (2001) 14429–14439.
[25] H. Lortat-Jacob, A. Grosdidier, A. Imberty, Structural diversity of heparan sulfate binding domains in chemokine, Proc. Natl. Acad. Sci. U. S. A. 99 (3) (2002) 1229–1234.
[26] H. Margalit, N. Fischer, S.A. Ben-Sasson, Comparative analysis of structurally defined heparin binding sequences reveals a distinct spatial distribution of basic residues, J. Biol. Chem. 268 (26) (1993) 19228–19231.
[27] A. Yayon, M. Klagsbrun, J.D. Esko, P. Leder, D.M. Ornitz, Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor, Cell 64 (4) (1991) 841–848.
[28] L. Pellegrini, D.F. Burke, F. von Delft, B. Mulloy, T.L. Blundell, Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin, Nature 407 (6807) (2000) 1029–1034.
[29] S. Sarrazin, D. Bonnaffé, A. Lubineau, H. Lortat-Jacob, Heparan sulfate mimicry: a synthetic glycoconjugate that recognizes the heparin binding domain of interferon-gamma inhibits the cytokine activity, J. Biol. Chem. 280 (45) (2005) 37558–37564.
[30] Z. Johnson, M.H. Kosco-Vilbois, S. Herren, R. Cirillo, V. Muzio, P. Zaratin, M. Carbonatto, M. Mack, A. Smailbegovic, M. Rose, R. Lever, C. Page, T.N. Wells, A.E. Proudfoot, Interference with heparin binding and oligomerization creates a novel anti-inflammatory strategy targeting the chemokine system, J. Immunol. 173 (9) (2004) 5776–5785.
[31] T.M. Handel, Z. Johnson, S.E. Crown, E.K. Lau, A.E. Proudfoot, Regulation of protein function by glycosaminoglycans — as exemplified by chemokines, Annu. Rev. Biochem. 74 (2005) 385–410.
[32] A.E. Proudfoot, The biological relevance of chemokine–proteoglycan interactions, Biochem. Soc. Trans. 34 (Pt 3) (2006) 422–426.
[33] T. Adage, A.M. Piccinini, A. Falsone, M. Trinker, J. Robinson, B. Gesslbauer, A.J. Kungl, Structure-based design of decoy chemokines as a way to explore the pharmacological potential of glycosaminoglycans, Br. J. Pharmacol. 167 (6) (2012) 1195–1205.
[34] R. Kisilevsky, L.J. Lemieux, P.E. Fraser, X. Kong, P.G. Hultin, W.A. Szarek, Arresting amyloidosis in vivo using small-molecule anionic sulphonates or sulphates: implications for Alzheimer's disease, Nat Med. 1 (2) (1995) 143–148.
[35] F. Zsila, G. Gedeon, Binding of anti-prion agents to glycosaminoglycans: evidence from electronic absorption and circular dichroism spectroscopy, Biochem. Biophys. Res. Commun. 346 (4) (2006) 1267–1274.
[36] M. Schuksz, M.M. Fuster, J.R. Brown, B.E. Crawford, D.P. Ditto, R. Lawrence, C.A. Glass, L. Wang, Y. Tor, J.D. Esko, Surfen, a small molecule antagonist of heparan sulfate, Proc. Natl. Acad. Sci. U. S. A. 105 (35) (2008) 13075–13080.
[37] S. Najjam, R.V. Gibbs, M.Y. Gordon, C.C. Rider, Characterization of human recombinant interleukin 2 binding to heparin and heparan sulfate using an ELISA approach, Cytokine 9 (12) (1997) 1013–1022.
[38] C. Foxall, S.R. Watson, D. Dowbenko, C. Fennie, L.A. Lasky, M. Kiso, A. Hasegawa, D. Asa, B.K. Brandley, The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis(x) oligosaccharide, J. Cell Biol. 117 (4) (1992) 895–902.
[39] X. Xie, A.S. Rivier, A. Zakrzewicz, M. Bernimoulin, X.L. Zeng, H.P. Wessel, M. Schapira, O. Spertini, Inhibition of selectin-mediated cell adhesion and prevention of acute inflammation by non-anticoagulant sulfated saccharides. Studies with carboxyl-reduced and sulfated heparin and with trestatin a sulfate, J. Biol. Chem. 34818–34825 (2000).
[40] B. Lange-Asschenfeldt, W. Weninger, P. Velasco, T.R. Kyriakides, U.H. von Andrian, P. Bornstein, M. Detmar, Increased and prolonged inflammation and angiogenesis in delayed-type hypersensitivity reactions elicited in the skin of thrombospondin-2-deficient mice, Blood 99 (538–545) (2002) 2002.
[41] S.R. Torres, T.S. Fröde, G.M. Nardi, N. Vita, R. Reeb, P. Ferrara, R.M. Ribeiro-do-Valle, R.C. Farges, Anti-inflammatory effects of peripheral benzodiazepine receptor ligands in two mouse models of inflammation, Eur. J. Pharmacol. 199–211 (2000).
[42] M.S. Co, N.F. Landolfi, J.O. Nagy, J.H. Tan, V. Vexler, M. Vasquez, L. Roark, S. Yuan, P.R. Hinton, J. Melrose, C. Klingbeil, C. Queen, E.L. Berg, Properties and pharmacokinetics of two humanized antibodies specific for L-selectin, Immunotechnology 4 (3–4) (1999) 253–266.
[43] R.H. Schirmer, H. Adler, M. Pickhardt, E. Mandelkow, "Lest we forget you–methylene blue…", Neurobiol. Aging 32 (12) (2011) 2325(e7–16).
[44] J. Suschke, R. Wilhelm, U.Z. Walther, Effect of chloroquine on polyanions, Rheumatology 39 (9–10) (1980) 314–321.
[45] R. Lüllmann-Rauch, R. Pods, B. von Witzendorff, The antimalarials quinacrine and chloroquine induce weak lysosomal storage of sulphated glycosaminoglycans in cell culture and in vivo, Toxicology 110 (1–3) (1996) 27–37.
[46] S. Yamazoe, H. Shimogawa, S. Sato, J.D. Esko, M. Uesugi, A dumbbell-shaped small molecule that promotes cell adhesion and growth, J. Chem. Biol. 16 (7) (2009) 773–782.
[47] M. Hossain, G. Suresh Kumar, DNA intercalation of methylene blue and quinacrine: new insights into base and sequence specificity from structural and thermodynamic studies with polynucleotides, Mol. Biosyst. 5 (11) (2009) 1311–1322(2009 Nov).
[48] F.E. Stanley, A.M. Warner, S.M. Gutierrez, A.M. Stalcup, Heparin-induced circular dichroism of chloroquine, Biochem. Biophys. Res. Commun. 388 (1) (2009) 28–30.
[49] O. Lider, L. Cahalon, D. Gilat, R. Hershkoviz, D. Siegel, R. Margalit, O. Shoseyov, I.R. Cohen, A disaccharide that inhibits tumor necrosis factor alpha is formed from the extracellular matrix by the enzyme heparanase, Proc. Natl. Acad. Sci. U. S. A. 92 (11) (1995) 5037–5041.
[50] R. Lafyatis, M. York, A. Marshak-Rothstein, Antimalarial agents: closing the gate on Toll-like receptors? Arthritis Rheum. 54 (10) (2006) 3068–3070.