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Discovery of a Small-Molecule Modulator of Glycosaminoglycan Sulfation

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Abstract

Glycosaminoglycans (GAGs) play critical roles in diverse processes ranging from viral infection to neuroregeneration. Their regiospecific sulfation patterns, which are generated by sulfotransferase enzymes, are key structural determinants that underlie their biological activity. Small-molecule modulators of these sulfotransferases could serve as powerful tools for understanding the physiological functions of GAGs, as well as potential therapeutic leads for human diseases. Here, we report the development of the first selective, cell-permeable small-molecule inhibitor of GAG sulfotransferases, which was obtained using a high-throughput screen targeted against Chst15, the sulfotransferase responsible for biosynthesis of the chondroitin sulfate-E (CS-E) motif. We demonstrate that the molecule specifically inhibits GAG sulfotransferases *in vitro*, decreases CS-E and overall sulfation levels on cell-surface and secreted chondroitin sulfate proteoglycans (CSPGs), and reverses CSPG-mediated inhibition of axonal growth. These studies pave the way toward a new set of pharmacological tools for interrogating GAG sulfation-dependent processes and may represent a novel potential therapeutic approach for neuroregeneration.

Graphical abstract

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Supporting Information

Experimental procedures, compound characterizations, supporting figures, tables and schemes. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

The authors declare no competing financial interests.

INTRODUCTION

Glycosaminoglycans (GAGs) play important roles in a myriad of cellular processes, including viral invasion,¹ cancer metastasis,^{1–3} cell development,^{1,4,5} and spinal cord injury.^{5–7} Their diverse biological activities are believed to stem from their complex structures, which allow them to interact with a wide range of proteins.^{1,8,9} GAGs are long, linear polysaccharides that display various patterns of sulfation. For example, chondroitin sulfate (CS) GAGs are assembled from glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc) disaccharide units that contain at least four distinct sulfation motifs: CS-A, CS-C, CS-D, and CS-E. Numerous studies suggest that specific sulfation motifs can serve as key structural elements for protein recognition and the regulation of downstream signaling.^{1,9–11}

The sulfation patterns of GAGs are controlled by a family of sulfotransferase enzymes.^{12–15} Two major classes of enzymes, the cytosolic and membrane-associated sulfotransferases, catalyze the transfer of a sulfonyl group from the donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to a variety of amine- and hydroxyl-containing substrates.^{12,15} The cytosolic sulfotransferases act on small-molecule substrates such as xenobiotics. With only one exception, the sulfotransferases responsible for modifying GAG polysaccharides are type II transmembrane proteins that reside in the Golgi apparatus. All sulfotransferases have a structurally conserved PAPS-binding site, but distinct acceptor binding sites.^{12,15} Although numerous cytosolic sulfotransferases have been crystallized, only limited structural and functional information on GAG sulfotransferases is available, which has hindered efforts to design tools to target these enzymes. Indeed, there are no cell permeable, small-molecule inhibitors of any GAG sulfotransferases.

GAG sulfotransferase inhibitors would provide valuable tools for manipulating GAG sulfation patterns and for understanding their roles in biology and disease. The induced expression of specific sulfation motifs is associated with human diseases, including certain cancers,^{16,17} Alzheimer's disease,¹⁸ osteoarthritis,^{19,20} malaria,^{21,22} herpes,²³ and macular corneal dystrophy,²⁴ but surprisingly, pharmacological tools for manipulating GAG sulfation have been lacking. Cellular sulfation levels can be modulated genetically or by using sodium chlorate, which inhibits PAPS synthetase.^{25–30} However, genetic approaches lack the ease and reversibility of small-molecule inhibitors, while sodium chlorate requires millimolar concentrations and globally affects all types of sulfation, inducing off-target effects.^{27–30}

Moreover, the toxicity of sodium chlorate due to oxidation of hemoglobin and other metalloproteins limits its biological applications and therapeutic potential.³¹

Here, we sought to develop selective inhibitors for GAG sulfotransferases. As a starting point, we targeted Chst15, the chondroitin sulfotransferase responsible for sulfating the 6-*O*-*N*-acetylgalactosamine position of CS-A to generate CS-E (Figure 1A).^{13,14,32} Previously our laboratory identified the CS-E motif as an important structural determinant in chondroitin sulfate proteoglycans (CSPGs) that inhibits axon regeneration after central nervous system (CNS) injury.³³ Blocking the CS-E motif using a monoclonal antibody specific for CS-E promoted optic nerve regeneration *in vivo*. As such, Chst15 may represent a novel target for neuronal repair after spinal cord injury, stroke, or other injuries.

In this report, we describe the first small-molecule inhibitors of Chst15 and other GAG sulfotransferases. Using a fluorescence-based, high-throughput screen (HTS), we identified two promising scaffolds that upon structure-activity relationship (SAR) optimization led to molecules with improved potency in biochemical and cellular assays. Kinetic analyses revealed a reversible-covalent mechanism of inhibition, along with good selectivity toward GAG sulfotransferases compared to cytosolic sulfotransferases. Notably, the most potent inhibitor decreased CS-E sulfation levels on CSPGs and reversed the inhibitory activity of CSPGs on axonal growth. These studies open the door to a new set of chemical genetic tools for manipulating and understanding GAG-mediated events, as well as a novel potential therapeutic strategy for neuroregeneration.

RESULTS AND DISCUSSION

To discover molecules that inhibit Chst15, we developed a fluorescence-based assay in which Chst15 activity was coupled to the enzyme Sult1c1 (Figure 1A). Sult1c1 converts 3'-phosphoadenosine-5'-phosphosulfate (PAP) to PAPS by using 4-methylumbelliferyl sulfate (MUS) as the sulfate donor, resulting in liberation of fluorescent 4-methylumbelliferone (MU).^{34,35} We optimized and validated the assay in 96-well plates to obtain a Z-factor value suitable for high-throughput screening (0.62; Figure S1). The assay was then miniaturized to a 1536-well plate format, and a diversity set of 70,000 compounds was screened at 12.5 μ M for its ability to inhibit Chst15. The hits obtained were counter-screened against Sult1c1, resulting in twenty-six compounds that inhibited Chst15 with little or no Sult1c1 inhibition (Figure S2A). To validate these compounds further, we used a direct, sensitive assay that monitored [³⁵S]-incorporation from [³⁵S]-PAPS into CS-A (Figure S2B). The top compounds, **5** and **19**, exhibited significant dose-dependent inhibition of Chst15 with IC₅₀ values of 23 μ M and 39 μ M, respectively (Figure 1B).

To further evaluate **5**, we re-tested its activity and that of several commercially available analogs using the [³⁵S]-labeling assay (Figure S3). None of the analogs showed improved inhibition relative to compound **5**, and **5** itself unexpectedly inhibited Sult1c1 when re-evaluated in the counter-screen (Figure S4). We therefore focused our attention on **19**. Thirty-one analogs of **19** were synthesized or obtained commercially (Tables 1 and 2; Figure S5). To facilitate diversification of the molecule, we devised two synthetic routes that allowed for modification of the anilide or vinylbenzene moieties (Scheme 1). Initial

formation of the α,β -unsaturated acid followed by an *N,N*-diisopropylcarbodiimide (DIC)-promoted amide bond coupling enabled functional groups around the anilide to be altered. Alternatively, synthesis of the cyanoacetamide in two steps permitted incorporation of different vinylbenzene substituents by a piperidinium acetate-catalyzed Knoevenagel condensation. These routes enabled the installation of a wide range of functionalities from a large pool of commercially available anilines and benzaldehydes.

We compared the inhibitory activities of the analogs using the [^{35}S]-labeling assay. Removal of either aromatic ring on **19** (**21**, **23**, **46**, Scheme 1 and Figure S5) abolished activity, and changing the anilide ring from di- to mono-substitution (**25-33**) greatly reduced its potency (Table 1 and Figure S5). Altering the position of mono substitution (**31**, **32**) did not change this trend, nor did substitution with larger groups such as *tert*-butyl (**30**), phenyl (**33**), or fused-phenyl substituents (**48**, Figure S5). The importance of disubstitution was highlighted further upon analysis of various dichloro-substituted analogs (**34-36**), which had comparable or greater potency than the original compound **19**. In particular, compound **34** showed three-fold greater potency when compared to **19** in the [^{35}S]-labeling assay.

We next explored the importance of the vinylbenzene substituents by synthesizing analogs derived from **34**. Removal of any of the three substituents on the vinylbenzene ring (**37**, **41**, **44**) greatly attenuated the inhibitory potency of the compound (Table 2). Replacement of the bromo substituent with other halogens (**38-40**) decreased the activity by up to 2.5-fold, suggesting that the stereoelectronic properties of the bromo group are well suited for binding the enzyme. By comparison, modifications to the methoxy group were more tolerated. For example, replacement of the methoxy with a bromo group (**42**) led to no significant change in potency, while replacement with an ethoxy group (**43**) enhanced potency by 21% as compared to **19**. However, neither of these analogs exhibited improved potency when compared to **34**. Meanwhile, modification of the phenol group to aniline (**45**) or indazole (**49**, Figure S5) decreased the potency by 3-fold and 26-fold, respectively. Methylation of the phenol functionality also drastically lowered the inhibitory potency for compounds with common scaffolds (*e.g.* **35** vs. **47**, Table 1 and Figure S5). Thus, the hydrogen bonding and electronic properties of the phenolic hydroxyl group are important for Chst15 inhibition.

We hypothesized that the α,β -unsaturated cyanoacrylamide moiety might serve as an electrophile for nucleophilic amino acid side chains. Indeed, cyanoacrylamide compounds have been reported to inhibit kinases via a “reversible covalent” mechanism by reacting with noncatalytic cysteine residues.^{36,37} Rotating frame nuclear Overhauser effect spectroscopy (ROESY) data suggested that the *E*-isomer is generated in **34** after the Knoevenagel condensation (Supporting Information). Specifically, correlations between the amide and alkene protons of **34** were detected, which is only possible in the *E*-configuration. Reduction of this *E*-olefin using Pd/C abolished the dose-dependent effects of **34** (**50**, Figure S5).

To investigate the electrophilic potential of **34**, we incubated it with a surrogate nucleophile, β -mercaptoethanol (β ME, Figure S6A). Consistent with the formation of a covalent thioether adduct,³⁶ we observed a decrease in the UV-visible absorption band corresponding to the cyanoacrylamide moiety ($\lambda_{\text{max}} = 378 \text{ nm}$; Figure S6B). Upon ten-fold dilution of the sample treated with β ME into phosphate buffered saline (PBS), the absorbance was restored

as compared to dilution into PBS containing β ME, suggesting that formation of the adduct was reversible (Figure S6C). Further evidence for a reversible covalent adduct was obtained by ^1H NMR spectroscopy (Figure S6D). Incubation of **34** with β ME led to partial loss of the olefinic proton ($\delta = 7.83$ ppm) and appearance of an aliphatic β -proton ($\delta = 4.49$ ppm). Peak integration indicated that ~57% of the population had formed the thioether adduct, and this population was reduced to ~37% upon two-fold dilution into PBS.

We also examined whether **34** inhibits Chst15 through a reversible covalent mechanism by pre-incubating the inhibitor with Chst15 for various lengths of time. Longer pre-incubation times lead to greater inhibition of the enzyme, consistent with a covalent mechanism of action (Figure S7A). However, unlike the inhibition typically observed with irreversible covalent inhibitors, the activity of Chst15 was rapidly restored in ten-fold dilution experiments even at high concentrations of **34** (up to 100 μM). These observations suggest dissociation of the enzyme-inhibitor complex, thus supporting its reversibility (Figure S7B).

To study the mechanism further, we performed detailed kinetic analyses with compound **34**. When the CS-A substrate concentration was varied under pseudo first-order PAPS concentration, we obtained K_i and K_i' values of 1.43 μM and 2.45 μM , respectively, and observed a shift in both V_{max} and K_m indicative of a mixed mode of inhibition (Figure 2A, Table S1, Figure S8). However, when the PAPS concentration was varied in presence of fixed CS-A acceptor substrate (10 mM), we observed a competitive mode of inhibition with a K_i value of 0.77 μM (Figure 2B, Table S1, Figure S8). Taken together, these results suggest that inhibitor **34** competitively interacts with the PAPS binding site and also likely engages part of the acceptor binding site.

We next investigated the specificity of **34** against a series of cytosolic and GAG sulfotransferases. Importantly, we observed a clear dichotomy in the ability of **34** to inhibit GAG sulfotransferases compared to cytosolic sulfotransferases (Figure 2C). While **34** inhibited Chst15 and other closely related GAG sulfotransferases such as the chondroitin 4-*O*-sulfotransferase Chst11, the chondroitin 2-*O*-sulfotransferase Ust, and the heparan 3-*O*-sulfotransferase Hs3st1 (IC_{50} values 2.0–2.5 μM), its activity against the cytosolic sulfotransferases Sult1e1, Sult2b1a, and Sult2b1b, which modify steroidal substrates, was significantly lower (IC_{50} values 19–42 μM). Moreover, **34** showed no activity against the cytosolic sulfotransferase Sult1c1 used in the enzyme-coupled assay at the concentrations tested (3–100 μM). Inhibition of other GAG sulfotransferases by **34** is consistent with the high sequence homology (25–81%)^{32,38,39} shared by GAG sulfotransferases and our observations that **34** engages the PAPS binding pocket.

The ability to inhibit GAG sulfation after CNS injury could have important implications for neural regeneration. After CNS injury, reactive astrocytes enter the site of injury and secrete CSPGs as a means to prevent further damage.^{5–7} Previous studies in our laboratory have established that the CS-E sulfation motif is upregulated on these CSPGs and inhibits the growth of sensory axons.³³ Thus, we first examined whether **34** could modulate CS-E expression levels on CSPG-secreting Neu7 astrocytes.⁴⁰ Indeed, astrocytes treated with 10 μM or 25 μM of **34** showed a significant, dose-dependent decrease in cell-surface CS-E expression, as indicated by immunostaining with a CS-E-specific antibody⁴¹ (Figure 3A and

3B). By comparison, sodium chlorate did not reduce CS-E levels to as large an extent, even at doses as high as 25 mM. Similar results were obtained using NIH3T3 fibroblasts, where significant loss of CS-E expression was observed upon treatment with **34**, and **34** had superior potency compared to sodium chlorate (Figure S9).

To characterize the effects of **34** on secreted CSPGs, we purified the CSPGs from Neu7-conditioned media, digested them with chondroitinase ABC (ChABC), and analyzed their disaccharide composition by reverse-phase ion-pair high-performance liquid chromatography (RPIP-HPLC). CSPGs secreted from Neu7 astrocytes treated with 25 μ M of **34** displayed a $62.9\% \pm 17.0\%$ increase in unsulfated chondroitin (uCS) content compared to CSPGs secreted from untreated astrocytes, indicating a decrease in overall sulfation (Figure 3C, Table S2). This overall decrease was accompanied by a specific $67.8 \pm 10.9\%$ reduction in the CS-E sulfation motif on secreted CSPGs. Although other CS sulfation motifs were also affected, the CS-E motif was reduced to the largest extent (Figure S10). By comparison, CSPGs secreted from sodium chlorate-treated cells also displayed lower sulfation compared to untreated cells, albeit to a lesser degree and only in the presence of high concentrations of sodium chlorate ($39.7 \pm 15.6\%$ increase in uCS; $31.6 \pm 10.9\%$ decrease in CS-E).

Having shown that **34** can effectively inhibit CS-E sulfation levels on both surface-expressed and secreted CSPGs, we next examined whether this mode of inhibition could be exploited to stimulate axonal growth. Sensory dorsal root ganglion neurons (DRGs) adjacent to the rat spinal cord were grown on a substratum coated with the CSPGs secreted from Neu7 astrocytes treated with or without **34**. The effects of the CSPGs on neurite outgrowth were determined by immunostaining for the neuronal-specific marker, β III-tubulin, and quantifying the average neurite length. To our delight, we observed a significant, dose-dependent promotion of neurite outgrowth ($30.7\% \pm 2.5\%$ at 25 μ M of **34**) when DRGs were grown on CSPGs secreted from treated astrocytes compared to untreated astrocytes (Figure 4A).

CNS injury induces tissue damage and leads to formation of a glial scar, which consists predominantly of reactive astrocytes and CSPGs.^{5–7} The glial scar serves as a major barrier to axon regeneration. We used an *in vitro* boundary assay to simulate the glial scar and assess the ability of **34** to reverse the inhibitory activity of CSPGs. In this assay, purified CSPGs from Neu7 astrocytes treated with or without **34** were mixed with Texas Red dye and spotted onto glass coverslips. DRG neurons were plated onto the coverslips, and the percentage of neurites that crossed the CSPG boundary was quantified. Whereas DRG neurites were strongly repelled by CSPGs from untreated astrocytes, they crossed more freely into boundaries of CSPGs from astrocytes treated with **34** (Figure 4B). Thus, compound **34** rendered CSPGs more permissive to axonal growth, demonstrating its ability to modulate sulfation-dependent cellular processes.

Finally, we evaluated the potential to use inhibitor **34** *in vivo* by profiling its absorption, distribution, metabolism, and elimination (ADME) properties. **34** showed good stability in human, mouse and rat liver microsomes based on intrinsic clearance (Table S3). The *in vivo* pharmacokinetic (PK) properties of **34** were also evaluated directly in rats. After intravenous injection of compound **34** at a dose of 3.0 mg/kg, the compound had a moderate clearance of

21 mL/min/kg moderate volume of distribution of 0.97 L/kg and short terminal half-life of 1.6 h (Table S4). These data suggest that direct application of the compound, as opposed to intravenous injection, may be more efficacious for future use *in vivo*. Taken together, our studies demonstrate that the sulfotransferase inhibitor **34** can diminish the inhibitory effects of CSPGs, and they highlight its promise to be used for the stimulation of neuronal repair.

CONCLUSION

GAG sulfotransferase inhibitors have the potential to be powerful pharmacological tools for elucidating the diverse physiological functions of GAGs and serve as potential therapeutic leads for spinal cord regeneration and human diseases. We report here the discovery of the first selective, cell-permeable, small-molecule inhibitor of GAG sulfotransferases. Inhibitor **34** was derived from a high-throughput screen targeted against the CS-E sulfotransferase Chst15 and potently inhibited the sulfation of surface-expressed and secreted CSPGs. Importantly, by decreasing sulfation levels on CSPGs, compound **34** reversed the growth inhibitory effects of CSPGs on sensory axons. These studies demonstrate the promise of using a sulfotransferase inhibitor-based strategy, and potentially **34** or its derivatives, for the stimulation of neuronal growth after injury, stroke, or neurodegenerative disease. Additionally, compound **34** provides a lead scaffold that may ultimately be developed to target individual sulfotransferases with high specificity. We envision that chemically-tuned small molecules capable of modulating the sulfation patterns of GAGs will be valuable for manipulating and understanding many GAG-dependent physiological and pathological processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Identification of two lead inhibitor scaffolds. (A) Enzyme-coupled fluorescence assay for the high-throughput screening of small-molecule inhibitors of Chst15. Chst15 activity was monitored by coupling to Sult1c1, which converted MUS to fluorescent MU. (B) Dose-dependent inhibition of Chst15 by compounds **5** and **19** using a $[^{35}\text{S}]$ -labeling assay.

Figure 2.

Kinetic analysis and specificity of compound **34**. Pseudo first-order studies using the enzyme-coupled fluorescence assay revealed a competitive and mixed mode of inhibition for the substrates (A) PAPS and (B) CS-A, respectively. (C) Compound **34** displayed 8- to 18-fold greater specificity for GAG sulfotransferases compared to cytosolic sulfotransferases.

Figure 3.

Treatment of Neu7 astrocytes with **34** reduces expression of CS-E on cell-surface and secreted CSPGs. (A) Representative images of Neu7 astrocytes treated with **34** or chlorate and co-stained with a CS-E-specific antibody (green) and the nuclear stain DAPI (blue). (B) Quantification of the average fluorescence from anti-CS-E immunostaining under each condition. Values represent the mean $\pm$ S.E.M. from three experiments. $n = 20$ -100 cells per experiment. (C) CS-E and uCS disaccharide composition of secreted CSPGs from Neu7 astrocytes treated with **34** or sodium chlorate. $n = \#3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s. = not significant. (Scale bar: 100 μ m.)

Figure 4.

Treatment of Neu7 astrocytes with **34** attenuates CSPG-mediated inhibition of sensory neurite outgrowth and repulsion. (A) Representative images and quantification of DRG neurite outgrowth recovery for CSPGs purified from treated astrocytes relative to CSPGs purified from untreated astrocytes. Data represent the mean $\pm$ S.E.M. from three experiments. $n = 20$ -80 cells per experiment. (B) Representative images and quantification of neurite crossing for DRG neurons grown in the presence of a boundary (shown in red) of CSPGs purified from treated or untreated astrocytes. Data represent the mean $\pm$ S.E.M. from five experiments. $n = 20$ -40 cells per experiment. $*P < 0.05$, $**P < 0.01$, n.s. = not significant. (Scale bars: 50 μ m.)

Scheme 1. Synthesis of Analogs of 19^a

^aReagents and conditions: (a) ethyl cyanoacetate, piperidine, AcOH, PhMe (91%); (b) NaOH, THF (77%); (c) Ar-NH, DIC, THF (17-56%); (d) cyanoacetic acid, DIC, THF (45%); (e) Ar-CHO, piperidine AcOH, PhMe (52-93%). See Table 1, Table 2, and Figure S5 for specific structures.

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Table 1

Relative Inhibition of Chst15 by Anilide Analogs^a

Compound	R ₁	R ₂	R ₃	R ₄	Relative Inhibition
19	H	F	Cl	H	1.00 ± 0.01
25	H	F		H	0.19 ± 0.00
26	H		H	H	0.30 ± 0.04
27	H		H	H	0.10 ± 0.00
28	H		H	H	0.08 ± 0.00
29	H		H	H	0.10 ± 0.00
30	H		H	H	0.35 ± 0.05
31	H	H	H		0.57 ± 0.02
32	H	H	H		0.37 ± 0.00
33	H	H		H	0.07 ± 0.01
34	H		H		1.35 ± 0.12
35		H		H	0.98 ± 0.10
36		H	H		1.21 ± 0.09

^aRelative inhibition values were determined from three independent measurements using 25 μM of inhibitor and normalized to the inhibition by compound 19.

Table 2Relative Inhibition of Chst15 by Vinylbenzene-Analogs^a

Compound	R ₅	R ₆	R ₇	Relative Inhibition
34	OMe	OH	Br	1.35 ± 0.12
37	OMe	OH		0.13 ± 0.00
38	OMe	OH		0.40 ± 0.03
39	OMe	OH		0.49 ± 0.01
40	OMe	OH		0.88 ± 0.02
41		OH	Br	0.32 ± 0.00
42		OH	Br	0.97 ± 0.10
43		OH	Br	1.26 ± 0.16
44	OMe		Br	0.00 ± 0.00
45	OMe		Br	0.32 ± 0.01

^aSee footnote from Table 1.