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Inhibitors of slit protein interactions with the heparan sulphate proteoglycan glypican-1: Potential agents for the treatment of spinal cord injury

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SUMMARY

1. The heparan sulphate proteoglycan glypican-1 is a major high-affinity ligand of the Slit proteins.
2. Messenger RNA for both Slit-2 and glypican-1 is strongly upregulated and coexpressed in the reactive astrocytes of injured adult brain, suggesting a possible function of Slit proteins and glypican-1 in the adult central nervous system as significant components of the inhibitory environment that prevents axonal regeneration after injury.
3. Based on the hypothesis that adverse effects on axonal regeneration may be due to a glypican–Slit complex or the retention of glypican-binding C-terminal proteolytic processing fragments of Slit at the injury site, we used ELISA to examine a number of small molecules and low molecular weight heparin analogues for their ability to inhibit glypican–Slit interactions.
4. Our studies have led to the identification of several potent inhibitors with a favourable therapeutic profile that can now be tested in a spinal cord injury model. Among the most promising of these are a low molecular weight heparin produced by periodate oxidation and having no significant anticoagulant activity, the chemically sulphonated yeast-derived phosphomannan PI-88 and a number of randomly derivatized water-soluble sulphated dextrans.

Keywords

axonal regeneration; glypican-1; heparan sulphate; PI-88; proteoglycans; Slit proteins; spinal cord injury; sulphated dextrans

INTRODUCTION

The approximately 200 kDa Slit proteins regulate axonal guidance, branching, dendritic development and neural migration.^{1–3} Previously, we demonstrated that the C-terminal portion of Slit, which can be released from the cell membrane by *in vivo* proteolytic processing, is a high-affinity ligand ($K_D = 80\text{--}110\text{ nmol/L}$) of glypican-1, the initial

member of a family of glycosylphosphatidylinositol-anchored heparan sulphate proteoglycans that is currently composed of six vertebrate proteins.⁴⁻⁶ Glypican-1 has a 56 kDa core protein and three to four heparan sulphate chains. *In situ* hybridization histochemistry has shown that both glypican-1 and the Slit proteins are synthesized by neurons, such as hippocampal pyramidal cells and cerebellar granule cells, and that they colocalize in the brain and spinal cord.^{4,5,7} The binding affinity of the glypican core protein to Slit is an order of magnitude lower than that of the glycanated protein and the *O*-sulphate groups on the heparan sulphate chains play a critical role in the interaction.⁵

In addition to evidence for the role of cell surface heparan sulphate in the repulsive guidance activities of Slit-2 protein,⁸⁻¹¹ it has been shown that mRNA for both Slit-2 and glypican-1 is strongly upregulated and coexpressed in the reactive astrocytes of injured adult brain.^{12,13} These studies were later extended by the demonstration that the particular Slit proteins affected (Slit-2 vs Slit-1 and -3) varied depending on the type of lesion (cryolesion vs traumatic injury) and the types of cells in which the Slit mRNAs were overexpressed (reactive astrocytes vs activated microglial cells or macrophages).¹⁴ It has also been shown that there are dynamic changes in glypican-1 expression in dorsal root ganglion neurons after peripheral and central axonal injury,¹⁵ including changes in the nuclear localization of glypican-1 consistent with our earlier demonstration that it is present in the nuclei of brain neurons and glioma cells, contains a functional nuclear localization signal and undergoes dynamic changes during the cell cycle.¹⁶

These findings suggest a possible function of Slit proteins and glypican-1 in the adult central nervous system (CNS; where few axon guidance events occur) because, by acting either alone or as a complex, they may be significant components of the inhibitory environment that prevents axonal regeneration in conditions such as spinal cord injury. The fact that many guidance molecules are expressed at high levels in the adult CNS indicates that their functions are not limited to the control of axonal pathfinding and target selection during development. Approaches to the treatment of spinal cord injury and the general failure of axonal regeneration in the CNS have mainly focused on the inhibitory effects of chondroitin sulphate proteoglycans in the glial scar produced by reactive astrocytes and on growth inhibitory proteins in myelin that are released as a consequence of axonal degeneration.¹⁷ Because even after removal or neutralization of these inhibitory molecules the number of severed axons that regenerate is low, it is likely that this is due to the presence of additional inhibitory factors and/or to the poor intrinsic ability of CNS neurons to regenerate.

Although significant amounts of full-length unprocessed Slit are present in nervous tissue (accounting for their original identification as glypican-1 ligands in the form of the 200 kDa proteins⁴), because the smaller C-terminal proteolytic processing product binds with high affinity to glypican-1 this would prevent its diffusion from sites of CNS injury. Regardless of whether any adverse effects on axonal regeneration are due to a glypican-Slit complex or the retention of C-terminal Slit protein fragments at the injury site, it is reasonable to hypothesize that by inhibiting their interaction, heparin-like compounds could limit the functional consequences of spinal cord injury. These premises led us to explore the possibility that relatively low molecular weight oligosaccharides of defined structure or other small polysulphated molecules may prove useful in inhibiting interactions between

glypican-1 and Slit proteins or other ligands and thereby serve as a pharmacological means for promoting axonal regeneration.

METHODS

Materials

Low molecular weight heparin analogues (SR series) were provided by Dr Maurice Petitou (Sanofi-Aventis, Toulouse, France). Dr Robert Linhardt (Rensselaer Polytechnic, Troy, NY, USA) provided periodate oxidized heparin (Astenose; Glycomed Inc., Alameda, CA, USA), fondaparinux, a septasulphated octyl 3-*O*-methyl-xylopyranosyl hexasaccharide (MW 1721), a nonasulphated fucose tetrasaccharide (MW 1633), a doubly branched persulphated D-glucose hexasaccharide¹⁸ (Ch2; MW 2942) and PI-88 hexa- and pentasaccharides. Mannopentasaccharide analogues of PI-88 were provided by Dr Yuguo Du (Chinese Academy of Sciences, Beijing, China). Sulphated dextrans were a gift from Dr Denis Barritault (CNRS / Université Paris-XII, Paris, France) and suramin analogues were from Dr Delwood Collins (University of Kentucky, Lexington, KY, USA). Synthetic polyaromatic phenols were made available to us by Dr Miriam Benezra (Mount Sinai School of Medicine, New York, NY, USA), the C3 oligosaccharide were from Dr Umberto Cornelli (Cornelli Consulting, Milan, Italy), GL-522-Y-1 and its methylated derivative were provided by Dr Carl Dietrich (Universidade Federal de São Paulo, São Paulo, Brazil) and the heparan sulphate mimetics KI 101–110 were provided by Dr Hiroyuki Osada (Riken, Tokyo, Japan).

Preparation of glypican-1–Fc and human Slit-2 fusion proteins

Human embryonic kidney (HEK) 293 cells were transfected with a glypican-1–Fc fusion protein construct¹⁶ using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and grown in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 1% ITS⁺. To separate the glycanated form of the proteoglycan (which was used for all studies) from unglycanated core protein, the conditioned medium was applied to a 0.9 × 8 cm column of DEAE-Sephacel equilibrated with 150 mmol / L NaCl and 50 mmol / L Tris–HCl, pH 8.0.¹⁹ After elution with 50 mmol / L Tris–HCl (pH 8.0) containing 0.6 mol / L NaCl, the glycanated glypican-1–Fc was bound to protein A Sepharose beads and eluted with 0.1 mol / L glycine, pH 3.0.

The HEK293 cells were transfected with the pSecTagB vector (Invitrogen, Carlsbad, CA, USA) containing cDNA for the His-tagged uncleavable variant of human full-length Slit-2, in which the nine amino acids encompassing the proteolytic processing site were deleted, producing an uncleavable full-length protein.²⁰ Then, 1 mol / L NaCl extracts of the HEK293 cells were incubated with nickel agarose beads for 2 h at 4°C and, after washing twice for 10 min each time with 10 volumes of 1 mol / L NaCl / 10 mmol / L HEPES / 10 mmol / L imidazole solution, bound protein was eluted with 10 mmol / L HEPES (pH 7.5) containing 250 mmol / L imidazole and 1 mol / L NaCl. Protein concentrations were determined by the Bradford method.²¹

ELISA assay

First, 96-well plates (9018; Corning Costar, Lowell, MA, USA) were coated overnight with the human full-length Slit 2 at a saturating concentration of 5 µg / well in phosphate-buffered saline (PBS). After removing the unbound protein by washing with TBST (10 mmol / L Tris-HCl, pH 7.4, 150 mmol / L NaCl, 0.1% Tween 20), the wells were blocked with 10% fetal bovine serum (FBS) in TBST for 2 h and then incubated for 18 h at room temperature with glypican-1-Fc (1 µg / mL) in PBS. Bound glypican was detected using a biotinylated anti-human Fc antibody (1 : 250 000 in TBST, for 2 h; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), followed by incubation for 20 min with horseradish peroxidase-conjugated streptavidin (1 : 20 000 in TBST). The colourimetric reaction product from the *o*-phenylenediamine substrate was measured at 450 nm using a Dynatech MRX ELISA plate reader (Dynatech Laboratories, Chantilly, VA, USA). Non-specific binding was calculated as the binding of glypican-1-Fc to wells coated with 100 µg bovine serum albumin (BSA). From serial dilutions of a known concentration of glypican-1-Fc directly coated onto the wells and the corresponding immunoreactivity absorbance, a standard curve was created and used to quantify the amount of glypican-1-Fc bound. Standard deviations of replicate determinations are indicated by the error bars and were calculated using the STDEV function of the Excel program (Microsoft, Redmond, WA, USA) used to graph the results of the ELISA assays.

RESULTS

Low molecular weight heparins

We first evaluated the inhibitory activities of two low molecular weight heparins, namely enoxaparin and dalteparin, which are in wide clinical use and are obtained by alkaline or nitrous acid depolymerization, respectively, of porcine intestinal mucosa heparin and have average molecular sizes of 4500–5000 Da. These produced a maximum 80–90% inhibition of glypican–Slit binding at concentrations of 1–5 µmol / L, whereas a smaller synthetic octasulphated heparan sulphate pentasaccharide (fondaparinux; MW 1728) was completely inactive at concentrations up to 50 µmol / L (Fig. 1a). Because heparin-like molecules can be chemically modified to greatly reduce or abolish anticoagulant activity, we considered these results to provide proof of principle that heparinoids in the MW 4500–5000 size range can function as potent inhibitors of glypican–Slit interactions.

Subsequent testing of a number of heparin analogues (Fig. 2a) demonstrated that a periodate-oxidized heparin with an average molecular size of approximately 6000 Da (SR80258A; prepared by mild alkaline hydrolysis followed by borohydride reduction²²) resulted in 70% inhibition of glypican binding to Slit at a concentration of 1 µmol / L (Fig. 1b; IC₅₀ = 0.2 µmol / L). Another sample of a periodate-oxidized heparin (astenosé) with an average molecular size of approximately 5000 Da gave essentially identical results. The hexanoyl acylated derivative (SR80037A), which is more lipophilic and could therefore be expected to cross the blood–brain barrier more easily, was significantly less potent and effective (IC₅₀ = 3.3 µmol / L; maximum inhibition = 53%). Because periodate treatment destroys the antithrombin binding site of heparin, these compounds are essentially devoid of anticoagulant activity, making such inhibitory molecules especially well suited for possible

therapeutic use in the treatment of spinal cord injury. As shown in Fig. 1b, SR80488A, SR80343A and SR80239A all inhibited glypican binding to Slit by approximately 50% at 5 $\mu\text{mol/L}$ (IC_{50} = 0.7, 4.2 and 4.8 $\mu\text{mol/L}$, respectively). SR80426A, SR80403A and SR122429A resulted in 24–37% inhibition at a concentration of 5 $\mu\text{mol/L}$, whereas 5 $\mu\text{mol/L}$ SR80536A and SR80027A had no significant inhibitory effect (data not shown).

Sulphated mannose oligosaccharides

PI-88 (Fig. 2b) is a chemically sulphonated yeast-derived phosphomannan that inhibits heparanase activity and functions as a heparan sulphate mimetic by inhibiting binding of growth factors such as Fibroblast growth factor (FGF)-2 and vascular endothelial growth factor to cell surface heparan sulphate chains. Because of its anti-angiogenic activity, PI-88 has been tested for the treatment of a variety of solid tumours.²³ Hexasaccharide and pentasaccharide fractions of PI-88 isolated by gel permeation chromatography,²⁴ with average molecular sizes of approximately 3000 and 2700 Da, were tested by ELISA for their ability to inhibit the binding of glypican-1 to Slit protein. At concentrations of 0.5–5 $\mu\text{mol/L}$ (approximately 1–10 $\mu\text{g/mL}$), which resulted in maximum 70–85% inhibition of glypican–Slit binding (see Fig. 1c for the pentasaccharide; IC_{50} = 0.1 $\mu\text{mol/L}$), PI-88 has no significant anticoagulant activity.²⁴ The PI-88 hexasaccharide fraction (Peak I in Yu *et al.*²⁴) showed maximum inhibition similar to that of the pentasaccharide (79–84% at 0.5–5 $\mu\text{mol/L}$).

We also evaluated a monosulphated mannopentasaccharide analogue of PI-88 and a multisulphated trimer of the pentasaccharide.²⁵ These produced significant inhibition of glypican–Slit binding (maximum 68–73% for the trimer at concentrations of 2–5 $\mu\text{mol/L}$ and 75% for the monosulphated pentasaccharide at a concentration of 50 $\mu\text{mol/L}$), but have a considerably lower potency than PI-88 itself (Fig. 1c; IC_{50} = 1.5 and 8 $\mu\text{mol/L}$, respectively).

Sulphated dextrans

In other studies, we evaluated the inhibitory activity of a series of randomly derivatized water-soluble synthetic dextrans with benzylamidesulphonate, carboxymethyl and other groups. These compounds have minimal anticoagulant activity (< 6% that of heparin), but can partially substitute for heparin with respect to FGF activation and stabilization²⁶ and have heparin/heparan sulphate-mimetic activity in a number of assays, including stimulating tissue repair in various *in vivo* wound healing models.^{27,28} The effects of nine sulphated hydrophilic and hydrophobic dextrans on glypican–Slit interactions are summarized in Fig. 3. Two unsulphated synthetic intermediates of the 20 kDa CR36 with and without the hydrophobic group resulted in no significant inhibition.

Sulphated carbohydrates and other compounds with mostly minimal or no inhibitory activity

A number of other compounds were tested and found to have relatively low inhibitory activity or to be entirely ineffective. These included the heparin-derived oligosaccharide C3, which has only very weak anticoagulant activity and is being explored as an inhibitor of amyloid interaction with anionic glycosaminoglycans.²⁹ It gave 22% inhibition at 5 $\mu\text{mol/L}$

and 53% at 50 $\mu\text{mol/L}$. *Myo*-inositol hexasulphate (hexapotassium salt; Sigma, St Louis, MO, USA; FW 889), a septasulphated octyl 3-*O*-methyl-xylopyranosyl hexasaccharide (MW 1721) and a nonasulphated fucose tetrasaccharide (MW 1633) gave no significant inhibition (3–9% at 50 $\mu\text{mol/L}$), but the doubly branched persulphated D-glucose hexasaccharide Ch2 (MW 2942) produced 51 and 64% inhibition at 5 and 50 $\mu\text{mol/L}$, respectively.

GL-522-Y-1 is a cyclic octaphenol-octasulphonic acid that has heparin-like antithrombotic activity on vascular endothelial cells, but no significant anticoagulant activity in whole blood.³⁰ This compound exhibited 61 and 67% inhibition at concentrations of 5 and 50 $\mu\text{mol/L}$, respectively; the corresponding values for its methylated derivatives were 29 and 50%. A series of 10 low molecular weight heparan sulphate mimetics (KI 101–KI 110) designed as potential antitumour agents³¹ showed no significant inhibition at 100 $\mu\text{mol/L}$.

From an extensive series of suramin analogues that had been synthesized and tested for their anti-angiogenic and antiproliferative effects,³² we evaluated nine of the larger analogues at a concentration of 5 $\mu\text{mol/L}$ for their ability to inhibit glypican-1 binding to Slit protein, but none had a greater effect than the polysulphonated naphthylurea suramin itself (53% inhibition).

A series of synthetic polyaromatic compounds synthesized by polymerization of aromatic ring monomers with formaldehyde to yield ordered backbones with different functional anionic groups (hydroxyl and carboxyl) on the phenol ring have demonstrated heparin-like activity in several functional assays.³³ Although most of the nine compounds tested had little or no effect on glypican–Slit interactions, compound no. 4 showed 79 and 82% inhibition at 1 and 5 $\mu\text{mol/L}$, respectively, indicating that it also may warrant further consideration.

DISCUSSION

We have evaluated a number of small sulphated and other molecules reported to have heparin-like activity, as well as sulphated oligosaccharides in the 2000–3000 MW range, various low molecular weight heparin analogues and sulphated dextrans for their ability to inhibit glypican–Slit interactions. This has allowed the identification of several molecules that are potent inhibitors of glypican–Slit interactions, can be economically produced in sufficient quantities and purity for human clinical use and for which available data indicate that they would not be toxic in the doses and by the routes of administration appropriate for the treatment of spinal cord injury. The time-course of upregulation of glypican-1 and Slit after injury is quite favourable, insofar as the expression of both mRNAs peaks at 1 week and becomes very weak (glypican-1) or undetectable (Slit-2) by 2 weeks after injury.^{12,13} These data, and those of Werhle *et al.*¹⁴ for Slit-1 and -3, indicate the possibility of a relatively short duration of therapy during an early ‘critical period’ that would minimize toxicity and allow the use of higher doses of systemically administered drug. Moreover, the blood–brain barrier to large and / or charged molecules also becomes more permeable at sites of CNS injury, factors that can be expected to aid drug penetration after systemic administration. The short-term increases in Slit and glypican-1 upregulation also provides

the option of intrathecal administration, either by infusion or from an implanted depot of a biocompatible, biodegradable and injectable biomaterial, such as collagen³⁴ or cross-linked hyaluronan. In this way, one can achieve higher drug concentrations at the injury site and limit systemic toxicity, as well as circumventing the blood–brain barrier. Although *in vitro* studies have shown that Slit proteins can repel and inhibit neurite extension of CNS neurons,³⁵ no cell culture system is available to reliably test the hypothesis that inhibitors of glypican–Slit interactions may promote axonal regeneration after spinal cord injury. The three compounds (periodate-oxidized heparin, PI-88 and the sulphated dextran D120) that we have identified in the present study as potent inhibitors of glypican–Slit interactions, with minimal or no anticoagulant activity, are therefore excellent candidates for testing in a spinal cord injury model to determine whether they promote axonal regeneration.

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Fig. 1.

(a) Effects of low molecular weight heparins on glypican-Slit interactions. (■), dalteparin; (●), enoxaparin; (◆), fondaparinux. (b) Inhibition by heparin analogues of glypican-1 binding to Slit protein. (◆), SR80488A; (■), SR80343A; (▲), SR80239A; (●), SR80258A; (*), SR80037A. (c) Inhibition of glypican-Slit binding by the PI-88 pentasaccharide (▲), the monosulphated mannopentasaccharide analogue of PI-88 (■) and a multisulphated trimer of the pentasaccharide analogue (◆).

Fig. 2.

Structures of heparin analogues from (a) Sanofi-Aventis (Toulouse, France) and (b) PI-88.

Fig. 3.

(a) Inhibition of glypican-1 binding to Slit protein by sulphated hydrophilic and hydrophobic dextrans. ($\blacklozenge$), sulphated hydrophilic D120 (80 kDa); ($\blacksquare$), sulphated hydrophilic CR17 (400 kDa); ($\blacktriangle$), sulphated hydrophilic CR21 (3500 kDa); ($\times$), sulphated hydrophobic RG94 (80 kDa); ($*$), sulphated hydrophobic DAC (80 kDa); ($\bullet$), sulphated hydrophobic CR27 (80 kDa); ($\circ$), sulphated hydrophobic CR29 (400 kDa); ($\square$), sulphated hydrophobic CR32 (4000 kDa); ($\triangle$), sulphated hydrophobic CR36 (20 kDa). (b) Inhibition by the 80 kDa sulphated hydrophilic dextran D120.