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Identification of Novel Inhibitors of Fibroblast Growth Factor (FGF-2) Binding to Heparin and Endothelial Cell Survival from a Structurally Diverse Carbohybrid Library

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Abstract—Inhibitors of FGF-2 binding to a heparin–albumin conjugate were identified by ELISA from a library of glucuronic acid derivatives. These compounds were also inhibitors of endothelial cell survival that is dependant on FGF-2 and heparin or heparan sulfate proteoglycans. The results indicate that these bioactive compounds may prove useful as lead structures for the further development of pharmaceutical agents capable of modulating biological activity of FGF-2.

The glycosaminoglycans (GAGs) are polyanionic linear carbohydrate polymers (e.g., heparin and heparan sulfate). They mediate numerous physiological processes including cell adhesion, activation of growth factors, blood coagulation, lipid metabolism, infection and inflammatory responses.[1–4] Heparin, used clinically as an anticoagulant, has a heterogeneous structure comprised of a repeating disaccharide unit with a hexuronic acid linked to D-glucosamine and a diverse and complex sulfation pattern. This structural diversity facilitates the interaction of heparin with many proteins[5] but limits heparin's clinical use as a drug due to the side effects that result from undesirable interactions. It would thus be interesting to develop compounds that could specifically target heparin binding proteins. These compounds could be useful tools for biologists and may have potential as drugs or as leads for drug development. It would be desirable, if possible, to develop low molecular weight modulators that could be attractive for drug development. Fibroblast growth factors (FGFs), have important roles in a variety of biological processes such as cell growth, differentiation, angiogenesis[6,7] and wound healing. The cellular receptors for FGFs are receptor tyrosine kinases (FGFR); these are activated by ligand-induced dimerisation and require heparin or heparan sulfate proteoglycans as co-receptors to promote high affinity binding.[8]

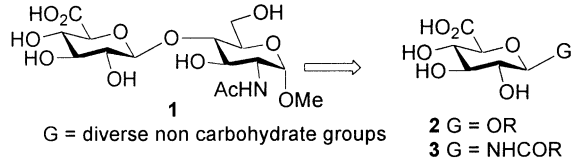

Figure 1. Design of library for evaluation in FGF-2 dependant assays.

The eventual consequence of exposing cells to growth factors in the presence of heparin/heparan sulfate proteoglycans can be cell movement, differentiation, proliferation or protection from death. There has been interest in developing antagonists of growth factor receptors (FGF, PDGF, EGF, TGF-β) due to their potential as angiogenesis inhibitors.[9] There has been a report by Ornitz and co-workers showing that non sulfated disaccharides and trisaccharides (e.g., **1**), have activity in a number of FGF dependant assays.[10] Although **1** and other oligosaccharides were not as potent as heparin/heparan sulfate they displayed bind-

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ing to FGF in competition with ^{125}I-heparin. They enhanced binding of FGF to FGFR and, in the presence of FGF, displayed potent mitogenic activity promoting the proliferation of cell lines expressing the FGF receptor. The cell lines that were used for these expermiments require both FGF and heparin/heparan sulfate for growth.[10] A recent report indicates that oligosaccharides containing Lewis X structural motifs have effects on proliferation of embryonic stem cells possibly through the modulation of FGF-2 (bFGF) further suggesting a potential role for smaller molecules in modulating FGF activity.[11] The results from both of these reports suggest that FGF can recognize structural features of smaller non-sulfated oligosaccharide components of heparin/heparan sulfate and provide a platform for the development of small molecule promoters or inhibitors of FGF function. Ornitz and co-workers carried out crystallography and cross-linking experiments that suggested that their oligosaccharides could, in principle, promote formation of FGF oligomers that may be biologically active.[10] Alternatively, crystallographic work reported by Mohammadi and co-workers suggest a more unifying mechanism by which both heparin and smaller oligosaccharides could activate cellular responses dependant on FGF and FGFR.[12] Although the oligosaccharides such as **1** were effectors of a biological response there was no evidence provided that they might inhibit signal transduction pathways. Thus, we have begun to investigate strategies for the design or identification of novel, small molecule inhibitors, which are reduced in carbohydrate character, of these FGF mediated signaling pathways. In this letter, we describe the identification of novel glucuronic acid derivatives that inhibited heparin binding to FGF-2 in enzyme-linked immunosorbant assays (ELISAs). These compounds also inhibited endothelial cell survival pathways that depend on interactions of heparan sulfate proteoglycans, FGF-2 and FGFR.

Previously Hindsgaul and co-workers have shown that evaluation of *carbohybrid* libraries composed of D-galactopyranose that carry a diverse range of small non-carbohydrate aglycon structures led to the identification of μM inhibitors of a galactose binding plant lectin.[13] We were interested to explore whether any compounds from a library composed of glucuronic acid (Fig. 1) and carrying a diverse range of non-carbohydrate groups (Fig. 2) would show evidence of binding to FGF-2. Glucuronic acid was chosen for these preliminary studies as it is readily available and is one of the simplest saccharides found in heparin structures. Also, it is a component of the active disaccharide **1** and we made the assumption, that a charged group is important for the biological activity displayed by **1**. A series of novel synthetic glucuronic acid derivatives (Fig. 2) as well as a number of commercially available glucuronides (**2c**, **2d**, **2g**, **2h**) and the 6-*O*-sulfo glucose derivative **4** were thus evaluated by ELISA to determine their ability to compete with heparin for binding to FGF-2. 96-well plates were coated with a heparin–albumin conjugate (HA) and then incubated with FGF-2 in the presence or absence of various compounds and then washed. The amount of FGF bound to the plates was detected using an anti-FGF-2 goat polyclonal IgG antibody and an alkaline phosphatase conjugated anti-goat IgG antibody. The assay was standardized using free HA which was found to inhibit binding of FGF to HA attached to the plate with an IC_{50} of 0.61 ng/mL and the maximum inhibition (I_{max}) achieved was 95% (at 10^6 ng/mL). Heparan sulfate and heparin were also evaluated (Table 1). Seventeen monosaccharides were assayed and six of the glucuronic acid conjugates were found to be inhibitors of FGF binding to HA. Results for the bioactive monosaccharides are summarised in Table 1. The compounds were less active than heparan sulfate and heparin. It is possible to determine the relative activity of the compounds with heparan sulfate (ave. M_t is 14,200) if it is assumed that the polysaccharide binds 13 FGF molecules.[14] The average weight of the oligosaccharide fraction on HS that binds one FGF is thus 1092; this would imply that the concentration of this oligosaccharide fraction that is required to inhibit FGF binding to HA by 35% is 126 nM (35% inhibition is observed at 136.5 ng/mL). Compound **2f** shows 25% inhibition of FGF binding to HA at 340 nM. The monosaccharides **3d** and **3h** show higher I_{max} values than **2f** (up to 45%) but this occurs at high concentrations (~3.3 mM).

Bovine arterial endothelial cell (BAEC) survival was next investigated as a model for testing the ability of the glucuronic acid conjugates to modulate signal transduction pathways. These cells express both the FGFR and heparan sulfate proteoglycans, and they release FGF-2. This release of FGF-2 not only drives cell proliferation (important in angiogenesis) but also potently suppresses apoptopic cell death; inhibition of FGF-2 activity using a neutralising anti-FGF-2 antibody resulted in increased apoptosis.[15]

The preliminary results (Fig. 3) indicate that compounds which show inhibitory activity in the ELISA assay also inhibit cell survival.[16] Other compounds (**2b** and **3g**) which do not show inhibition in the binding assay do not alter cell viability. This indicates that **3d** and **3h** inhibit survival activity of the endothelial cells at the concentrations used (~33 μM) through their inhibition of the interaction of heparan sulfate proteoglycans with FGF-2. We believe that it is unlikely that the compounds are cytotoxic as the structurally related compound **3g** was inactive and the cell morphology was unchanged. Further work is underway to explore in more detail the mechanism of action of these compounds and to improve their potency and to determine whether they have any mitogenic properties. The synthesis of the series of compounds[17] (Fig. 2) was straightforward and can be carried out (Scheme 1) from the azide **5**, amine **6**,[18] or the bromide/imidate **7**, all of which can be prepared from glucurono-3,6-lactone.

In conclusion, carbohybrid libraries can be used to identify inhibitors of FGF binding to heparin. These compounds also inhibit cell survival pathways and are thus worth consideration for further development as

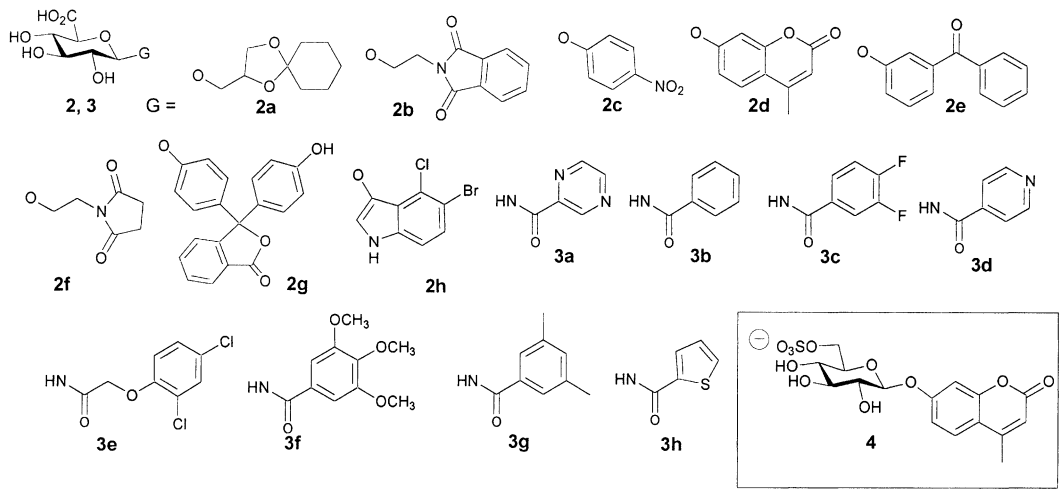


Figure 2. Structure of compounds evaluated in ELISA binding assay.

Table 1. Inhibition of binding of heparin–albumin to FGF-2 determined by ELISA[a]

Compd	I_{max}[b] (%)	IC_{50} (ng/mL)
Heparin–albumin	95	0.6
Heparan sulfate	70	136.5
Heparin	95	1.61
2f	25	<100 (340 nM)
3c	39	11,000
3d	45 at 10^6 ng/mL	—
3e	24	10
3f	23	58
3h	40 at 10^6 ng/mL	—

[a]All other compounds in Figure 2 were not inhibitors.
[b]IC_{50} is defined as concentration of carbohydrate required to obtain 50% of maximum inhibition (I_{max}). Heparan sulfate was obtained from Sigma (cat No. H9902, ave. M_r 14,200) and heparin from Fluka

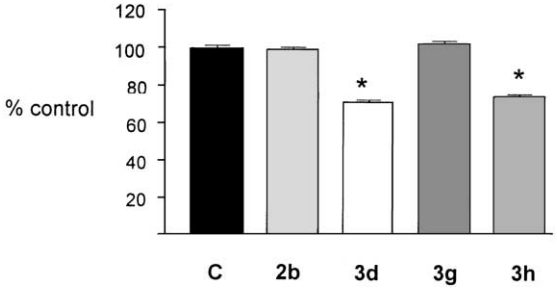
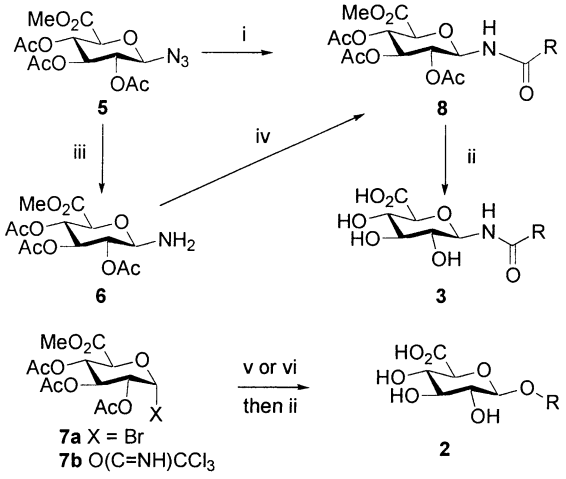


Figure 3. Effect of compounds on bovine aortic endothelial cell viability on a confluent monolayer. Cells were treated for 24 h with 10 μg/mL (29.2–36.5 μL) of the compound and number of viable cells determined by MTT assay. Control cells treated with complete medium only. Experiment performed in triplicate on 2–4 separate occasions; $n = 6$–12. $^{**}p < 0.01$, ANOVA followed by Dunnett's post-ANOVA test. Conjugates **3d** and **3h**, inhibitors in the ELISA assay, alter cell viability whereas compounds that are not inhibitors **2b** and **3g** have no effect.

modulators of FGF mediated processes. The development of potent small molecule inhibitors of the heparin–FGF-2 interaction is challenging given the complex nature of the saccharide structures required for high affinity binding to FGF (octasaccharides at minimum)



Scheme 1. Reagents and conditions: (i) RCOCl, CH_3CN then Ph_3P or diphenylphosphinopolystyrene; (ii) LiOH, H_2O, THF, MeOH; (iii) Pd/C, H_2, −15 °C, THF, 2 h; (iv) RCO_2H, DCC, HOBT, DMAP, THF; (v) $AgCO_3$, $AgClO_4$, mol. sieves, dry CH_2Cl_2, ROH; (vi) BF_3OEt_2, CH_2Cl_2, ROH.

but the results described herein indicate it worthwhile to further explore this goal. The synthesis of monosaccharide conjugates is more straightforward than disaccharide or higher order oligosaccharide conjugates and this should facilitate a more rapid investigation of this possibility. Further work to determine the potential of the inhibitors described herein as leads for angiogenesis related therapy and the establishment of structure–activity relationships are underway. The results will be reported in due course.

Acknowledgements

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References and Notes

1. Lander, A. D. *Chem. Biol.* **1994**, *1*, 73.
2. Capila, I.; Linhardt, R. *J. Angew. Chem. Int. Ed.* **2002**, *41*, 390.
3. Turnbull, J.; Powell, A.; Guimond, S. *Trends Cell Biol.* **2001**, *11*, 75.
4. Lever, R.; Page, C. P. *Nat. Drug Discov.* **2002**, *1*, 140.
5. Conrad, H. E. *Heparin Binding Proteins*; Academic: San Diego, 1998.
6. Cristofanilli, M.; Charnsangavej, C.; Hortobagyi, G. N. *Nat. Drug Discov.* **2002**, *1*, 415.
7. Lundin, L.; Larsson, H.; Kreuger, J.; Kanda, S.; Lindahl, U.; Salmivirta, M.; Claesson-Welsh, L. *J. Biol. Chem.* **2000**, *275*, 24653.
8. Ornitz, D. M.; Yayon, A.; Flanagan, J. G.; Svahn, C. M.; Levi, E.; Leder, P. *Mol. Cell. Biol.* **1992**, *12*, 240.
9. For a recent example see: Stahl, P.; Kissau, L.; Mazitschek, R.; Giannis, A.; Waldmann, H. *Angew. Chem. Int. Ed.* **2002**, *41*, 1174 and cited references.
10. Ornitz, D. M.; Herr, A. B.; Nilsson, M.; Westman, J.; Svahn, C.-M.; Waksman, G. *Science* **1995**, *268*, 432.
11. Jirmanova, L.; Pacholikova, J.; Krejci, P.; Hampl, A.; Dvorak, P. *Int. J. Dev. Biol.* **1999**, *43*, 555.
12. Schlessinger, J.; Plotnikov, A. N.; Ibrahimi, O. A.; Eliseenkova, A. V.; Yeh, B. K.; Yayon, A.; Linhardt, R. J.; Mohammadi, M. *Mol. Cell* **2000**, *6*, 743.
13. Nilsson, U. J.; Fournier, E. J. L.; Fryz, E. J.; Hindsgaul, O. *Comb. Chem. High Throughput Screening* **1999**, *2*, 335.
14. It is estimated there are 15 FGF binding sites per heparin molecule if the average molecular weight of heparin is 16,000. See ref 10 and Mach H.; Volkin, D. B.; Burke, C. J.; Middaugh, C. R.; Linhardt, R. J.; Fromm, J. R.; Loganathan, D.; Mattsson, L. *Biochemistry* **1993** *32*, 5480
15. Garcia, C. M.; D'Amore, P. A. *Invest. Opthalmol. Vis. Sci.* **1999**, *40*, 2945.
16. Mosmann, T. *J. Immunol. Meth.* **1983**, *65*, 55.
17. Selected experimental procedure, synthesis of **3h**. 2-Thiophenoyl chloride (0.5 g, 3.4 mmol) and azide **5** (0.41 g, 1.14 mmol) were added to anhydrous acetonitrile (4 mL), and triphenylphoshine polystyrene (0.5 g, 3.4 mmol, from Novabiochem) was added at room temperature. The reaction was allowed to stir for 12 h, then filtered, diluted with 20 mL CH_2Cl_2, washed with a saturated solution of $NaHCO_3$ (5 mL) and then with water. After drying over Na_2SO_4, the solution was filtered, evaporated to dryness and then purified by chromatography, to afford **8h** (0.26 g, 53%), $[\alpha]_D^{20}$ −15.7° (*c* 0.134, $CHCl_3$), ^{1}H NMR ($CDCl_3$, 300 MHz) δ, 7.88–7.05 (ms, 3H, aromatic), 7.40 (d, 1H, $J=9.0$ Hz, NH), 5.54 (overlapping signals, 2H, H-1 and H-3), 5.19 (apt t, 1H, $J_{3,4}=J_{4,5}=9.5$ Hz, H-4), 5.09 (apt t, 1H, $J_{2,1}=J_{2,3}=9.5$ Hz, H-2), 4.27 (d, 1H, $J_{4,5}$ 9.5 Hz, H-5), 3.71 (s, 3H, OMe), 2.07, 2.05, 2.04 (3s, each 3H, 3×OAc), ^{13}C NMR ($CDCl_3$, multiplicity in parenthesis as obtained by DEPT) δ, 171.3, 169.8, 169.3, 167.2, 161.9 (each s, each C=O), 137.4 (s, aromatic C), 131.8, 129.4, 128.0 (3C, each d, aromatic CH), 78.5 (d, C-1), 73.7, 71.8, 70.4, 69.6 (each d, C-2–5), 52.9 (q, OMe), 20.7, 20.5, 20.4 (each q), IR (liquid film): ε, 3354 (NH), 2955, 2358, 1750, 1659, 1541, 1372, 1232, 1037, 734 cm^{-1}, HRMS-CI (*m*/*z*, positive ion mode): Found 444.0961, required 444.0964 $[M+H]^+$. This intermediate (159 mg, 0.4 mmol) was treated with 6 equiv of 0.05 M LiOH in MeOH/water/THF (2.5/1.0/0.5) and the resulting solution allowed to stir until TLC analysis (3:1 EtOAc/MeOH) indicated the disappearance of starting material. The solution was then diluted with water and neutralised by adding acidic amberlite (IR 120+). THF was then added to homogenise the suspension. The amberlite was removed by filtration and solid Na_2CO_3 was added. The solvent was removed under reduced pressure and the residue was purified by column chromatography (EtOAc/MeOH) to afford the title compound as an off-white solid (90 mg, 83%). Further purification of a portion of this material by C-4 RP-HPLC (95:5H_2O/MeCN, isocratic) yielded, after lyophilization, 3 h as an amorphous white solid which was then used for biological evaluation, ^{1}H NMR (D_2O, 270 MHz): δ, 7.47 (m, 3H, Ar–H), 5.15 (d, 1H, $J_{1,2}=8.4$ Hz, H-1), 3.82 (d, 1H, $J_{4,5}=9.0$ Hz, H-5), 3.56 (m, 3H, H-2, H-3 and H-4), ^{13}C NMR (D_2O, 75 MHz): δ, 178.4 and 168.0 (each s, each C=O), 139.1 (s, aromatic C), 135.4, 133.5 and 131.0 (3C, each d, aromatic CH), 82.4, 80.9, 78.9, 74.5 and 74.4 (5C, C-1–5), ESMS (*m*/*z*, negative ion mode): Found 301, required 301 $[M-H]^-$.
18. von Roedern, E. G.; Lohof, E.; Hessler, G.; Hoffmann, M.; Kessler, H. *J. Am. Chem. Soc.* **1996**, *118*, 10156.