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Bioorganic & Medicinal Chemistry Letters 17 (2007) 6321–6325
Bioorganic & Medicinal Chemistry Letters

Novel heparin/heparan sulfate mimics as inhibitors of HGF/SF-induced MET activation

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Received 14 May 2007; revised 30 August 2007; accepted 31 August 2007
Available online 4 September 2007

Abstract—The synthesis of simple, non-sugar glycosaminoglycan (GAG) mimics has been achieved and the analogues evaluated for their ability to inhibit the activation of the MET receptor by hepatocyte growth factor/scatter factor (HGF/SF).

Cancer progression involves the proliferation and invasion of cancer cells, the stimulation of tumor angiogenesis, and the metastasis of tumor cells through the vasculature. Key factors in these cellular processes involve growth factors such as hepatocyte growth factor/scatter factor (HGF/SF).[1] HGF/SF activates a single, unique tyrosine kinase receptor, MET, which is the product of the *c-met* proto-oncogene.[2,3] There is strong evidence that over-expression or dysregulation of the HGF/SF-MET system in a large variety of tumors is a major contributor to their growth, invasion, and metastasis. Moreover, it has been demonstrated that efficient activation of the MET receptor requires HGF/SF engagement with a GAG co-receptor, which can be heparin (H), heparan sulfate (HS), or dermatan sulfate (DS),[4] and probably subsequent formation of a signaling ternary complex.[5] Therefore disrupting productive HGF/SF-MET interactions, via inhibition of the co-receptor role of GAGs, may be useful in preventing tumor invasion and metastasis.

Previous studies of H/HS interaction with HGF/SF have shown that a tetrasaccharide is the minimal active species.[6,7] There is also evidence that trisaccharides may be capable of interacting with HGF/SF.[8] Previous studies suggested that neither *N*-sulfates[9] nor 2-*O*-sulfates[10] were specifically required in H/HS binding, but that hexosamine *O*-sulfation might be most important.[6] However, more recent work has indicated that overall sulfate density is more critical than strict sulfate positioning.[11] Specific enzymatic and chemical scission of H/HS, together with chemical *N*-desulfation, suggested that the hexosamine 6-*O*-sulfate might be the most critical sulfate group in H/HS for HGF/SF recognition.

A crystal structure (PDB:1GMN) of heparin bound to NK1, a truncated form of HGF/SF, has recently provided structural insight into the function of heparin.[12] Heparin is positioned on a positively charged surface on the N domain, possibly extending to the K1 Kringle domain. The GAG-binding properties of individual domains of HGF/SF have confirmed the major binding site in the N-domain, and also pointed to a secondary site in K1.[13] Key interactions are mainly observed with a tetrasaccharide segment and involve both hydrogen and ionic bonds with sulfate and carboxylic groups. Given the potential significance of therapies based on control of GAG–protein interactions, the development of structurally simpler GAG mimics is an attractive area for investigation.

Keywords: Heparin/heparan sulfate (H/HS); Hepatocyte growth factor/scatter factor (HGF/SF); Tyrosine kinase receptor MET; Glycosaminoglycan (GAG); Non-carbohydrate mimic.
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0960-894X/$ - see front matter
doi:10.1016/j.bmcl.2007.08.074

The successful use of non-carbohydrate compounds such as suramin and its analogues for inhibitory binding to the fibroblast growth factor (FGF) suggests that the synthesis of aromatic, non-sugar compounds as potential inhibitors is an attractive alternative to relatively complex sugar synthesis.[14] Here we present the synthesis and biological evaluation of a series of simple, non-sugar mimics of the trisaccharide **1** as inhibitors of growth factor HGF/SF-activation of receptor (MET).

The design of the first generation of mimics **2** was based on the trisaccharide structure with terminal hexosamines and an internal uronate (Fig. 1). We envisaged an aromatic skeleton to mimic the central carbohydrate residue, bearing diametrically opposite aliphatic chains with variable patterns of sulfate groups that would be able to occupy similar spatial orientations as the sulfates in the model trisaccharide.

The synthesis of heparin mimics was straightforward. It was designed to introduce different degrees of sulfation, demonstrated to be an important feature for binding affinity (Scheme 1). Briefly, methylation and allylation (steps i, ii or (v) a) of 2,5-dihydroxybenzoic acid **3** led to mono- and di-allyl methyl esters. The Sharpless asymmetric dihydroxylation.[15,16] (AD steps (iii) a and (iv) a) was used to enantioselectively prepare 1,2-diols which were sulfated[17] (steps (iii) b and (iv) b) to afford analogues **6** and **7**. Mono-allyl ester was subjected to reaction with 4-bromobutyl acetate (step (v) b) to afford compound **8**, followed by AD and sulfation (step vi) to give analogue **9**. Finally analogues **11** and **12** were obtained using similar sequences (steps viii and ix) from compound **10**.

The inhibitory properties of the heparin mimics **6**, **7**, **9**, **11**, and **12** were first determined in wild type Chinese hamster ovary (CHO) cells, which naturally express both MET and FGF receptors, as well as GAG co-receptors. FGF activity is also dependent upon H/HS co-receptors. Receptors are activated upon productive binding of the respective growth factors leading to phosphorylation of their intrinsic tyrosine kinase domains. This event initiates an intracellular signaling cascade resulting in cellular responses such as proliferation, migration, and differentiation. One important component of this cascade is the activation by di-phosphorylation of the ERK mitogen activated protein kinase (MAPK) which then translocates to the nucleus. Phosphorylation of ERK can be monitored by Western blotting as a downstream indicator of receptor activation by growth factor.

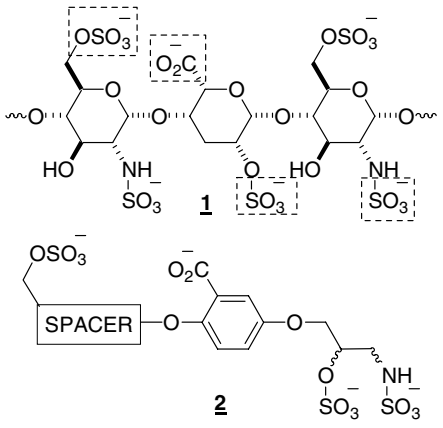


Figure 1. Design of non-carbohydrate heparin mimics.

The five analogues were evaluated for their ability to inhibit ERK phosphorylation elicited by two growth factors: HGF/SF and FGF-2 (Fig. 2).

Two compounds (**7** and **12**) were found to have weak but differential inhibitory activities. Both compounds were able to completely inhibit HGF/SF activity (Fig. 2a) but not FGF-2 activity (Fig. 2b) at concentrations of 100 μg/mL. At lower concentrations neither compound **7** (Fig. 3a) nor **12** (Fig. 3b) showed any inhibitory activity toward ERK phosphorylation induced by HGF/SF.

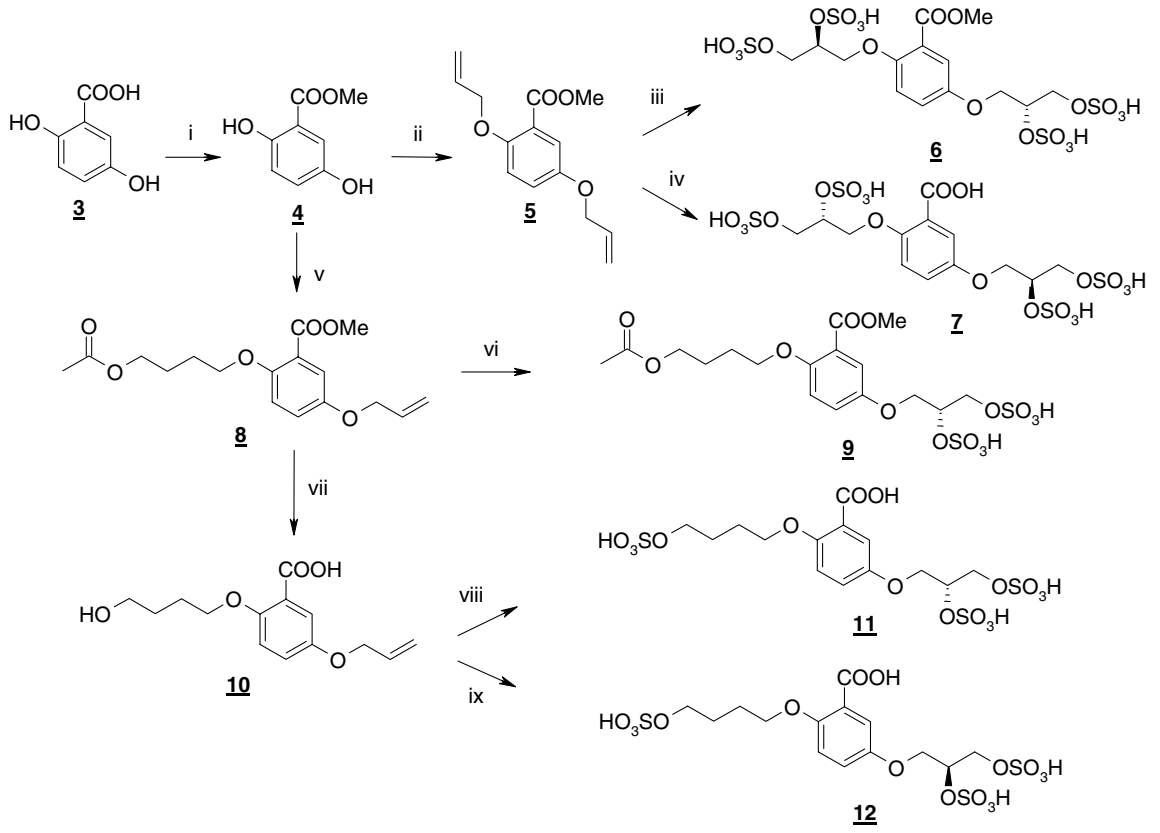
The discrimination in the inhibition of different GAG-dependent growth factors demonstrates the potential for selective clinical targeting via antagonism of the co-receptor mechanism.

Madin–Darby canine kidney (MDCK) cells are commonly used for assaying HGF/SF activity in vitro. These cells will actively migrate across a porous Transwell membrane in a chemotactic response to HGF/SF.[18] Compounds **6**, **7**, **9**, **11**, and **12** were evaluated in the migration assay (Fig. 4). Compound **7** was able to inhibit HGF/SF-induced cell migration by 57% while compound **12** inhibited it by 70%.

Although compounds **7** and **12** showed only a micromolar inhibitory activity toward HGF/SF, they are the first sugar mimetic compounds described to demonstrate significant inhibition of both HGF/SF-induced ERK activation and HGF/SF-induced migration in MDCK cells.

A computational study was undertaken to predict possible binding modes for heparin mimic **12**. The model was built based on the X-ray crystal structures of NK1 (PDB: 1BHT, 2.0 Å resolution) and NK1-heparin (PDB: 1GMN, 3.0 Å resolution). In order to check that the selected *blind docking* process was adequate for finding the correct binding mode of compound **12**, the heparin tetradecasaccharide was extracted from the original structure 1GMN and docked into the better resolved 1BHT using AUTODOCK 3.0 (100 runs).[19] The docked structures were clustered at 1.0 Å rmsd. The most populated cluster (62 conformations) contained the heparin conformation with the lowest docking energy ($E = -589.59$ kJ mol^{-1}). The binding mode of the lowest energy complex was submitted to energy minimization and was compared to the crystal structure. The docking study results in a binding mode for heparin that is almost identical to that found in the original X-ray structure (rms 1.0 Å).

Compound **12** was docked using the methodology described above (250 runs).[20] The final complexes were



Scheme 1. Reagents and conditions: (i) MeOH, H_2SO_4, reflux, 24 h, 95%; (ii) NaH (3 eq), AllylBr (3 equiv), *t*-Bu_4NI (0.2 equiv), DMF, rt, 12 h, 92%; (iii) a—AD mix α, $MeSO_2NH_2$ (1 equiv), *t*-BuOH/H_2O, 0 °C, 12 h, 61%; b—SO_3·NMe_3, DMF, 40 °C, 12 h, 55%; (iv) a—KOH (2 equiv), water/ethanol, 6 h, 60 °C, 70%; b—AD mix β, $MeSO_2NH_2$ (1 equiv), *t*-BuOH/H_2O, 12 h, 0 °C, 58%; c—SO_3·NMe_3, DMF, 40 °C, 12 h, 42%; (v) a—K_2CO_3 (1.5 equiv), AllylBr (1.5 equiv), *n*-Bu_4NI (0.2 equiv), acetone, rt, 12 h, 68%; b—NaH, 60% (1.5 equiv), $Br(CH_2)_4OAc$ (1.5 equiv), *n*-Bu_4NI (0.2 equiv), DMF, 12 h, 71%; (vi) a—AD mix α, $MeSO_2NH_2$ (1 equiv), *t*-BuOH/H_2O, 0 °C, 12 h, 58%; b—SO_3·NMe_3, DMF, 40 °C, 12 h, 73%; (vii) KOH (2 equiv), water/ethanol, rt, 60 °C, 6 h, 70%; (viii) a—AD mix α, $MeSO_2NH_2$ (1 equiv), *t*-BuOH/H_2O, 0 °C, 12 h, 86%; b—SO_3·NMe_3, DMF, 40 °C, 12 h, 83%; (ix) a—AD mix β, $MeSO_2NH_2$ (1 equiv), *t*-BuOH/H_2O, 12 h, 0 °C, 80%; b—SO_3·NMe_3, DMF, 40 °C, 12 h, 79%.

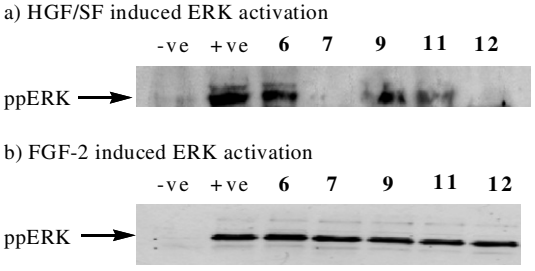


Figure 2. Effect of heparin mimics **6**, **7**, **9**, **11**, and **12** on growth factor-induced ERK activation. CHO cells were treated for 20 min with either (a) HGF/SF or (b) FGF-2 and candidate analogues (100 μg/mL). Cell extracts resolved by SDS–PAGE were probed for activated ERK by Western blotting.

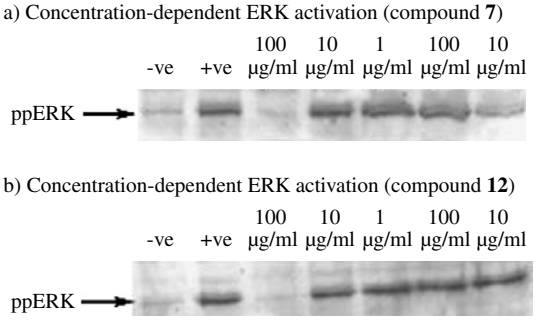


Figure 3. Concentration-dependent effect of compounds **7** and **12** on HGF/SF-induced ERK activation.

optimized by energy minimization using Macromodel.[21] Finally, the docked structures were clustered at 4.0 Å rmsd. Thirty-eight distinct conformational clusters were found. The most populated cluster (73 conformations) contained the conformation with the lowest docking energy ($E = -14.67$ kcal mol^{-1}).

After minimization of the docked complex, amino acid residues involved in the interaction between the protein and compound **12** could be identified (Fig. 5). Compound **12** interacts with residues Arg35, Arg76, Arg73, Lys78, Lys60, and Lys62 through hydrogen/ionic interactions. Residues Arg73, Lys60, and Lys62 are known to play an important role in heparin-HGF/SF binding.

In summary, a series of structurally-related heparin mimics were synthesized in 4–6 steps. These mimics bear sulfate groups responsible for the binding of the H/HS to the growth factor. They were evaluated for their

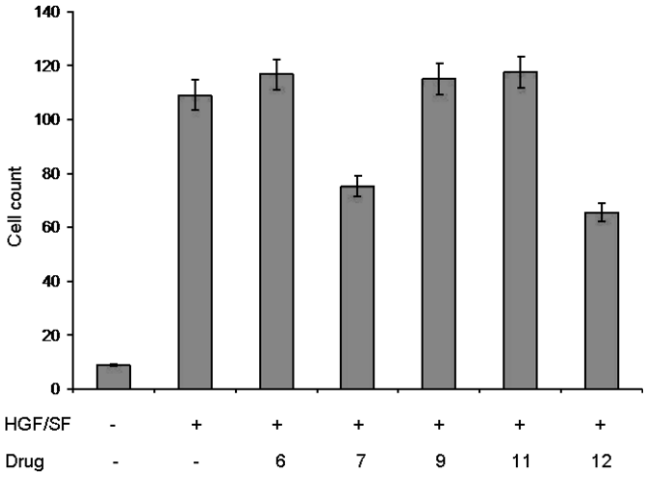


Figure 4. Effect of analogues **6**, **7**, **9**, **11**, and **12** on the Transwell migration of MDCK cells. MDCK cells (2×10^5 cells/well) were seeded onto Transwell membrane (pore size 8 μm) and incubated for 4 h at 37 °C in the absence (−) or presence (+) of HGF/SF and heparin mimics (100 μg/mL). Migrated cells were stained with Crystal Violet and counted, to give a mean cell count.

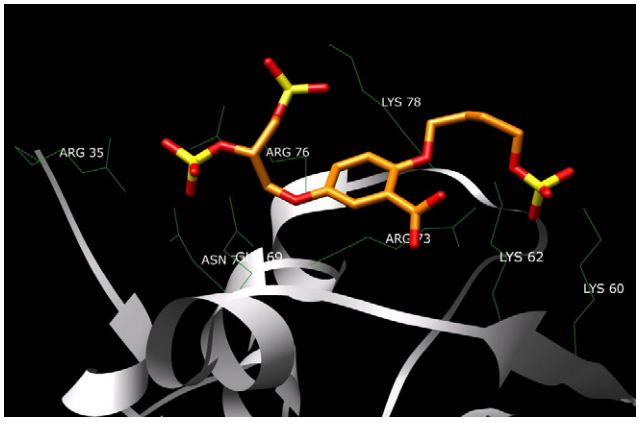


Figure 5. Proposed binding mode for compound **12** (orange) with HGF/SF (gray).

ability to inhibit HGF/SF- or FGF-2-mediated activation of ERK MAPK in wild type CHO cells. Two compounds **7** and **12** were found to have good, selective inhibitory activity. Both compounds were also able to significantly inhibit HGF/SF-induced cell migration in MDCK cells. Simulated docking experiments suggest that compound **12** may compete for binding to part of the H/HS binding site in HGF/SF. These results are promising and demonstrate the potential for these aromatic sugar mimetics to selectively inhibit HGF/SF. We now intend to design a second generation of H/HS mimics based upon our preliminary docking model.

Acknowledgments

The Biomedical Science Research Institute at Salford and Cancer Research, UK, have generously supported our work throughout this time. We would like to thank Boehringer Ingelheim for a travel fund for E-AR. We acknowledge EPSRC for provision of accurate mass spectral data.

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16. *General procedure for Sharpless AD*: AD α mix (5.54 g) was dissolved in a mixture of *t*-BuOH/water (20 mL/20 mL) and cooled down to 0 °C. The alkene (2.0 mmol) and methansulfonamide (2.0 mmol) were added and the reaction mixture was stirred at 0 °C for 12 h. The reaction was quenched with sodium sulfite (5 g) and the solvent was removed in vacuo. Ethanol (15 mL) was added to the crude mixture and the mixture was heated under reflux for 1 h. The salt was filtered off and the crude compound purified by flash column chromatography on silica.
17. *General procedure for sulfation*: The alcohol (0.3 mmol) was dissolved in *N*,*N*-dimethylformamide (3.8 mL). Sulfur trioxide-trimethylamine complex (1.68 mmol) was added and the reaction mixture was stirred at 40 °C for 12 h. The solvent was removed in vacuo and the crude product was purified by flash column chromatography.
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20. *Autodock parameters*: Translation, Quaternion and Torsion reduction factor 1/cycle; No. of top inividudals that

automatically survive 1; Rate of gene mutation 0.02; Rate of crossover 0.8; No. of generations for picking worst individual 10; Mean of Cauchy distribution for gene mutation 0; Variance of Cauchy distribution for gene mutation 1; No. of iterations of Solis and Wets local search 300; No. of consecutive successes before changing p 4; No. of consecutive failures before changing p 4; Size of local search space to sample 1; Lower bound on p 0.01; Probability of performing local search on an individual 0.06; Total number of energy evaluations 10 million; Populations in the generic algorithm 250.

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