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Different protein-binding selectivities for *N*-acyl heparin derivatives having *N*-phenylacetyl and heterocycle analogs of *N*-phenylacetyl substituted in place of *N*-sulfo groups

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Abstract—Replacing *N*-sulfo groups in heparin with *N*-arylacyl moieties has been shown to afford charge-reduced heparin derivatives that maintain affinity for select heparin-binding proteins. In this study 50% and 100% N-desulfonated heparins were selectively N-acylated with phenylacetic acid and four phenylacetic acid analogs where the phenyl ring was replaced by a heterocycle. Protein-binding studies reveal that structural differences in the ring systems of the *N*-acyl groups appended to heparin afford significant affects on affinity and selectivity for different heparin-binding proteins.

Heparin is a linear, negatively charged microheterogeneous polysaccharide that binds hundreds of proteins.[1] Many heparin-binding proteins play a critical role in biological processes by virtue of binding to endogenous heparin-like cell surface glycosaminoglycans such as heparan sulfate and dermatan sulfate.[2] Numerous potential therapeutic applications have been proposed for heparin and heparin-like polyanionic oligosaccharides.[3,4] Significant problems thwarting therapeutic applications of polyanionic saccharides include poor pharmacological properties and promiscuous binding to many proteins. Strategies to identify charge-reduced heparin derivatives that selectively bind individual heparin-binding proteins with equivalent or increased affinity compared to parent heparin are needed to overcome non-specific protein binding observed with most polyanionic saccharides, and to begin tapping potential therapeutic application of heparinoids as selective bind-and-block antagonists of endogenous glycosaminoglycan–protein interactions.[4]

We recently outlined a rationale for replacing *N*-sulfo groups in heparin with non-anionic moieties, toward identifying heparinoids that bind heparin-binding proteins with improved selectivity.[5] The goal of replacing anionic groups on heparin with structurally diverse non-anionic moieties capable of providing binding contacts other than charge–charge interactions is to minimize charge and optimize spatially more stringent saccharide–protein binding contacts, thus affording reduced-charge lead structures as more selective bind-and-block antagonists of HS–protein interactions. Indeed, *N*-sulfo groups in heparin previously shown to be required for high affinity binding of heparin to specific proteins can, in fact, be replaced with select aromatic *N*-acyl moieties to yield charge-reduced heparin derivatives that more selectively bind a protein with equivalent or increased affinity compared to unmodified heparin.[5]

Previous studies of N-desulfonated/N-acylated heparin derivatives employing graded N-desulfonation of heparin followed by N-acylation with 19 structurally diverse *N*-acyl moieties revealed N-desulfonated/N-acylated heparin derivatives that maintained affinity for select growth factors and/or α-thrombin. A consistent trend with the N-acylated heparin derivatives that retained affinity for select heparin-binding proteins was the presence of an aromatic ring in the *N*-acyl moiety. In the work reported here, 50% and 100% N-desulfonated heparin was prepared and N-acylated with phenylacetic acid or one of four heterocycle-containing phenylacetic acid analogs (see Fig. 1). These 10 N-desulfonated/N-acylated heparin derivatives were screened against a randomly selected panel of heparin-binding proteins to determine

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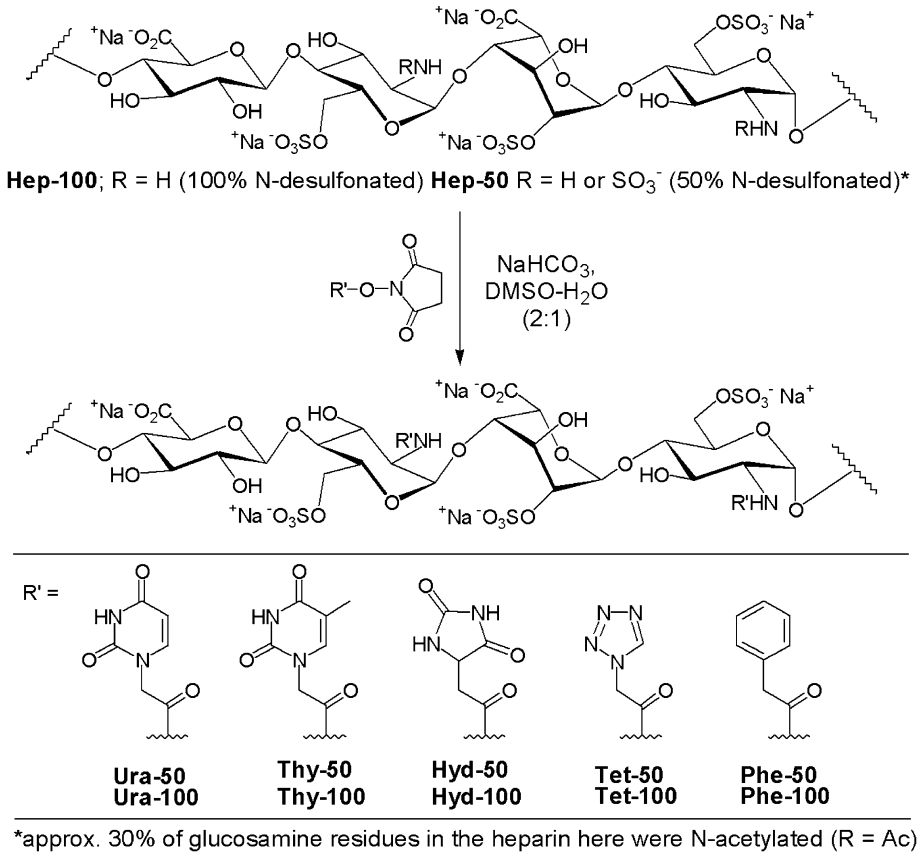

Figure 1. Synthesis of N-acylated heparin derivatives having 50% and 100% of the *N*-sulfo groups replaced with ring-analog *N*-acyl moieties.

the degree of protein-binding selectivity imparted by modest structural changes in the acyl ring moieties. In addition, by using an expanded panel of heparin-binding proteins we looked to test if non-aromatic *N*-acyl groups capable of multiple hydrogen bond contacts could replace *N*-sulfo groups to afford heparin derivatives that maintained affinity for one or more heparin-binding proteins.

Heparin was selectively N-desulfonated at 50 °C as previously described to afford heparin fractions having 50% (**Hep-50**) and 100% (**Hep-100**) of the *N*-sulfo group converted to free amine.[5,6] Selective N-acylation of **Hep-50** and **Hep-100** fractions was achieved using *N*-hydroxysuccinimide (NHS) esters of uracil-1-acetic acid, thymine-1-acetic acid, hydantoin-5-acetic acid, 1*H*-tetrazole-1-acetic acid, and phenylacetic acid (Fig. 1).[7] Complete acylation of all amine groups was achieved using no more than two coupling cycles as confirmed by 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay (Fig. 2).[6]

The binding of each N-acylated heparin to one or more heparin-binding proteins in a panel of proteins was initially determined using a competition-binding assay (Fig. 3).[8] Affinity of each N-acylated heparin derivative relative to heparin was determined by comparing displacement of fluorescent-labeled heparin (FL-heparin) from each heparin-binding protein at 10 μg/mL. All of the N-acylated heparin derivatives here showed decreased affinity, diminished ability to displace FL-heparin, against α-thrombin, secretory leukocyte protease inhibitor (SLPI), and fibroblast growth factor 2 (FGF2).

For each of the remaining proteins (myeloperoxidase (MPO), FGF1, laminin, and lactoferrin), *N*-phenylacetyl heparins **Phe-50** and **Phe-100** showed equivalent or increased displacement of FL-heparin from protein as compared to heparin (Fig. 3). Significantly, each protein that retained binding affinity for heparin bearing *N*-phenylacetyl groups in place of *N*-sulfo groups also showed equivalent or increased affinity for select N-acylated heparins where the *N*-acyl group contained certain heterocycle moieties. Indeed, different heterocycle-containing *N*-acyl groups imparted unique structure-dependent increases or decreases in their ability to displace FL-HP from each protein, which is a measure of relative affinity. For example, **Hyd-50** and **Hyd-100** displayed greater affinity than heparin for FGF1, while **Thy-50** and **Thy-100** displayed lower affinity than heparin for FGF1. The opposite trend in selectivity was observed with laminin, where **Thy-50** and **Thy-100** displayed higher affinity than heparin while **Hyd-50** and **Hyd-100** displayed equivalent or lower affinity. **Phe-50** and **Phe-100** bound with slightly higher or equivalent affinity to lactoferrin, respectively, and with equivalent or lower affinity as compared to the heterocycle-containing N-acylated heparin derivatives.

Using lactoferrin as the representative protein, concentration-dependent displacement of FL-heparin from lactoferrin was performed to further validate the

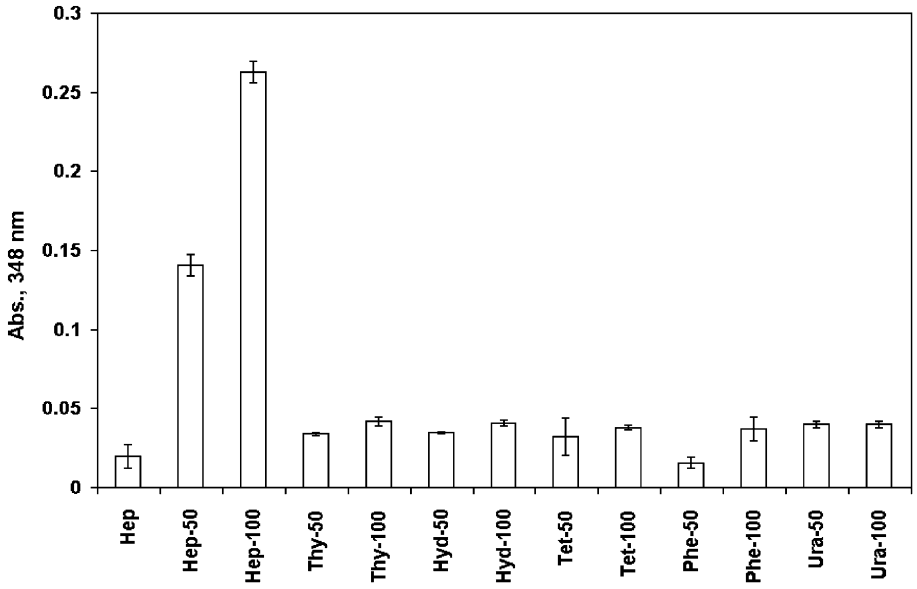

Figure 2. Uncorrected absorbance values from the TNBS assay for amine content. Hep (parent heparin); **Hep-50** (50% N-desulfonated heparin); **Hep-100** (100% N-desulfonated heparin); and corresponding N-acylated heparin derivatives after two coupling cycles.

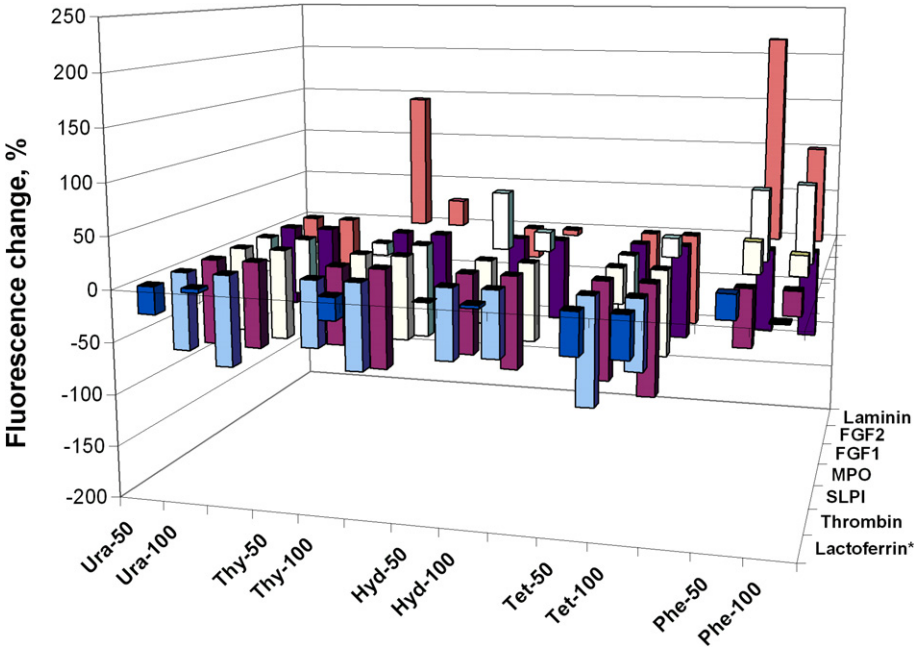

Figure 3. Competition-binding screen comparing relative affinity of each *N*-acyl heparin derivative to heparin for each protein as determined by displacement of FL-heparin.[8] Percent fluorescence change was calculated as the percent increase or decrease in fluorescence intensity (increase or decrease in FL-heparin displaced from protein) as compared to heparin at an equivalent concentration of heparin and the N-acylated heparin (10 μg/mL). Heparin is thus 0 on the *Z*-axis. Data shown is from a single sample screen. Replicates of select individual samples were consistent with data shown; observed differences ranged from 1% to 10%. See references and notes for a discussion of mass differences.[11]

single-concentration screening data reported in Figure 3, and to further characterize the degree to which affinity of the different *N*-acyl heparin derivatives is maintained or lost against an individual protein (Fig. 4). The single concentration affinity data at 10 μg/mL showed that **Phe-50** possessed a 23% increase in displace FL-heparin over parent heparin, while **Phe-100** and parent heparin were equivalent. Concentration-dependent displacement of FL-heparin from lactoferrin by **Phe-50** and **Phe-100** clearly demonstrates that each of these N-desulfonated/N-phenylacetyl heparin derivatives maintained affinity for lactoferrin (Fig. 4).

In contrast to *N*-phenylacetyl heparin derivatives maintaining affinity for lactoferrin, single concentration affinity data revealed that most heparin derivatives substituted with *N*-acyl groups bearing heterocyclic rings instead of the phenyl ring possessed lower affinity

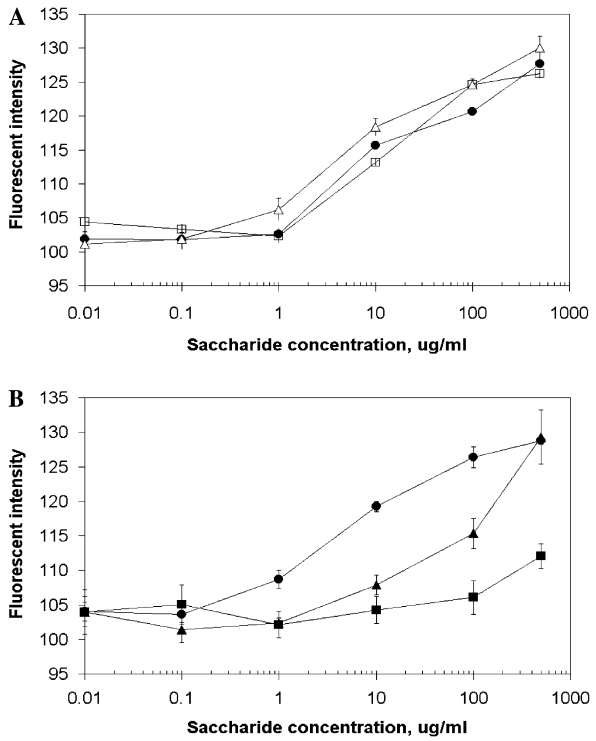

Figure 4. Concentration-dependent binding of select N-desulfonated/N-acylated heparin derivatives to human lactoferrin. Fluorescence intensity is uncorrected and correlates to the amount of unbound (displaced) FL-heparin. (A) Concentration-dependent displacement of FL-heparin from lactoferrin by **Phe-100** (□) and **Phe-50** (Δ) as compared to parent heparin (●). (B) Concentration-dependent displacement of FL-heparin from lactoferrin by Tet-50 (■) and Thy-100 (▲) as compared to heparin (●). See references and notes for a discussion mass differences for the different N-acylated heparins.[11]

Table 1. Comparison of clotting times for heparin and N-desulfonated/N-acylated heparin derivatives in the aPPT assay as a measure of anticoagulation activity

Heparin derivative (0.2 μg/mL plasma)	% N-desulfonation/ N-acylation	Clotting time (s)
Unmodified heparin	0	57.2
No heparin (controls)	—	45–47
Ura-50	50	45.2
Ura-100	100	44.9
Thy-50	50	44.7
Thy-100	100	41.2
Hyd-50	50	43.2
Hyd-100	100	44.7
Tet-50	50	44.2
Tet-100	100	45.7
Phe-50	50	43.9
Phe-100	100	42.2

than heparin for lactoferrin. Concentration-dependent displacement of FL-heparin from lactoferrin by **Thy-100** and **Tet-50** confirms this loss in affinity, where approximately 10- and 100-fold increased concentration of saccharide is required to displace 50% of FL-heparin, respectively. Removal of *N*-sulfo groups from heparin was previously reported to afford an approximate 10-fold increase in the concentration of heparin required to displace 50% of a radiolabeled heparin from lactoferrin.[9] Here, it is shown that replacing *N*-sulfo groups on heparin with *N*-phenylacetyl groups has no significant effect on affinity for lactoferrin, while the corresponding heterocycle analogs of the *N*-phenylacetyl group afford a significant decrease in affinity for lactoferrin.

An ultimate goal of this work is to develop strategies to prepare non-anticoagulant heparin derivatives that have increased selectivity for binding individual, or a more limited number of, heparin-binding proteins. To this end we looked to evaluate anticoagulant activity of the N-acylated heparin derivatives used in this study. Clotting times for heparin and each N-acylated heparin derivative were compared in the activated partial thromboplastin time (aPTT) assay (Table 1).[10,11] All N-acylated heparin derivatives showed diminished anticoagulant activity in this assay, as indicated by shorter clotting times. It is interesting to note that little difference in clotting time is observed between 100% and 50% *N*-acyl heparins. This result suggests that N-desulfonation of glucosamine residues within key anticoagulant sequences of heparin is likely occurring early in the desulfonation reaction, before 50% N-desulfonation is reached.

Results of this study demonstrate that relatively small changes to the size of ring-containing *N*-acyl moieties substituted into heparin in place of *N*-sulfo groups, but significant differences in electronic nature and hydrogen bonding capabilities, afford dramatic differences in whether an N-desulfonated/N-acylated heparin derivative will have increased affinity, decreased affinity, or maintain affinity for a specific heparin-binding protein. Comparison in protein binding affinity between *N*-phenylacetyl heparin derivatives and corresponding heterocycle analogs indicates that aromatic character as well as hydrogen-bond forming capability likely play a direct role in protein-binding selectivity. This suggests that specific binding contacts between protein and *N*-acyl group on the heparin chain are being formed, although other explanations for variable saccharide–protein affinity such as conformation changes to the saccharide chain as a consequence of different *N*-acyl groups cannot be ruled out. We are currently working to determine if specific binding contacts such as cation–π interactions or hydrogen-bond networks are responsible for the observed differences in protein-binding affinity. Ultimately understanding the molecular interactions responsible for select charge-reduced, non-anticoagulant *N*-acyl heparin derivatives binding to select heparin-binding proteins with equivalent or increased affinity is anticipated to facilitate the future design and synthesis of more selective bind-and-block antagonists of glycosaminoglycan-binding proteins.

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6. Amine content of the N-desulfonated heparin fractions and N-acylated heparin derivatives was quantified using the trinitrobenzenesulfonic acid (TNBS) assay and sulfate analysis as described in Ref. 5. 100% N-desulfonation of **Hep-100** was confirmed by ^{1}H NMR showing complete shift of glucosamine H-2 proton from 3.21 (C-2 *N*-sulfo) to 3.34 (C-2 NH_2).
7. The NHS esters of thymine-1-acetic acid, hydantoin-5-acetic acid, 1*H*-tetrazole-1-acetic acid, and phenylacetic acid were purchased or prepared and used in coupling reactions essentially as described.[5] The NHS ester of uracil-1-acetic acid was prepared and employed for coupling reactions as follows to give **Ura-50** and **Ura-100**. Uracil-1-acetic acid (85.5 mg, 0.5 mmol) and NHS (115 mg, 1.0 mmol) were azeotroped with toluene (3 × 6 mL), dissolved in 2 mL DMF, and cooled with an ice-bath. DCC (104 mg, 0.5 mmol) was azeotroped with toluene, dissolved in 0.5 mL DMF, and added to the above-cooled solution. The ice-bath was removed after 5 min. White precipitate (DCU) appeared in 20 min with formation of NHS ester followed by TLC (EtOAc/MeOH/HOAc, 15:5:1, R_f = 0.75). After stirring for 2 h at RT, the reaction mixture was transferred to a centrifuge tube and centrifuged. The supernatant was employed directly as a stock solution for N-acylation of heparin fractions. Each N-desulfonated heparin (10 mg) was dissolved in 1 mL DMSO–H_2O (2:1) saturated with $NaHCO_3$ and treated with the NHS ester solution by addition (0.2, 0.1, 0.1, and 0.1 mL) at 30 min intervals. Coupling reactions were then stirred at RT overnight after the final addition of NHS ester. Reaction mixtures were precipitated by addition to 40 mL cold acetone/ethyl ether (1:1). The white precipitates were centrifuged, supernatant decanted, and dried. Residues were dissolved in 1 mL H_2O, filtered, transferred to dialysis tubing (MWCO, 3500), and dialyzed. Lyophilization afforded products as white cotton-like solids. TNBS analysis showed **Ura-100** still contained approximately 10% of possible free amine, and thus a second, identical coupling cycle was performed. ^{1}H NMR (300 MHz, D_2O): δ 2.03 (s, *N*-Ac), 3.2–5.5 (core heparin signals), 5.86 (d, uracil), 7.59 (d, uracil).
8. Screening of heparin and *N*-acyl heparin derivatives for relative protein-binding affinity at 10 μg/ml against α-thrombin, SLPI, MPO, FGF1, FGF2, and laminin) employed fluorescein-labeled heparin in a 96-well plate filtration-based competition binding assay as previously described.[5] Screening and concentration-dependent binding studies for lactoferrin similarly employed the methods previously reported, with the exception that bodipy-labeled instead of fluorescein-labeled heparin was employed.
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11. MW differences imparted by 70% *N*-acyl versus *N*-sulfo groups translate MW_{ave} = 15,000 for heparin to MW_{ave} = 16,134 for **Thy-100**. Molar concentrations at 10 μg/mL thus range from 0.67 μM for heparin to 0.62 μM for **Thy-100** (Fig. 1). Similarly, Table 1 concentrations for 0.2 μg range from 44 nM for heparin to 41 nM for **Thy-100**.