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Polycationic lipophilic-core dendrons as penetration enhancers for the oral administration of low molecular weight heparin

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Abstract—Two polycationic lipophilic-core carbohydrate-based dendrons **2a–b** and five polycationic lipophilic-core peptide dendrons **3–6**, containing four arginine or lysine terminal residues, were synthesized and then tested in rats as penetration enhancers for the oral delivery of low molecular weight heparin. Better results were obtained with dendrons containing terminal lysine residues than terminal arginine. A significant anti-factor Xa activity was obtained when low molecular weight heparin was coadministered with dendron **5**.

1. Introduction

Unfractionated heparin (UFH, avg. MW ca. 12,000 Da) is a heterogeneous mixture of polysaccharides comprising alternating 1-to-4 linked sulfated monosaccharide residues of D-glucosamine and a uronic acid (90% L-iduronic acid, 10% D-glucuronic acid) (Fig. 1A).[1–3] The pentasaccharide binding site (Fig. 1B) for antithrombin occurs in approximately one-third of heparin chains.[2,3] UFH is used for the prevention and treatment of thrombotic disorders and in the treatment of acute coronary syndromes.[4,5] Formed from the chemical or enzymatic digestion of UFH, low molecular weight heparin (LMWH) has replaced UFH as the drug of choice for the surgical prophylaxis of deep vein thrombosis and the management of acute coronary syndromes.[5] LMWH was recently approved for the treatment of thrombotic disorders and is being developed for therapeutic applications in cancer, transplantation, and immunology.[5] LMWH has an average molecular weight of ~4500 Da, and compared to UFH (~12,000 Da), it has improved pharmacokinetic properties and a decreased toxicity profile.[4,6,7] Nevertheless, LMWH is a high molecular weight, hydrophilic polyanion, which can be degraded in the gastrointestinal tract by bacteria[8] and to a lesser extent by acid;[9] and therefore it exhibits poor oral bioavailability. Consequently, it must be delivered via the less desirable parenteral route, which is expensive, inconvenient, and limits use by outpatients.[10–12] In this paper, we describe the synthesis and in vivo evaluation of a series of penetration enhancers[13,14] designed to improve LMWH oral bioavailability.

Intensive effort has focused on developing an oral heparin formulation. Ubrich et al.[15] reviewed the literature prior to October 2002, pertaining to formulations designed to increase heparin intestinal absorption via the oral, intragastric, intraduodenal, or rectal routes of administration. Ross and Toth[16] examined the literature, mostly published between 2000 and 2005, focusing on delivery systems that improved the gastrointestinal absorption of heparin. These review articles noted that promising delivery agents included microspheres, Carbopol®, chitosan, deoxycholic acid, fatty acids and surfactants, saponins, and polymeric micro- and nanoparticles.

The most advanced studies relating to the development of oral heparin are attributed to Emisphere Technologies Inc., which used sodium *N*-[8-(2-hydroxybenzoyl)amino]caprylate (SNAC) and sodium *N*-[10-(2-hydroxybenzoyl)amino]decanoate (SNAD), as penetration enhancers for the oral delivery of UFH and LMWH. SNAC and SNAD were identified as leads from a series of compounds according to their ability to enhance

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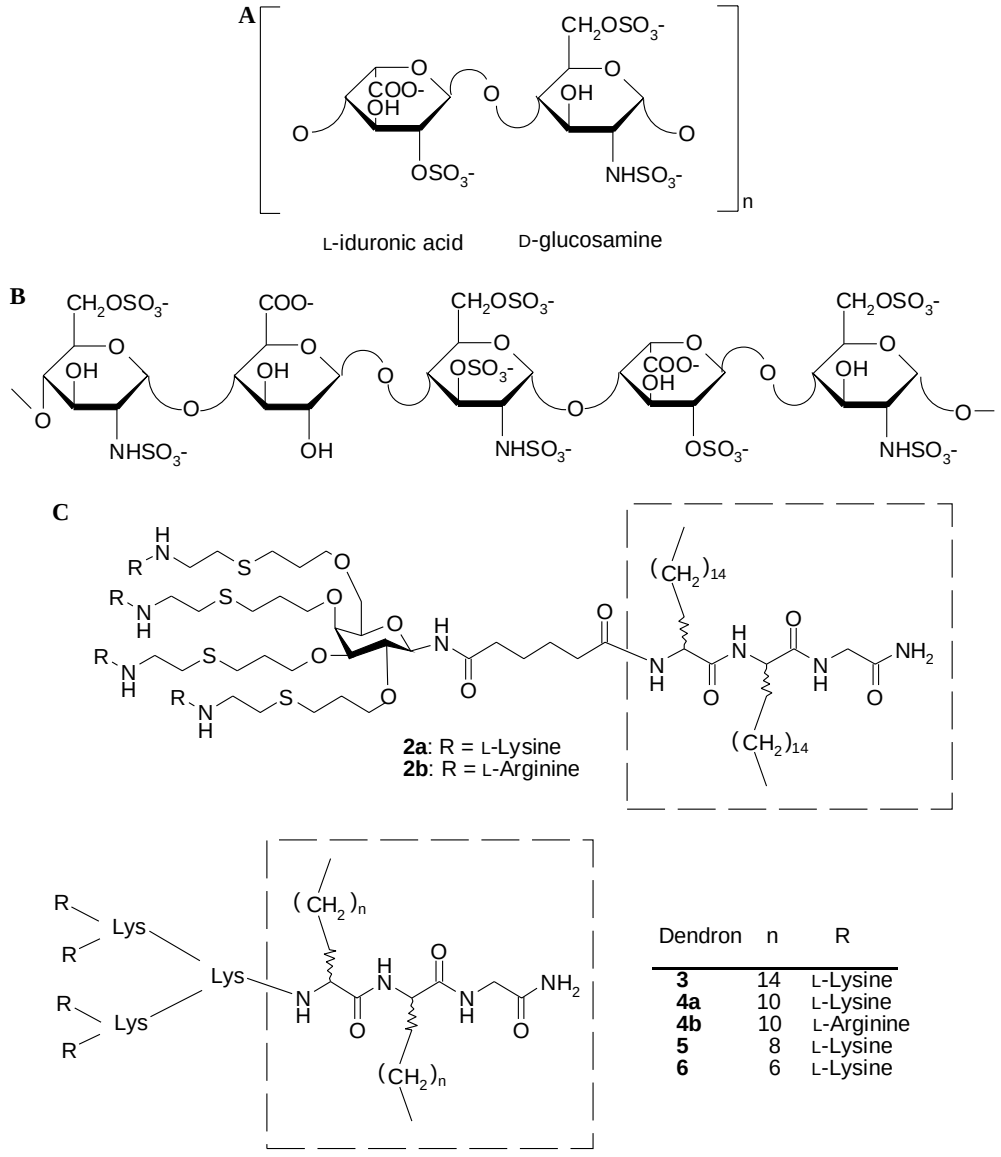


Figure 1. Heparin is a heterogeneous mixture of polysaccharides comprising alternating 1-to-4-linked sulfated monosaccharide residues. (A) The most common type of disaccharide unit. (B) The pentasaccharide binding site for antithrombin. (C) Polycationic lipophilic-core dendrons **2**–**6**. The lipophilic-core moiety is enclosed by the box. (Lys = L-Lysine.) [(A) and (B) were modified from chromogenix monograph series: heparin;[2] Rang et al.;[3] and Samama et al.[67]].

colonic absorption of UFH in rats.[17,18] Subsequent studies in rats and monkeys demonstrated the effectiveness of SNAC for the oral delivery of UFH.[19] The UFH and SNAC formulation was successful in phase I and II clinical trials; [20,21] however, phase III trials were negatively influenced by poor patient compliance associated with the taste of the liquid formulation.[22,23] SNAD was found to be about twice as effective as SNAC for facilitating oral LMWH absorption in rats.[24] In monkeys, SNAD was ~10-fold more effective than SNAC for oral LMWH delivery.[24] The bioavailability of a SNAD and LMWH combination, administered orally to dogs as a solid dose form, relative to subcutaneous injection, was 3%.[24]

In this study, we examined polycationic lipophilic-core dendrons (**2**–**6**) as penetration enhancers for the oral delivery of LMWH. Dendrimers and dendrons (partial dendrimers) have been a topic of extensive research since they were first prepared by Tomalia et al.[25] and Newkome et al.,[26] in the 1980s. Some of the most promising applications of these molecules lie in the fields of drug and gene delivery.[27–30] For example, dendrons structurally similar to **3**–**6** (Fig. 1C) mediated the transfection of Cos-7[31] and BHK-21 cells[32] with plasmid DNA. Recently, human cells (D-407) were successfully transfected with an oligodeoxynucleotide in vitro, using a dendron related to **5** (Fig. 1C), as a vector.[33] In these examples, the polycationic nature of the dendrons was designed to enable ion-pair formation with polyanionic DNA. The formation of ion-paired complexes was studied by examining the dendron mediated exclusion of ethidium bromide (EB) from DNA–EB complex,[34] and also the electrophoretic retardation of DNA–dendron complexes in

agarose gel electrophoresis.[31,32] The lipophilic-core moieties [the moiety of **2**–**6** enclosed by the box (Fig. 1C)], which contained lipoamino acids[35] (LAAs), were designed to increase the lipophilicity of the complex and thereby facilitate membrane partitioning. LAAs are versatile compounds, which have been incorporated into penetration enhancers,[36,37] and poor absorbed drugs,[38–40] to enhance membrane permeability and reduce enzymatic degradation. LAAs, in the form of the lipophilic-core moiety, have also found application in experimental vaccines.[41,42] An advantage of LAAs is that they are bifunctional molecules that are amenable to solid-phase synthesis techniques (e.g., Scheme 2).

We postulated that polycationic lipophilic-core dendrons **2**–**6** could be used as penetration enhancers, for the oral delivery of LMWH, by forming lipophilic ion-pairs with the polyanionic LMWH. Lipophilic ion-pairing has previously been examined as a method to enhance heparin bioavailability. Complexation with a diamine improved the intraduodenal absorption of UFH and LMWH, in the rat.[43] Monoamines, and a more hydrophilic diamine, were ineffective. In dogs, the oral absorption of UFH was enhanced by coadministration with a diamine, using enteric-coated pellets.[44] Di- and triamines, and to a lesser extent monoamines, were effective at improving the intraduodenal absorption of UFH in rabbits.[45,46]

The ability of heparin to bind with polycationic molecules is exemplified by its interaction with many proteins, a topic that was reviewed by Capila and Linhardt.[47] They stated that the most prominent type of interaction between heparin and a protein is ionic, whereby clusters of positively charged basic amino acids on proteins form ion-pairs with negatively charged sulfo or carboxyl groups on the heparin chain. Recently, Kasai et al.[48] synthesized two arginine-rich (polycationic) dendrimers, which were designed to act as antiangiogenic agents via a heparin-binding mechanism.

The synthesis and in vivo evaluation of the series of dendrons **2**–**6** (Fig. 1C) as penetration enhancers for the oral delivery of LMWH, are described below.

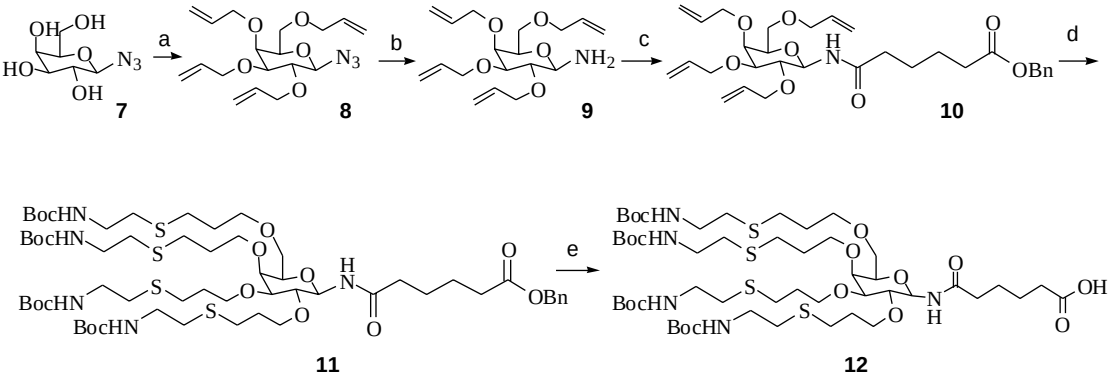
2. Results and discussion

2.1. Synthesis

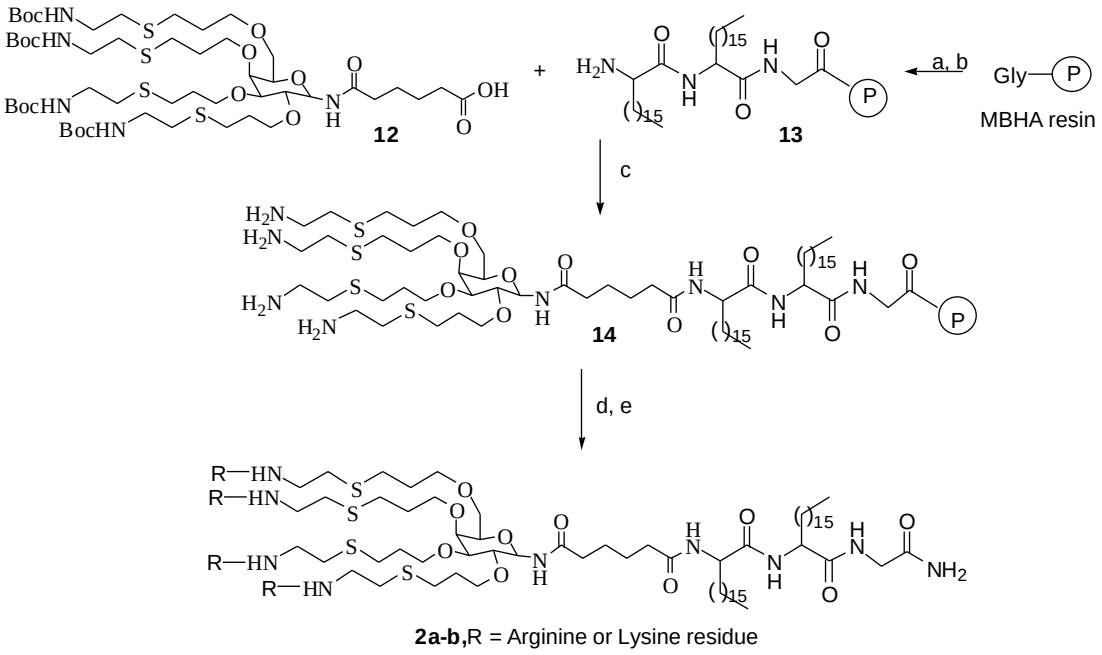
The carbohydrate cores of **2a** and **2b** were synthesized, as shown in Scheme 1. The β-galactosyl azide **7** was synthesized from β-D-galactose pentaacetate following a literature procedure.[49] The tetraallyl derivative **8** was obtained by reaction of **7** with allyl bromide/NaH in DMF[50] in 60% yield. Selective reduction of the azido group was achieved in 52% yield using tin (II) chloride in methanol. The corresponding amine **9**[51] was quickly purified and coupled with adipic acid mono benzyl ester using HBTU/DIPEA activation to afford **10** in 62% yield. Irradiation of **10** at 254 nm for 4 h[52] in dried and degassed 1,4-dioxane in the presence of 4 equiv of *tert*-butyl-*N*-(2-mercaptoethyl) carbamate afforded the tetra-Boc-protected derivative **11** in good yield, which was then saponified with aqueous lithium hydroxide solution to give the free acid **12** in 88% yield.

The tetra-Boc-protected acid **12** is a substrate suitable for solid-phase peptide synthesis and the synthesis of the carbohydrate-based polycationic lipophilic-core dendrons **2a** and **2b** is illustrated in Scheme 2. Compound **14** was obtained using solid-phase synthesis by coupling Boc-glycine to MBHA resin using HBTU/HOBt/DIPEA coupling reagents in DMF, followed by removal of the N-terminal Boc group with trifluoroacetic acid (TFA). Two 2-*tert*-butoxycarbonylamino-octadecanoic acid[53] residues were then coupled following the same protocol. The Boc-protected tetraamine **12** was then attached to the solid phase (HBTU/HOBt/DIPEA in DMF) and after removal of the Boc groups with TFA, Boc-Lys(ClZ) (**2a**) or Boc-ArgTsOH (**2b**) residues were coupled to the solid phase. Removal of the Boc groups (TFA) and cleavage of the peptides from the resin using HF methodology (10% of cresol as scavenger at 0 °C for 2 h) afforded the dendrons **2a** and **2b** in 61% and 47% yields, respectively, after purification by preparative RP-HPLC. These two dendrons were characterized by electrospray ionization mass spectrometry (ES-MS).

Dendrimers **3**–**6** were synthesized in 35% to 68% yields using standard solid-phase synthesis techniques[33,54] and purified by preparative RP-HPLC.



Scheme 1. Reagents and conditions: (a) allylbromide, NaH, DMF, 60%; (b) $SnCl_2{\cdot}2H_2O$, MeOH, 52%; (c) HBTU, DIPEA, THF, adipic acid monobenzyl ester, 62%; (d) Boc-Cysteamine, 1,4-dioxane, 254 nm, 4 h, 90%; (e) $LiOH{\cdot}H_2O$ in aqueous MeOH, pH 13, 88%.



Scheme 2. Reagents and conditions: (a) i—2-amino-octadecanoic acid, HBTU, DIPEA, DMF, rt, 30 min; ii—TFA, rt, 2× 1 min; (b) i—2-Amino-octadecanoic acid, HBTU, DIPEA, DMF, rt, 30 min; ii—TFA, rt, 2× 1 min; (c) i—**12**, HBTU, DIPEA, DMF, rt, 30 min; ii—TFA, rt, 2× 1 min; (d) Boc-Lys(ClZ) (**2a**) or Boc-ArgTsOH (**2b**), HBTU, DIPEA, DMF, rt; ii—TFA, rt, 2× 1 min; (e) HF-cleavage.

2.2. Microcalorimetry

Isothermal titration microcalorimetry (ITC)[55,56] was used to determine the optimal ratio of dendron:LMWH. Fig. 2 shows the ΔH versus c plot for the injection of aliquots of a solution of dendron into the microcalorimeter sample cell, which contained a solution of LMWH. The initial portion of the curves (molar ratio < 5:1) indicates

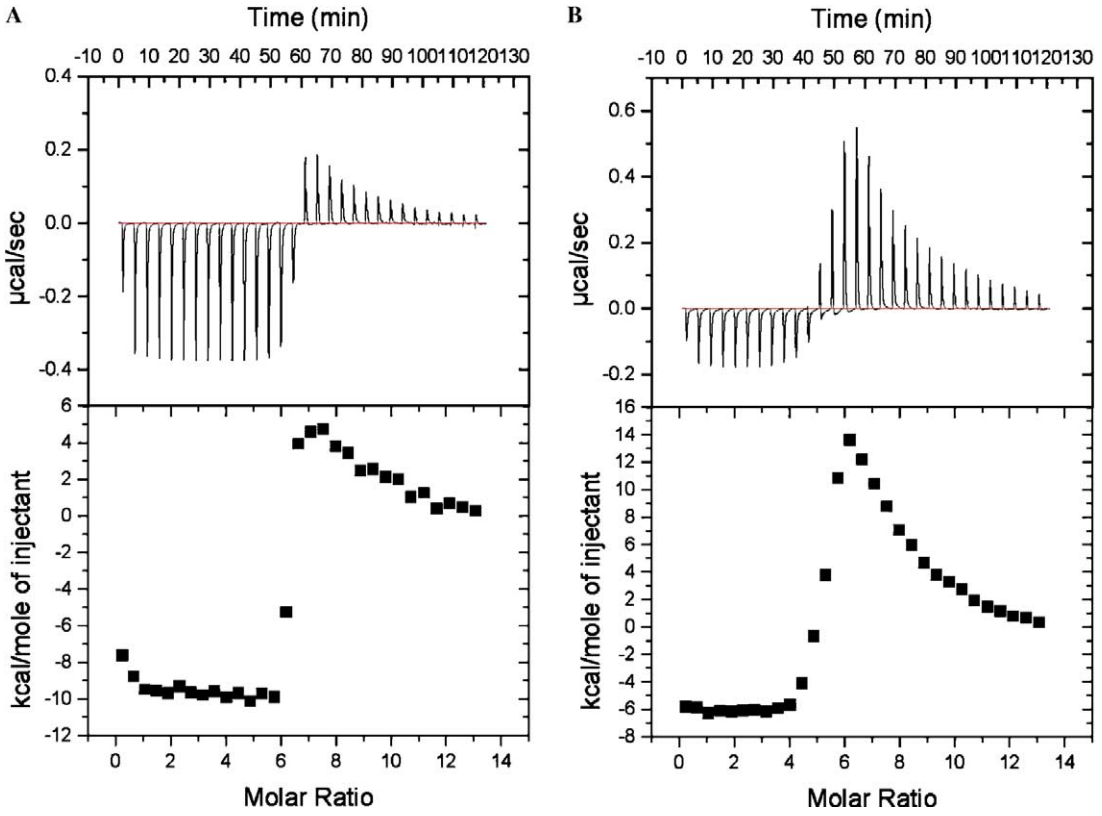


Figure 2. Isothermal titration calorimetry curve for the complex formation between LMWH and (A) **2b**; (B) **4a**.

complex formation between the dendron and LMWH, which is an exothermic process. The complex formation was complete at a molar ratio of ~7:1 (dendron:LMWH), since beyond that point only an endothermic enthalpy of dendron dilution was observed. A similar process was used by Wimmer et al.[33] to determine the optimum ratio of dendron:oligodeoxynucleotide, for the purpose of facilitating in vitro cell transfection.

2.3. Pharmacodynamic study

The ability of the polycationic lipophilic-core dendrons to enhance the oral absorption of LMWH was examined in vivo, using rats. The rat is commonly used in studies of oral LMWH absorption.[24] LMWH (7500 IU/kg) was administered alone or admixed with a penetration enhancer (7 mol equiv). The doses were delivered as suspensions, via oral gavage, using a vehicle of propylene glycol and water (1:1, 1 mL) (Propylene glycol was used as a cosolvent in formulations containing SNAC and SNAD.).[24] The mean plasma anti-factor Xa activities as a function of time are shown in Figures 3 and 4, and the pharmacodynamic parameters are listed in Table 1.

Initial work examined the influence of the branching moiety (either polylysine or a monosaccharide) and the cationic group (either lysine or arginine) on the effectiveness of the penetration enhancers. The coadministration of LMWH and dendrons bearing terminal arginine moieties (**2b** and **4b**) did not improve the plasma anti-factor Xa activity when compared to control (Fig. 3, Table 1). However, the dendrons with terminal lysines (**2a** and **4a**) increased the C_{max} and AUC values, relative to control (Fig. 3, Table 1). Considering the results of Fromm et al.[57] who found that arginine bound more tightly than lysine to heparin, it is possible that once absorbed, the arginine dendrons may not release bound heparin as readily as lysine dendrons, and consequently they are less effective as delivery agents. Of the compounds **2a**–**b** and **4a**–**b**, the largest C_{max} (0.13 ± 0.02 IU/mL) and AUC (19.9 ± 3.0 IU min/mL) occurred when LMWH was coadministered with the peptide dendron **4a**. Therefore, peptide dendrons containing terminal lysines were examined further, as penetration enhancers.

A series of dendrons (**3**, **4a**, **5**, and **6**) which contained a variety of LAAs was synthesized to determine whether the LAA alkyl chain length influenced penetration enhancer effectiveness. Figure 4 illustrates the plasma anti-factor Xa activity following oral administration of LMWH (7500 IU/kg) alone and in combination with the penetration enhancers **3**, **4a**, **5**, or **6** (7 mol equiv). A statistically significant ($P < 0.05$) increase in the C_{max} value was observed when LMWH was administered with the penetration enhancer **3** or **5** (Fig. 4 and Table 1).

The largest AUC value (24.5 ± 4.6 IU min/mL) occurred when LMWH was coadministered with **5** and represented a 2-fold improvement in AUC when compared to the control AUC (12.3 ± 2.5 IU min/mL). The t_{max} values

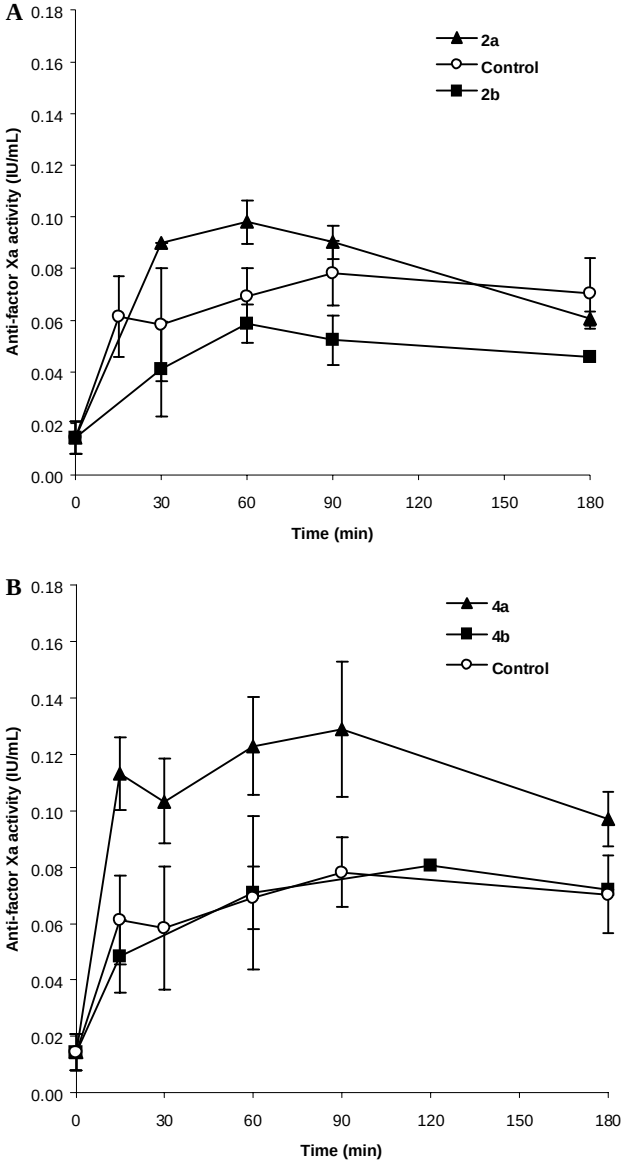


Figure 3. Plasma anti-factor Xa activity following oral administration of LMWH (7500 IU/kg) with or without penetration enhancer [7 mol equiv; (A) **2a** and **2b**; (B) **4a** and **4b**]. Each data point is the means ± SEM of 1–5 rats. Compound **4a** is also illustrated in Figure 4.

ranged from 15 to 120 min, and **5** and **3** (the enhancers with significantly increased C_{max} values) had t_{max} values of 30 and 15 min, respectively. Although variation of the LAA moieties in the lipophilic-core did alter penetration enhancer effectiveness, there was no apparent correlation between the LAA alkyl chain length and penetration enhancer effectiveness (which was measured as an increase in either C_{max} and/or AUC).

The highest C_{max} value of 0.24 ± 0.11 IU/mL (attained using **5**) was a significant ($P < 0.01$) 3.4-times greater than the control C_{max} of 0.07 ± 0.01 IU/mL. Bianchini et al.[58] reported that a plasma anti-factor Xa activity of 0.12 IU/mL was necessary to induce a 50% antithrombotic effect in rats, and concentrations exceeding 0.2 IU/mL always afforded an evident antithrombotic effect. Therefore, a therapeutically relevant anti-factor

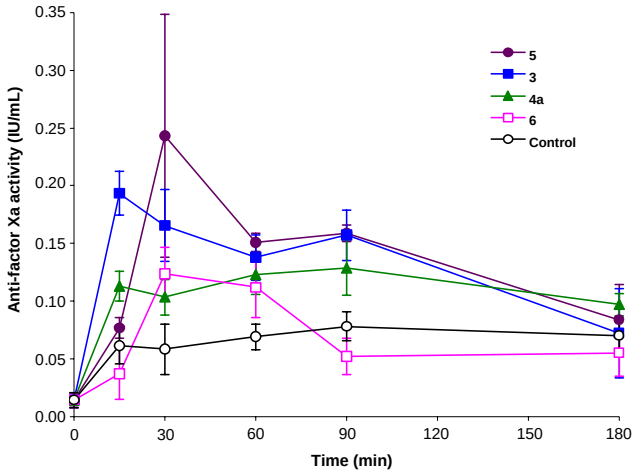


Figure 4. Plasma anti-factor Xa activity following oral administration of LMWH (7500 IU/kg) with or without penetration enhancer (**3**, **4a**, **5** and **6**; 7 mol equiv). Each data point is the means ± SEM of 2–5 rats. Compound **4a** is also illustrated in Figure 3.

Table 1. Pharmacodynamic parameters

Penetration enhancer	C_{max}[a,b] (IU/mL)	t_{max}[c] (min)	AUC_{180}[b,d] (IU min/mL)
Control	0.07 ± 0.01	90	12.3 ± 2.5
2b	0.06 ± 0.01	60	8.40 ± 1.44
4b	0.08 ± 0.00	120	12.3 ± 1.9
6	0.12 ± 0.02	30	12.4 ± 3.6
2a	0.10 ± 0.01	60	14.0 ± 0.9
4a	0.13 ± 0.02	90	19.9 ± 3.0
3	0.19 ± 0.02[e] (2.7)[f]	15	23.6 ± 4.7
5	0.24 ± 0.11[g] (3.4)[f]	30	24.5 ± 4.6[h] (2.0)[i]

[a] Maximum plasma anti-factor Xa activity.
[b] Values are means ± SEM.
[c] Time to reach C_{max}.
[d] Area under the curve.
[e] $P < 0.05$ cf. control.
[f] C_{max}/control C_{max}.
[g] $P < 0.01$ cf. control.
[h] $P < 0.1$ cf. control.
[i] $AUC/AUC_{control}$.

Xa activity was attained by coadministration of LMWH with the penetration enhancer **5**. However, the concentration was only maintained above 0.2 IU/mL for ~20 min, which is a shorter time period than that observed in some studies.[24]

The precise mechanism by which polycationic lipophilic-core dendrons improve LMWH absorption is unknown. The compounds were designed to act as penetration enhancers by forming lipophilic ion-pairs with the polyanionic LMWH. This ion-pair model of absorption assumes that the dendrons are absorbed as a complex with LMWH. Ion-pairing has been used as a means to enhance the absorption of various drugs,[59–61] and di- and triamines were previously used to enhance heparin bioavailability.[43–46] The dendrons examined in this study are amphipathic molecules, which are likely to possess surfactant properties. Similar dendrons were found to adsorb at the air/water interface[62] and to self-associate to form supramolecular aggregates such as vesicles.[62,63] Therefore, the dendrons could act as surfactants and enhance drug absorption by perturbing membrane lipids and proteins.[64]

In vivo studies of LMWH absorption may be classified into two groups: those that examine oral absorption (i.e., administration via oral gavage)[19,24] and those that examine in situ intestinal absorption (e.g., intraduodenal administration).[43,45,65,66] It has been stated[65] that heparin is unstable under acidic conditions such as in the stomach, and this has been used as one reason to reject oral gavage and to examine only in situ intestinal administration.[43] Nevertheless, the current 'gold standard' penetration enhancers for oral heparin delivery are SNAC and SNAD, which were studied in numerous animal models[19,24] and in clinical trials,[20,21] using the oral route of administration. Additionally, the literature indicates that in oral studies the detrimental effects of the oral route (e.g., degradation and dilution) are typically compensated for; by using a heparin dose at least one order of magnitude greater than that used for in situ intestinal studies. Therefore, we chose the oral route of delivery, and comparison to the penetration enhancers SNAC and SNAD is appropriate. Such comparison with the literature reveals that although dendrons can enhance LMWH absorption, they are approximately one order of magnitude less effective than SNAC or SNAD, for oral LMWH delivery.[24]

3. Conclusion

Several polycationic lipophilic-core dendrons **2**–**6** were synthesized and screened as penetration enhancers for the oral administration of LMWH. The results obtained show that the dendrons with terminal lysine were more effective than the dendrons with terminal arginine residues. Dendrons **3** and **5** were the most effective for the oral absorption of LMHW in rats. An important factor limiting the absorption of the dendron–LMWH complex may be poor aqueous solubility, which meant that the compounds in this study were administered as suspensions. Continuing efforts in this research are aimed at solubilizing the dendron/LMWH complex.

4. Experimental

4.1. Synthesis

Mass spectra were run on a Perkin-Elmer API 3000 LC/MS/MS electrospray instrument or with a VG Analytical ZAB-SE instrument using fast atom bombardment (FAB) techniques (20 kV Cs+ ion bombardment). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 500 instrument. Preparative separations were carried out by column chromatography using Merk silica gel (230–400 mesh). Analytical RP-HPLC were performed on a LC-10AT Shimadzu liquid chromatograph (SCL-10A system controller, SPD-6A UV detector, and a SIL-6B auto injector with a SCL-6B system controller) and C4 or C18 columns (Vydac, 25 cm, with 5 μm pore

size and 4.6 mm internal diameter), using an acetonitrile/water gradient. Preparative RP-HPLC were performed using a Waters HPLC system (Model 600 controller, 490E UV detector, F pump, and TSK-GEL C4 column, 25 cm, with 10 μm pore size and 22 mm internal diameter) using an acetonitrile/water gradient.

4.1.1. 2,3,4,6-Tetra-*O*-(2-propenyl)-β-D-galactopyranosyl azide (8). Sodium hydride (60% in dispersion oil, 7.76 g, 194 mmol) was slowly added to a solution of β-D-galactopyranosyl azide **7** (7.91 g, 38.6 mmol) at 0 °C in dry DMF (250 mL). After 45 min, allyl bromide (19.8 mL, 234 mmol) was added dropwise and the temperature was raised to 50 °C. After 4 h, the reaction was cooled to room temperature and the excess NaH was quenched by addition of methanol (80 mL). The solvent was then removed under vacuum and the residue was then dissolved in DCM (300 mL) and washed with water (3× 150 mL). After drying over $MgSO_4$, the organic layer was filtered and then concentrated in vacuo. The residue was then purified by flash chromatography [Silica, Ethyl acetate–hexane gradient (2–20%)] to yield the tetra allyl ether (8.52 g, 60%) as a colorless oil. ES-MS (*m*/*z*): 366 (MH^+), 383 $(M+NH_4)^+$, 388 $(M+Na)^+$. ^1H NMR (500 MHz, $CDCl_3$): 3.34 (dd, *J* 3.0, 7.7, 1H), 3.51 (dd, *J* 9.5, 1H), 3.58–3.64 (m, 3H), 3.80 (d, *J* 2.7, 1H), 3.99–4.28 (m, 8H), 4.50 (d, *J* 8.5, 1H), 5.13–5.33 (m, 8H), 5.91 (m, 4H). ^{13}C NMR (125 MHz, $CDCl_3$): 68.2, 71.6, 72.5, 73.0, 73.9, 74.2, 75.4, 78.3, 82.0, 90.4, 116.8, 116.9, 117.1, 134.3, 134.7, 134.8, 135.2.

4.1.2. 2,3,4,6-Tetra-*O*-(2-propenyl)-β-D-galactopyranosyl amine (9). A solution of $SnCl_2{\cdot}2H_20$ (14.91 g, 66 mmol) in MeOH (100 mL) with a few drops of deionized water was added to a flask containing azide **8** (2.42 g, 6.6 mmol) and the mixture was stirred overnight at room temperature under an inert atmosphere. The solvent was then removed under vacuum and the residue was diluted with ethyl acetate (200 mL) and basified by addition of saturated $NaHCO_3$ solution (2× 200 mL). The combined layers were filtered to remove the solid precipitate. The organic phase was isolated and dried over $MgSO_4$ and evaporated. The crude product was then purified by flash chromatography (50% ethyl acetate in hexane in the presence of 1% of triethylamine) to yield the desired amine **9** as a colorless oil (2.34 g, 52%). ES-MS (*m*/*z*): 340 (MH^+), 357 $(M+NH_4)^+$, 362 $(M+Na)^+$. ^1H NMR (500 MHz, $CDCl_3$): 3.34 (dd, *J* 3.0, 7.7, 1H), 3.53 (dd, *J* 9.5, 1H), 3.58–3.64 (m, 3H), 3.80 (d, *J* 2.7, 1H), 3.99–4.28 (m, 8H), 4.26 (dt, *J* 1.28.5, 1H), 5.13–5.33 (m, 8H), 5.91 (m, 4H), 5.91 (m, 4H). ^{13}C NMR (125 MHz, $CDCl_3$): 69.1, 71.8, 72.6, 74.0, 74.3, 74.4, 80.5, 83.0, 86.6, 116.7, 117.0, 117.3, 117.7, 134.6, 135.2, 134.5, 135.7.

4.1.3. 6-(2,3,4,6-Tetra-*O*-(2-propenyl)-β-D-galactopyranosylamino)-6-oxohexanoic acid benzyl ester (10). Adipic acid monobenzyl ester (1.88 g, 8.07 mmol), HBTU (3.04 g, 8.07 mmol), and DIPEA (1.38 mL, 8.07 mmol) in dry THF (50 mL) were added to a stirred solution of **9** (2.25 g, 6.61 mmol) in THF (150 mL). The reaction mixture was then stirred overnight under an inert atmosphere. The solvent was removed under vacuum and the residue was dissolved in ethyl acetate (200 mL). The organic phase was washed with 5% HCl solution (2× 150 mL), saturated $NaHCO_3$ solution (2× 150 mL), and brine (100 mL). After drying over $MgSO_4$, the solvent was removed under vacuum and the residue was purified by flash chromatography (Silica, hexane/ethyl acetate 3:2) to give **10** as a colorless oil (2.3 g, 62%). ES-MS (*m*/*z*): 558 (MH^+), 575 $(M+NH_4)^+$, 580 $(M+Na)^+$. ^1H NMR (500 MHz, $CDCl_3$): 1.63–1.70 (m, 4H), 2.24–2.30 (m, 2H), 2.36 (t, *J* 6.9, 2H), 3.43 (dd, J 2.8, 9.4, 1H), 3.46–3.53 (m, 4H), 3.58–3.64 (m, 1H), 3.80 (br s, 1H), 3.93–4.33 (m, 8H), 5.05 (t, *J* 9.1, 1H), 5.08 (s, 2H), 5.13–5.33 (m, 8H), 5.78–5.97 (m, 4H), 6.06 (d, *J* 9.2, 1H), 7.28–7.36 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$): 24.3, 24.6, 33.8, 36.2, 66.1, 67.7, 71.3, 72.3, 73.1, 73.7, 73.9, 74.5, 79.0, 79.1, 82.7, 116.7, 116.8, 117.0, 117.3, 128.1, 128.5, 134.3, 134.7, 135.0, 135.3, 136.0, 172.4, 173.1.

4.1.4. 6-[(2,3,4,6-Tetra-*O*-(2-propenyl)-β-D-galactopyranosylamino]-6-oxohexanoic acid benzyl ester (11). A mixture of **10** (200 mg, 0.36 mmol) and *tert*-butyl-*N*-(2-mercaptoethyl) carbamate (Boc-cysteamine) (1.02 g, 5.5 mmol, 16 equiv) in dried and degassed 1,4-dioxane (3 mL) was irradiated at 254 nm for 4 h. The solvent was then removed under vacuum and the residue was purified by flash chromatography (ethyl acetate/hexane 3/2, 5% Et_3N) to afford 400 mg (88%) of **11** as a colorless oil. ES-MS (*m*/*z*): 1266 (MH^+), 1283 $(M+NH_4)^+$, 1288 $(M+Na)^+$. ^1H NMR (500 MHz, $CDCl_3$) 1.44 (s, 36H), 1.62–1.69 (m, 4H), 1.74–1.83 (m, 8H), 2.20–2.26 (m, 2H), 2.32–2.35 (m, 2H), 2.53–2.63 (m, 8H), 2.70–2.76 (m, 8H), 3.19–3.28 (m, 8H), 3.35–3.41 (m, 8H), 3.42–3.90 (m, 6H), 5.01 (t, 5H), 5.10 (s, 2H), 6.03 (br s, 1H), 7.26–7.32 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$): 23.1, 23.9, 24.5, 24.6, 24.8, 28.3, 28.4, 28.5, 28.6, 29.1, 29.8, 30.3, 30.5, 31.0, 31.2, 31.9, 32.2, 32.3, 32.4, 34.1, 36.3, 38.9, 40.0, 40.1, 40.2, 66.3, 68.3, 68.8, 69.0, 69.7, 71.1, 71.5, 72.5, 74.3, 74.5, 77.4, 78.7, 79.3, 79.5, 79.6, 83.7, 128.3, 128.4, 128.7, 128.9, 136.2, 155.8, 155.9, 156.0, 156.1, 173.0, 173.3. HRMS (TOF): Calculated for $C_{59}H_{104}N_5O_{16}S_4$ $(M+H)^+$: 1266.6361, Found: 1266.6328.

4.1.5. 6-[(2,3,4,6-Tetra-*O*-(2-propenyl)-β-D-galactopyranosylamino]-6-oxohexanoic acid (12). A solution of **11** (380 mg, 0.3 mmol) in H_2O/methanol mixture (20/80) in which the pH had been adjusted to 13 by addition of solid $LiOH.H_2O$ was stirred at room temperature overnight. The solvent was then removed under vacuum and the residue was dissolved in ethyl acetate (150 mL). The organic layer was washed with cold 0.01 M citric acid solution (2× 100 mL). After drying over $MgSO_4$, the solvent was removed and the residue was washed via a plug of silica gel using 10% MeOH in $CHCl_3$ as eluent to give the pure acid **12** (312 mg, 88%) as a colorless oil. ES-MS (*m*/*z*): 1176 (MH^+), 1193 $(M+NH_4)^+$, 1198 $(M+Na)^+$. ^1H NMR (500 MHz, $CDCl_3$) 1.44 (s, 36H), 1.62–1.69 (m, 4H), 1.74–1.83 (m, 8H), 2.20–2.26 (m, 2H), 2.32–2.35 (m, 2H), 2.53–2.63 (m, 8H), 2.70–2.76 (m, 8H), 3.19–3.28 (m, 8H), 3.35–3.41 (m, 8H), 3.42–3.90 (m, 6H), 5.01 (t, 5H), 6.03 (br s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): 23.1, 23.9, 24.5, 24.6, 24.8,

28.3, 28.4, 28.5, 28.6, 29.1, 29.8, 30.3, 30.5, 31.0, 31.2, 31.9, 32.2, 32.3, 32.4, 34.1, 36.3, 38.9, 40.0, 40.1, 40.2, 66.3, 68.3, 68.8, 69.0, 69.7, 71.1, 71.5, 72.5, 74.3, 74.5, 77.4, 78.7, 79.3, 79.5, 79.6, 83.7, 155.8, 155.9, 156.0, 156.1, 173.0, 173.3. HRMS (TOF): Calculated for $C_{52}H_{98}N_5O_{16}S_4$ $(MH)^+$: 1176.5891, Found: 1176.5861.

4.1.6. Dendron 2a. MBHA resin (926 mg, 0.25 mmol)) was swollen in DMF in a solid-phase peptide synthesis reaction vessel for 90 min. This resin was washed with DIPEA (500 μL) in DMF by shaking the resin 2× 1 min. The resin was thoroughly washed with DMF, and then the resin was shaken for 15 min with Boc-glycine (350.2 mg, 2 mmol), HBTU (4 mL of a 0.5 M solution in DMF), and DIPEA (460 μL), resulting in a negative ninhydrin test. After washing well the resin with DMF, the Boc group was removed by treatment with neat TFA (2× 1 min), and then the resin was again washed well with DMF. 2-*tert*-Butoxycarbonylamino-octadecanoic acid was coupled twice (239 mg, 0.6 mmol each time) on the resin following the same protocol. The sugar derivative **12** (705 mg, 0.6 mmol) was coupled on the resin following the same protocol (shaking time 2 h and 30 min). Finally, Boc-Lys(ClZ) (831.4 mg, 2 mmol, twice) was coupled on the resin following the same protocol with 1 h shaking time, resulting in a negative ninhydrin test.

The four Boc groups were removed by treatment with neat TFA (2× 1 min), and the resin was washed well with DMF, then MeOH, and finally DCM, and then dried under vacuum to give 1.128 g of the loaded resin. The peptide was then cleaved from the resin using anhydrous hydrofluoric acid using 10% cresol as scavenger at 0 °C for 1 h. The product was precipitated with diethyl ether and then lyophilized from water/acetonitrile to give the peptide as a white powder. The crude peptide was then purified by preparative HPLC using an acetonitrile/water gradient. The purified peptide was then lyophilized from water/acetonitrile to give the pure glycodendrimer as a white powder (244 mg, 17%). ES-MS: 1907 (M+H), 954, 637, 478, 382.

4.1.7. Dendron 2b. This dendron (1 mmol) was synthesized, cleaved, and purified, following the same protocol as that for dendron **2a**. The purified peptide was then lyophilized from water/acetonitrile to give the pure glycodendrimer as a white powder (275 mg, 27.5%). ES-MS: 2020 (M+2H), 1010, 674, 506, 405.

4.1.8. General procedure for the synthesis of dendrons 3–6. MBHA resin (1.85 g, 1 mmol)) was swollen in DMF in a solid-phase peptide synthesis reaction vessel for 90 min. This resin was washed with DIPEA (500 μL) in DMF by shaking the resin 2× 1 min. The resin was thoroughly washed with DMF, and then the resin was shaken for 15 min with Boc-Glycine (700 mg, 4 mmol), HBTU (8 mL of a 0.5 M solution in DMF), and DIPEA (920 μL), resulting in a negative ninhydrin test. After washing the resin with DMF well, the Boc group was removed by treatment with neat TFA (2× 1 min), and then the resin was again washed well with DMF. The appropriate LAA, C_{10} to C_{18} was coupled twice on the resin (2 mmol each time) using the same protocol followed, by TFA deprotection, and DMF flow-wash. Boc_2Lys (692 mg, 2 mmol) was coupled on the resin following the same protocol (shaking time: 30 min, TFA deprotection and DMF flow-wash). This step was repeated for the coupling of the second lysine residue (using 4 mmol of Boc_2Lys). Finally, Boc-Lys(ClZ) (8 mmol) or Boc-ArgTsOH (8 mmol, for **4b**) was coupled on the resin following the same protocol with 1 h shaking time resulting in a negative ninhydrin test. The 4 Boc groups were removed by treatment with neat TFA (2× 1 min), and the resin was washed well with DMF, then MeOH, and finally DCM, and then dried under vacuum to give the loaded resin. The peptides (1 mmol) were then cleaved from the resin using anhydrous hydrofluoric acid using 10% cresol as scavenger at 0 °C for 1 h. The products were precipitated with diethyl ether and then lyophilized from water/acetonitrile to give the peptides as a white powder. Purification by preparative HPLC (using an acetonitrile/water gradient) afforded the purified peptides after lyophilization as white powder. **3** (64%). ES-MS: 1535 (M+H), 768, 512, 254. Compound **4a**: (35%). ES-MS: 1422 (M+H), 711, 285. Compound **4b** (40% yield). ES-MS: 1534 (M+H), 767, 512. **5** (55%). ES-MS: 1366 (M+H), 683,456, 194, 170. Compound **6** (68%). ES-MS: 1310 (M+H), 655, 437.

4.2. Microcalorimetry

Isothermal titration microcalorimetry (ITC) was performed using a MicroCal VP-ITC titration calorimeter (MicroCal, Northampton, MA). A solution of LMWH (1.70 μm) was placed in the sample cell (1.4395 mL) and a solution of dendron **2b** (62.5 μm) was placed in the syringe. The cell temperature was maintained at 30 °C and dendron **2b** was added to the solution of LMWH using 1× 5 μL and then 29× 8 μL injections, each injection being 4 min apart. The experiment was performed in triplicate and the software Origin (version 5.0, Microcal) was used for data analysis.

4.3. Pharmacodynamic study

LMWH (sodium salt, from porcine intestinal mucosa, produced by oxidative depolymerization, avg. MW = 4125 Da, anti-factor Xa activity = 105 IU/mg) was purchased from MP Biomedicals (Seven Hills, Australia; catalog #194113). Male Sprague–Dawley rats (200–300 g, Herston Medical Research Centre, Brisbane, Australia) were fasted for 12 h prior to dosing. Each rat was dosed by oral gavage, using a blunt-tipped feeding needle, while the animal was under light anesthesia (O_2/CO_2, 1:1). The doses consisted of LMWH (7500 IU/kg) alone or in combination with a penetration enhancer (7 mol equiv). The delivery vehicle was propylene glycol and water (1:1, 1 mL), and LMWH alone was a clear solution, whereas doses containing a penetration enhancer were suspensions. Two blood samples were collected in series from each rat using cardiac puncture, while the animal was anesthetized (O_2/CO_2, 1:1). After the second blood collection, the anaesthetized animal was promptly euthanized by cervical dislocation. Blood samples (450 μL) were collected in tubes containing

3.8% sodium citrate solution (50 μL). The blood samples were centrifuged (2000*g*, 15 min, 20 °C) within 1 h of collection, and the plasma was harvested and stored frozen (−70 °C) until analysis. Plasma samples were analyzed for anti-factor Xa activity using a chromogenic assay (Coatest® LMW Heparin/Heparin, Chromogenix Instrumentation Laboratory S.p.A., Milan, Italy). All animal experiments were approved by The University of Queensland Animal Ethics Committee.

4.4. Analysis of pharmacodynamic data

The maximum plasma anti-factor Xa activity (C_{max}) and time to reach C_{max} (t_{max}) are observed values taken directly from analytical data. The area under the plasma anti-factor Xa activity versus time curve (AUC_{180}) was estimated using the linear trapezoidal rule for the time period 0–180 min. The means of the C_{max} data were compared by one-way analysis of variance (ANOVA) combined with the Tukey multiple comparison procedure. *AUC* for the treatment group that received dendron **5** was compared to control *AUC* using a two-tailed unpaired *t*-test with unequal variance (Welch's approximate *t*).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.08.004.

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