Heparin derivatives for the targeting of multiple activities in the inflammatory response

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Abstract

An attractive strategy for ameliorating symptoms arising from the multi-faceted processes of excessive and/or continual inflammation would be to identify compounds able to interfere with multiple effectors of inflammation. The well-tolerated pharmaceutical, heparin, is capable of acting through several proteins in the inflammatory cascade, but its use is prevented by strong anticoagulant activity. Derivatives of heparin involving the periodate cleavage of 2,3 vicinal diols in non-sulfated uronate residues (glycol-split) and replacement of *N*-sulphamido- with *N*-acetamido- groups in glucosamine residues, capable of inhibiting neutrophil elastase activity *in vitro*, while exhibiting attenuated anticoagulant properties, have been identified and characterised. These also interact with two other important modulators of the inflammatory response, IL-8 and TNF-alpha. It is therefore feasible in principle to modulate several activities, while minimising anticoagulant side effects, providing a platform from which improved anti-inflammatory agents might be developed.

Keywords:
Neutrophil elastase IL-8 TNF-alpha Inflammatory network Chemically modified heparin Glycol-split

1. Introduction

Inflammation is the result of the biological response to injury or harmful stimuli, e.g. irritants, pathogens or damaged cells, in an attempt by the organism to defend and heal itself. The inflammatory response involves a complex network of cellular changes, cytokine release and cellular infiltration. Neutrophils play an important role in host defence against fungal and bacterial infections. They are primed, activated and engaged in bacterial phagocytosis releasing large amounts of oxidants and intracellular stored proteases, which include neutrophil elastase (NE) and matrix metalloprotease-9 (MMP-9) (Downey, Bell, & Elborn, 2009). Recently, recognition of NE as a promising therapeutic target in chronic inflammatory diseases has increased (Mitsuhashi et al., 1999; Yoshimura et al., 2003). The imbalance between NE and its inhibitors is implicated in many inflammatory diseases, including rheumatoid arthritis, respiratory distress syndrome, pulmonary emphysema and acute lung injury. Their importance in inflammation can be seen, for example, in cystic fibrosis (CF) patients, who suffer continual bacterial infection. Interleukin-8 (IL-8) is responsible for neutrophil recruitment to sites of infection, contributes to neutrophil transendothelial migration into CF airways and its expression is prolonged following bacterial, e.g. Pseudomonas aeruginosa, stimulation (Joseph, Look, & Ferkol, 2005). This results in excessive cellular recruitment, further triggering the release of pro-inflammatory mediators and chemoattractants (Cosgrove, Chotirmall, Greene, & McElvaney, 2011; Wilmott, Frenzke, Kociela, & Peng, 1994). Among the released proteases, NE has the most potential to cause undesired tissue injury (Suzuki et al., 1996) by escaping from cells and degrading structural proteins, such as elastin and fibronectin, while interfering with the innate airway immunity by impairing opsonophagocytosis (Tosi, Zakem, & Berger, 1990).

Another cytokine, TNF-alpha (TNFα), lying at a node in networks of interactions in the inflammatory pathways of many diseases, is involved in the promotion of macrophage transformation to T-helper cells and neutrophil activation. One possible route to the suppression of excessive inflammation could be the targeting of multiple activities, for example, the inhibition of NE activity and the sequestering of interleukins of central importance to neutrophil activation, such as IL-8 and TNFα.

Heparin, the polydisperse, heterogeneous, linear sulfated polysaccharide, which is used widely in the clinic as an antithrombotic agent and is generally well tolerated, interacts with and inhibits the activity of many regulatory proteins (Ori, Wilkinson, & Fernig, 2008; Ori, Wilkinson, & Fernig, 2011) including IL-8 and elastase. Furthermore, heparin has been shown capable of modulating growth factor receptor binding and activity (Jayson & Gallagher, 1997), inhibiting the enzyme heparanase (Brown, Lever, Jones, & Page, 2003; Lever & Page, 2002; Vlodavsky et al., 1994) and reducing selectin-mediated interactions (Borsig et al., 2001; Varki & Varki, 2002). It has been suggested that heparin may have the potential to relieve symptoms in lung conditions, ranging from the excess of NE in CF airways to asthma (Diamant et al., 1996) or even respiratory distress syndrome. Nevertheless, although several studies reported that heparin did not cause bleeding (Yip, Lim, & Chan, 2011) the effective dose of heparin (or low molecular weight heparin) required to achieve prolonged anti-inflammatory effects could result in anticoagulant complications.

A solution to the problem of anticoagulant side-effects may be offered by chemically modified derivatives of heparin, which retain the desired activities, but exhibit attenuated anticoagulant activity (Lever & Page, 2002). Many such derivatives have been studied and characterised (Mulloy, Forster, Jones, & Davies, 1993; Rudd et al., 2009; Yates et al., 1996) and some of their activities, such as angiogenesis inhibition (Casu et al., 2004), antimetastatic activity and antagonism of P-selectins (Hostettler et al., 2007), have been reported. Heparin and its derivatives serve as proxies for the naturally occurring GAG, heparan sulfate (HS), known to interact with hundreds of proteins, many of them involved in regulation of the extracellular matrix (Ori et al., 2008, 2011). The chemical modification of

heparin, especially de-*O*- and de-*N*-sulfation, which usually results in a net reduction of the overall charge density and also tend to reduce structural complexity, provides the means by which biochemical processes can be influenced, while attenuating undesired anticoagulant activities.

Since NE (Fryer et al., 1997; Redini et al., 1988; Walsh, Dillon, Scicchitano, & McLennan, 1991) and IL-8 (Goger et al., 2002) have been identified as relevant to chronic inflammatory conditions and interact with HS, the ability of a series of chemically modified heparin derivatives to exhibit a combination of favourable activities in vitro was explored. The aim was to generate a series of compounds able to inhibit NE and to bind to key modulators of the inflammatory network, IL-8 and TNF-, while exhibiting severely attenuated potential side-effects, particularly anticoagulant activity (AT/factor Xa, PT and APTT).

2. Methods

2.1. Preparation and characterisation of heparin derivatives

The N-acetylated heparin and glycol-split derivatives were prepared as described previously (Casu et al., 2004; Naggi et al., 2005) starting from unmodified pig mucosal heparin (PMH compound 1, Bioiberica S.A., Spain) and characterised by ^{13}C NMR (see Supplementary data). The weight average molecular weights (M_w) were determined in sodium nitrate at a concentration of 5 mg/mL and at 313K employing Viscotek HP-SEC–TDA (Table 1) equipped with a SEC column coupled with three detectors, light scattering, refractometer and viscosimeter (Bertini, Bisio, Torri, Bensi, & Terbojevich, 2005).

Table 1 Structural characteristics of the compounds originating from heparin (series A; 1–1c) and glycol-split heparin (series B; 2–2d) derivatives. The table contains the materials weight average molecular weight (M_w), percentage of N-acetyl substitution in glucosamine residues and percentage of glycol-split uronate residues (cleavage by periodate oxidation of vicinal diols in unsubstituted d-GlcA and l-IdoA residues). Compound 1 is unmodified PMH, used as reference for series A, while compound 2 is glycol-split heparin, used as reference for series B.

Series	Compound	M_w (kDa)	% *N*-acetyl	% Glycol-split
A	1 (PMH)	20.0	15	0
	1a	21.0	45	0
	1b	22.0	64	0
	1c	17.0	100	0
B	2	16.5	15	20
	2a	17.0	27	27
	2b	13.0	49	35
	2c	15.0	64	25
	2d	16.0	100	25

2.2. Anticoagulant assay

The anticoagulant activity of the derivatives was assayed using the COATEST® heparin (chromogenix) following the manufacturer's instructions. Briefly, heparin reacts with antithrombin and an excess of factor Xa was added leading to the formation of a ternary complex. Free factor Xa cleaves a chromogenic substrate and the absorbance is read at 405 nm. Several concentrations of standard heparin were tested and a standard curve was obtained from 0 to 0.35 µg/mL. Then, the heparin derivatives (0.25 µg/mL) were tested and compared to the same concentration of standard heparin. The test was performed twice in

duplicate in a 96-well plate and the colour read photometrically (VersaMaxmicroplate reader, Molecular Devices, USA).

The PT assay was performed as per the manufacturer's instructions with some minor modifications. Pooled (normal) human plasma was obtained from Technoclone Ltd (UK). Briefly, the test sample was incubated with plasma prior to the addition of Thromborel S (Siemens, at 2X concentration). The time taken for clot formation was monitored using a thrombotic coagulometer (Stage Diagnostics) and recorded if the clot formation occurred before 120 s.

APTT assays were performed essentially according to the manufacturer's instructions. Briefly, human plasma test sample and Pathromtin SL (Siemens) were incubated for 2 min at 37 ◦C, 50 mM CaCl2 was then added to initiate coagulation. The time taken for clot formation was observed as per PT assay.

2.3. Competitive ELISA to measure IL-8 displacement from heparin

The test performed was a modified version of the classic competitive ELISA. Streptavidin (30 g/mL, Sigma Aldrich, USA) was used to coat a Maxibinding 96-well plate (SPL Lifesciences) overnight (4 ◦C). The plate was then incubated with heparin–biotin (0.1 mg/mL, Sigma Aldrich, USA) at room temperature, followed by blocking (2% BSA in DPBS overnight at 4 ◦C). The next step was the binding of 1.5 µg/mL IL-8 (Millipore, USA) to the plate, in the absence and presence of a heparin derivative ranging from 0 to 10 µM and subsequent incubation with a rabbit anti-human IL8 primary (Millipore, Bedford, MA) (1:500, 50 L, 1 h) and a goat anti-rabbit-HRP-conjugated secondary (Millipore, Bedford, MA) (1:1000, 50 µL, 1 h) antibody. All incubations were followed by triplicate washes in DPBS with the addition of 0.05% Tween20. Finally, 0.4 mg/mL o-phenylenediamine (Sigma Aldrich, USA) in 50 mM citrate/dibasic sodium phosphate and 0.0004% hydrogen peroxide (Sigma Aldrich, USA) was added. The enzymatic reaction was terminated by addition of 1 M H_2SO_4. Absorbance measurements were made at 492 nm (VersaMaxmicroplate reader, Molecular Devices, USA). A calibration curve of IL-8 from 0 to 1.5 µg/mL was set up for each experiment.

2.4. Elastase inhibition assays

The inhibition test of human leukocyte elastase (EPC, Owensville, USA) was based on the release of ρ-nitroaniline from the chromogenic substrate MeO–Suc–Ala–Ala–Val–pNO2-anilide (EPC, Owensville, USA). The reaction was conducted at 37 ◦C in 96- well plates and monitored by reading at 405 nm continuously for 25 min (VersaMaxmicroplate reader, Molecular Devices, USA). The substrate (240 µM final; 2.1 mM stock in phosphate buffer 50 mM, pH 7.4, 15 mM DMSO, 0.13 M NaCl) was incubated for 10 min in the presence or absence of increasing concentrations of derivatives before adding the enzyme (60 nM; 800 nM stock in sodium acetate 70 mM, pH 4.5). As control, the enzyme was inactivated with sulphuric acid (1 M, to a final concentration of 0.5 M) prior to the addition to the reaction mix.

The ability of the derivatives to inhibit α-elastin digestion by human sputum elastase (EPC, Owensville, USA) was tested using a FastinTM Elastin kit (Biocolor, Carrickfergus, N. Ireland), employing an adaptation of the method recommended by the manufacturer. The modified procedure involved the digestion of a solution of α-elastin (20 µL, 1 mg/mL) in 50 mM phosphate buffer pH 7.4 by elastase (100 µL final volume, 0.54 µM, 37 ◦C, 1 h) in the presence or absence of 0.54 M derivatives (1:1 molar ratio to elastase), inactivation with oxalic acid (1 M, to a final concentration of 0.25 M) and staining with the FastinTM kit, according to the manufacturer's instruction. Briefly, α-elastin was precipitated using 50 µL of Precipitating Reagent and the tubes were then centrifuged (10,000 × g, 10 min). After the removal of the liquid, 500 µL of dye were added and the tubes were mixed (90 min, room

temperature). After centrifugation and careful removal of the liquid, 250 µL of the Dye Dissociation Reagent were added. After mixing, the contents of the tubes were transferred to a 96-well plate and read at 513 nm (VersaMaxmicroplate reader, Molecular devices, USA). A calibration curve of α-elastin from 0 to 50 µg was obtained to verify the efficacy of the assay. As control, the enzyme was inactivated with oxalic acid (1 M, to a final concentration of 0.25 M) prior to the addition of the substrate.

2.5. Nuclear magnetic resonance

The ^{13}C NMR spectra of the heparin chemical derivatives (100 mg/mL) were recorded in D_2O at 313K on Bruker AC300 and AMX400 spectrometers. The data were processed using Topspin 2.0 software.

2.6. Circular dichroism

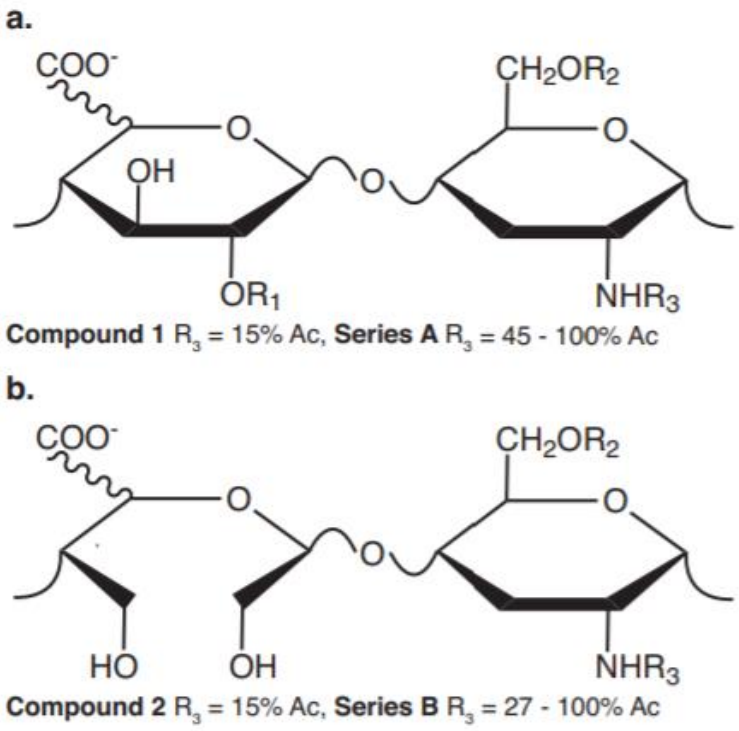
The synchrotron radiation circular dichroism (SRCD) spectra were recorded (180 to 260 nm) on a purpose-built CD beam line (B23 at Diamond Light Source, Didcot, Oxfordshire, UK) using a CaF_2 sample cell with 0.02 cm path length, employing 1 nm resolution. The slit widths for all spectra were 0.5 mm, 1 s integration time was used and the storage ring current was 250 mA for the duration of the experiments. SRCD spectra of the derivatives alone were also recorded (Supplementary data). Spectra of IL-8 were recorded at a concentration of 0.5 mg/mL in 17.5 mM phosphate buffer, the concentration of the derivatives were at equal weight ratios for all samples. TNF-α spectra were recorded at 10 mg/mL, with 0.1 mg/mL derivatives, in 17.5 mM phosphate buffer.

3. Results

3.1. Preparation and characterisation of heparin derivatives

A number of heparin derivatives were generated, based on a porcine mucosal heparin (PMH, compound 1) scaffold as described previously (Casu et al., 2004; Naggi et al., 2005). Heparin derivatives have been shown previously to exhibit strongly reduced anticoagulation activities, while maintaining the ability to interact with other proteins (Guimond, Turnbull, & Yates, 2006). Two series of compounds were produced for this study and compared; series A (1a-1c) and B (2 and 2a-2d). Both sets of compounds contained varying amounts of *N*-acetylation. In series B, the non-sulfated uronate residues containing 2,3 vic-diols underwent periodate oxidation, forming a glycol-split uronic acid residue (Casu et al., 2004). Furthermore, prior to N-acetylation of compound 2b the sample was partially de-2-*O*-sulfated, thereby providing a higher percentage of glycol-split uronic acids in this derivative. Scheme 1 contains the major repeating disaccharide unit of heparin and the structure of the glycol-split uronic acid.

The compounds in series A and B are listed in Table 1. It should be noted that, as the degree of *N*-acetylation increases, the overall level of sulfation decreases, as expected. The degree of *N*-acetylation and periodate oxidation in the heparin compounds was quantified by ^{13}C NMR (see Supplementary data), while SEC–TDA was used to determine the weight average molecular weight of the compounds (Bertini et al., 2005). Size can be an influential parameter on the binding of polysaccharides to proteins, especially the minimum length that is required to establish an interaction. The molecular weight of the test compounds ranged from 13 to 22 kDa, assuring an interaction with both IL-8 and human leukocyte elastase (HLE).



Scheme 1 (A) The repeating disaccharide unit of the compounds comprising series A and B, based on R_1 and R_2 = H/SO_3^-, R_3 = SO_3^-/$COCH_3$. The uronic acid in these compounds (derived from PIMH) is predominantly in the form l-iduronic acid (lIdoA and l-IdoA-2-*O*-sulfate; ~ 80%) with d-glucuronic acid (d-GlcA; ~20%) making up the remainder. (B) Series B contains the glycol-split uronic acid residue (see Table 1 for full details of series A and B compounds).

3.2. Neutrophil elastase inhibition

Neutrophil elastase is a key enzyme involved in the excessive inflammation response, for example, in the lungs of CF patients. The ability of heparin derivatives to inhibit elastase was measured using two assays. The first involved measuring the release of - nitroaniline from a low molecular weight chromogenic substrate following the cleavage by human leukocyte elastase. The ability of the heparin derivatives to inhibit this enzyme is shown for 1a–1c in Fig. 1A and for 2a–2d in Fig. 1B.

The second involved measurement of the inhibition of digestion of α-elastin by human leukocyte elastase by 1a–1c, shown in Fig. 1C and for 2a–2d shown in Fig. 1D. All heparin derivatives proved able to inhibit digestion of the synthetic peptide, similar to standard heparin, 1, at concentrations above 20 nM, while only minor differences were observed at lower concentrations, probably due to different modes of interaction. The glycol-split modification had no effect in either of the assays. The inhibitory effect of 1a–1c increased with the degree of *N*-acetylation using the peptide substrate, but no significant differences were found when α-elastin was used. Elastase inhibition was only partial, however; neither a 3–4 fold increase in the concentration of heparin (up to 200 nM, data not shown) nor any of the derivatives proved able to inhibit the enzyme completely. The activities of the series A and B compounds are summarised in Table 2. The anticoagulant activities (anti-factor Xa, APTT, PT) and the major potential side-effects of heparin derivatives are shown, together with values for the inhibition of HLE and IL-8 displacement. The aPTT and PTT results confirmed the decrease in anticoagulant activity of the derivatives, which correlates with the

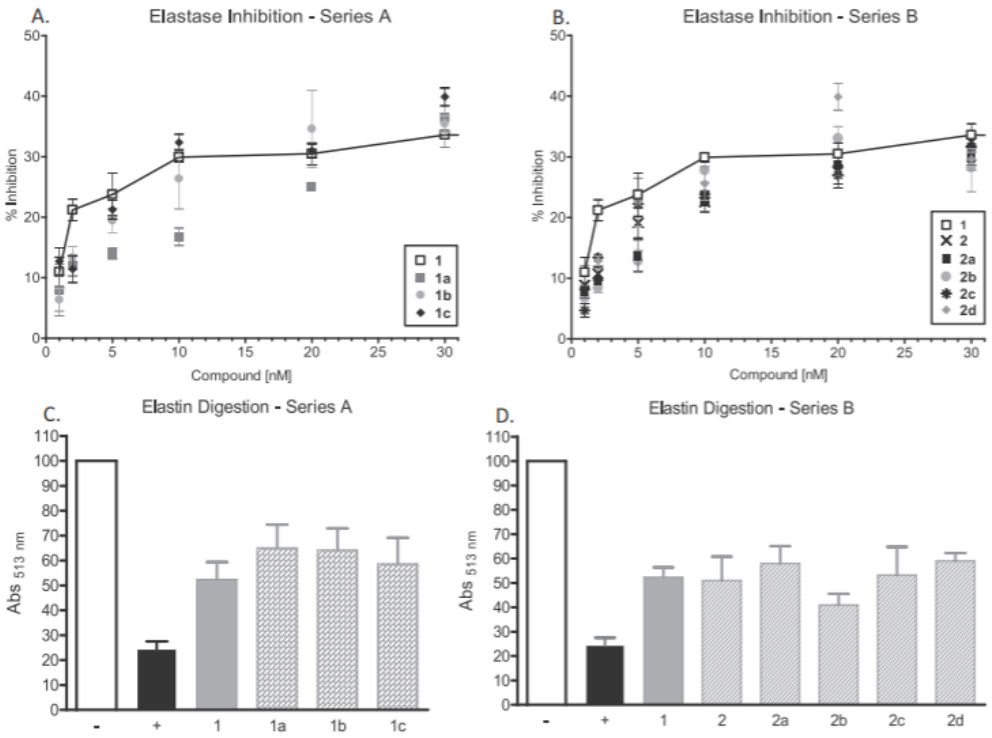


Figure 1 Inhibition of human leukocyte elastase by heparin derivatives in assays measuring release of -nitroaniline from a chromogenic peptide substrate, (A) (1a–1c) and (B) (2a–2d). The increase in absorbance at 513 nm reports the inhibition of digestion of the natural substrate (-elastin), (C) (compounds 1, 1a–1c) and (D) (compounds 2, 2a–2d). The negative control is the level of digestion in the presence of inactive enzyme, while the positive control is the digestion in the absence of inhibitors. Results shown are averages of 3 independent experiments, each with 2 replicates, ± standard errors. Error bars on the negative controls are negligible.

decrease of sulfation degree, while the glycol-split modification, whilst present in only 25–35% of disaccharides, abolished the anticoagulant activities.

3.3. Anticoagulant activity screening using anti-factor Xa, APTT and PT assays

The major potential side-effect associated with heparin derivatives relates to their ability to interfere with the blood clotting cascade, via interactions with a number of proteins. The best-known activity of intact heparin, which is exploited in its application as a pharmaceutical anticoagulant, involves an interaction with antithrombin and subsequently with factor Xa, the region of the clotting cascade termed the common pathway. However, there are several other points in the clotting cascade, at which heparin derivatives can also exert influence. These activities occur in two branches, one termed the extrinsic (tissue factor) pathway and the other the intrinsic (contact activation) pathway. These activities can be measured conveniently by straightforward assays, using the prothrombin time (PT) and the activated prothrombin time (APTT), respectively, in addition to the antithrombin activity of the common pathway (anti-factor Xa assay). The results of these three assays for 1a–1c and 2–2d, are shown in Table 2.

3.4. Interaction with interleukin-8

The ability of the heparin derivatives to interact with IL-8, a key modulator of inflammation, was also tested by two independent methods. The first measured their ability to displace IL-8

Table 2 Anticoagulant properties and the percentage inhibition of HLE for compound series A and B. The table contains, from left to right, anticoagulant (percentage factor Xa inhibition), activated partial thromboplastin time (APTT), prothrombin time (PT) and inhibition of elastase (chromogenic peptide method and elastin assay) for heparin and its chemical derivatives.

Series	Compound	% Factor Xa inhibition	APTT	PT	% HLE inhibition (peptide)[#] (10 nM)	% HLE inhibition (α-elastin)[◊] (0.54 μM)
A	1 (PMH)	79.6	1.0	2.0	30.0	35
	1a	24.1	4.0	2.5	19.0	48**
	1b	22.4	2.0	3.0	26.0	46**
	1c	6.0	27.3	14.7	32.4	42*
B	2	17.0	8.7	9.1	22.6	39
	2a	10.5	7.3	91.2	22.7	41*
	2b	12.8	15.2	90.8	27.7	28
	2c	11.0	178.8	237.0	24.0	41*
	2d	13.5	264.0	>237	25.6	39*

§ Reported values are normalised relative to control heparin (1), which had APTT and PT activities (EC_{50}) of 2.1 and 23.9 μg/mL. Higher values denote a weaker anticoagulant activity than heparin.
# The percentage reported is relative to a specific concentration of the compounds as indicated in the headings of each column, for a range of concentrations see Fig. 1.
◊ Comparison with positive control (absence of inhibitor): one way ANOVA—Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$).

in solution from surface-bound heparin using a competitive ELISA (Fig. 2A and B). While, the second monitored direct binding in solution to IL-8 through changes in IL-8 protein secondary structure, detected via chiral chromophores present in the protein backbone which are sensitive to conformational changes when probed using synchrotron radiation circular dichroism (SRCD) spectroscopy (Fig. 3).

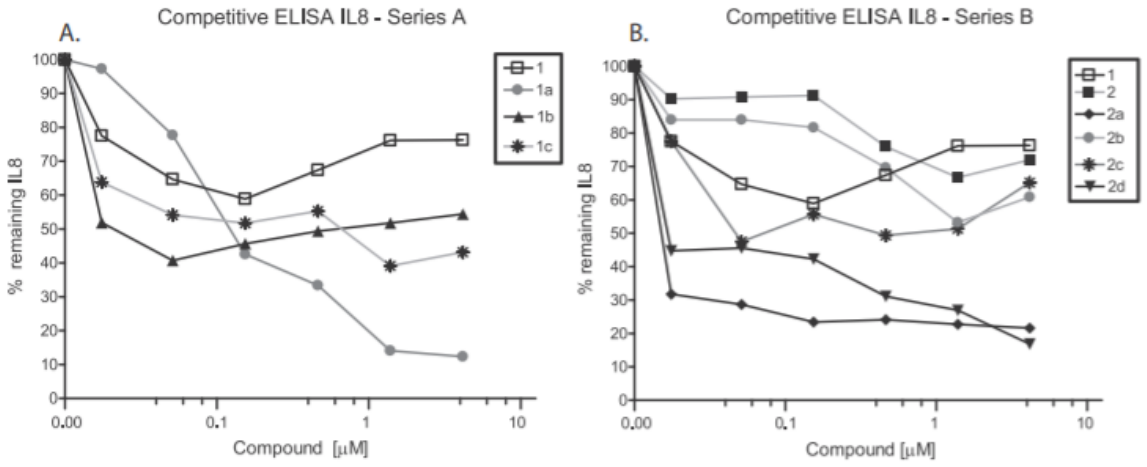


Figure 2 Competitive ELISA showing displacement of bound IL-8 from surface immobilised heparin by 1a–1c (A) and 2a–2d (B). Reference heparin 1 is shown on both graphs.

All of the heparin derivatives were able to compete with heparin for IL-8 binding and were, therefore, able to displace it. Several were more active than the reference heparin (1), which was able to displace less than 50%, before reaching a plateau. In series A, 1a promoted a more effective displacement up to almost 90% and in a different manner to both 1 and 1b or 1c, suggesting distinct modes of interaction. The differences between 1b and 1c, 2 and 2b were not statistically significant. The glycol-split modification of heparin 2 did not improve its

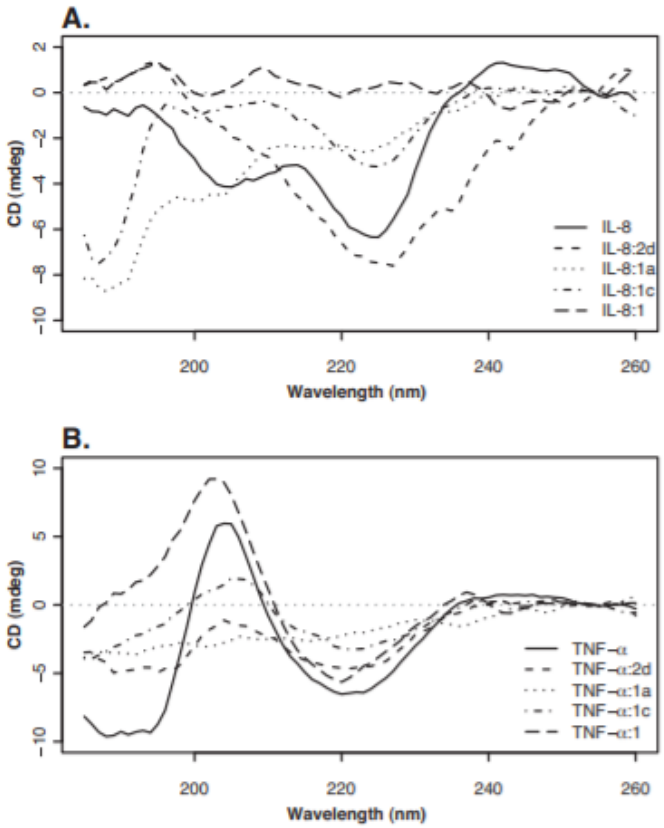


Figure 3 . (A) Interaction of heparin and derivatives in solution with IL-8 demonstrated through changes in protein secondary structure. SRCD spectra (185–260 nm) of IL-8 in the presence of selected test compounds (1a, 1c, 2d), unmodified porcine mucosal heparin (1) and the natural ligand heparan sulfate (HS) in a 1:1 molar ratio. The spectra of heparin and derivatives alone were subtracted. (B) SRCD spectra (185–260 nm) of TNF-α in the presence of heparin derivatives 1a, 1c, 2d and unmodified porcine mucosal heparin 1, which induces changes in the secondary structure of the protein, demonstrating a direct interaction in solution.

ability to compete with the standard heparin bound to the plate, although a concentration-dependent effect was observed, distinct from that observed for unmodified heparin (1). Compound 2b was less efficient than the N-acetylated counterpart in detaching IL-8, in contrast to compounds 2c and 2d. Synchrotron radiation circular dichroism spectroscopy in the range 185–260 nm, which is sensitive to secondary structure changes in proteins, was used to establish, unequivocally, the interaction between IL-8 and heparin derivatives in solution. Profound structural changes in IL-8 were observed in the presence of both standard heparin and selected derivatives (Fig. 3). The CD spectrum of IL-8 (Fig. 3) resembles that of a previously reported spectrum of IL-8 (amino acids 1–66) (Fernando, Nagle, & Rajarathnam, 2007). The two minima located at ~204 and ~224 nm are characteristic of the chemokine as a monomer (Fernando et al., 2007). The addition of the heparin derivatives, in molar ratio of ~1:1, to IL-8 caused structural rearrangement of the protein, with the resulting spectra resembling that of a protein with a random-coil structure.

3.5. Interaction of heparin derivatives with TNF-α, detected in solution by SRCD

The interaction of selected heparin derivatives with TNF-α, which is involved in the inflammatory response, was also explored in solution. The SRCD spectra of TNF-α, alone, and in the presence of either unmodified heparin (1), or heparin derivatives 1a, 1c and 2d, which all exhibit favourable IL-8 binding and elastase inhibition are shown in Fig. 3.

The TNF-α active form is a 51 kDa trimer held together by non-covalent interactions and its secondary structure comprises predominantly β-sheet and turns as evinced by the positive peak at 205 nm and a negative peak between 216 and 225 nm, although little α-helix is present. These data are consistent with previous CD and IR studies indicating that TNF-α contains about 60% β-sheet or turns and a significant amount of irregular structures (Eck & Sprang, 1989; Jones, Stuart, & Walker, 1989; Narhi et al., 1996). Binding of both standard heparin and derivatives, although present only in a 1:10 molar ratio, caused significant and distinct changes in the CD spectra of TNF-α but, while 1 caused a 40% increase in β-sheet, its derivatives influenced the secondary structure of the protein in different ways.

4. Discussion

The chemical modification of heparin can generate compounds with a wide-range of activities, some of which depend on their overall charge but, more often, exhibit subtle variation as a function of substitution pattern. To help allay fears that any desirable activities were dependent only on charge density, two series of chemically modified heparin derivatives were compared.

The principal anticoagulant activity of heparin, through activation of antithrombin and inhibition of factor Xa, can be measured readily, enabling derivatives to be screened. Highly charged polysaccharides, generated either by deliberate modification, or introduced through adulteration, can activate the extrinsic (tissue factor) or intrinsic (contact activation) pathways, causing further unwanted coagulation (Guerrini et al., 2008;Kishimoto et al., 2008). The glycol-split modification of *N*-acetylated compounds, 2a–2d, resulted in reduction of the anticoagulation activity (Table 2), demonstrating that this type of modification can be useful to reduce undesired side effects without altering the substitution pattern of heparin. For example, 1c and 2d contain the same level of *N*-acetylation, but 2d showed much lower anticoagulant activity than 1c, and this trend obtains for all glycol-split compounds.

Compounds were screened for their ability to inhibit neutrophil elastase. From these two series of derivatives, the most promising are 1c from series A, and both 2c and 2d from series B, that inhibit NE and exhibit anticoagulant activity more than 10 times lower than heparin. Although the inhibition level did not exceed 50%, complete inhibition of elastase activity may impair the ability to combat bacterial infection, while significant attenuation of its activity is desirable.

Compounds from both series were able to bind and displace IL-8 from the surface of the ELISA plate (Fig. 2A and B) and interaction was confirmed in solution independently using SRCD (Fig. 3). It is apparent from the SRCD spectra of IL-8 with the modified polysaccharides, that each derivative induces distinct structural changes in IL-8, implying either distinct binding modes, the induction of different structural changes by distinct structures binding the same binding site, or a mixture of both. The ELISA results (Fig. 2A and B) showed that 1a was able to displace up to 90% of IL-8 from the plate at 3.3 μM, while its glycol-split counterpart, 2b, despite increased structural flexibility (Casu et al., 2002), was a less effective inhibitor. In contrast, the glycol-split modification of 1c increased the affinity for IL-8. From another perspective, with the exception of 2b in which partial 2-O desulfation did not promote further activity, the increase of *N*-acetylation in glycol-split compounds promoted affinity for IL-8, suggesting that factors besides pure electrostatics were responsible for the interaction (Rudd et al., 2010).

The interaction of IL-8 with intact heparin has been mapped, and involves the C-terminal -helix and residues 18–23, including a lysine at position 20 (Kuschert et al., 1998) while

several other lysine residues were identified as critical, including K64 and R68 and the IL-8 sequence contains others with which heparin derivatives may interact, including at positions 20, 60 and 67. It is also known that removal of sulfates can have dramatic effects on the conformation of heparin derivatives, affecting both glycosidic linkage geometry and uronate residue conformations (Rudd et al., 2007). It is noteworthy that, while the compounds showed lowered anticoagulant activity as a consequence of the structural modifications made, which reduced their overall charge, they still retained the desired target activities, some comparable to, or better than, unmodified heparin (Figs. 1 and 2 and Table 2). Many heparin chemical derivatives have been studied and characterised and some of their activities, such as angiogenesis inhibition, anti-metastatic activity and antagonism of P-selectins, have been recorded. Particularly, the glycol-split heparin derivatives, with various levels of *N*-acetylation, have previously been reported to inhibit P-selectin and heparanase, which are also involved in inflammation (Borsig et al., 2001; Naggi et al., 2005). The results presented here suggest that compounds with moderate levels of N-acetylation and glycol split (e.g. 2a and 2c) could also retain the ability to inhibit heparanase, as well as binding IL-8.

Given that unmodified heparin is known to interact with several hundred proteins (Ori et al., 2008), many of them involved in cell signalling and regulation, it is perhaps surprising that heparin administration does not elicit more side-effects in patients undergoing anticoagulant therapy (Cestac et al., 2003; Quinlan et al., 2012). While this is encouraging for those aiming to develop alternative treatments employing a heparin scaffold, it does present something of a puzzle, and the explanation may lie in the interconnected nature of these signalling and regulatory systems. For example, in recent work on the FGF–FGFR signalling system, analysed in terms of the effects of intact heparin, it has been observed that a very well-connected and highly redundant signalling system exists (Xu et al., 2013). This robustness may be a characteristic of such highly connected networks (of which the inflammation response is another example (Calvano et al., 2005)). Binding by heparin to several proteins in the network may simply result in a strongly dampened effect throughout. Reducing the sulfation level of derivatives, which can also have the overall effect of lowering the heterogeneity of the heparin, may focus some of these activities into fewer interactions, resulting in higher selectivity, hence provide agents with distinct activities, yet capable of effective intervention.

Significant structural changes in TNF- upon binding to heparin and derivatives (1a) (Fig. 3) were observed using SRCD. It is also possible that other proteins involved in the inflammatory response interact with, and are affected by, these compounds. Key players such as P-selectin (O'Sullivan et al., 2005), whose dependent cell rolling in the vascular lumen is prevented by heparin (Borsig et al., 2001), or L-selectin, whose effects are also inhibited by heparin (Hostettler et al., 2007; Koenig, Norgard-Sumnicht, Linhardt, & Varki, 1998), metalloproteases (Morishima et al., 2001; Warner et al., 2004) and other interleukins are obvious candidates to investigate.

5. Conclusions

The heparin derivatives prepared here interacted with IL-8 and TNF-α and partially inhibited cleavage of both a synthetic peptide and a natural substrate by human sputum elastase in vitro. The partial, rather than the complete inhibition of key elements involved in the excessive inflammation response could return the immune response towards normal levels, thereby reducing damage to lung tissue, while maintaining some capacity to combat infection. The compounds identified offer a starting point for future drug development, opening-up the possibility of the synthesis of polysaccharides or analogues with lower molecular weight, capable of acting on multiple cytokines with the ability to decrease

inflammation through several targets simultaneously, while minimising unwanted side-effects.

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References

Bertini, S., Bisio, A., Torri, G., Bensi, D., & Terbojevich, M. (2005). Molecular weight determination of heparin and dermatan sulfate by size exclusion chromatography with a triple detector array. *Biomacromolecules, 6*(1), 168–173.

Borsig, L., Wong, R., Feramisco, J., Nadeau, D. R., Varki, N. M., & Varki, A. (2001). Heparin and cancer revisited: Mechanistic connections involving platelets, Pselectin, carcinoma mucins, and tumor metastasis. *Proceedings of the National Academy of Sciences of the United States of America, 98*(6), 3352–3357.

Brown, R. A., Lever, R., Jones, N. A., & Page, C. P. (2003). Effects of heparin and related molecules upon neutrophil aggregation and elastase release in vitro. *British Journal of Pharmacology, 139*(4), 845–853.

Calvano, S. E., Xiao, W., Richards, D. R., Felciano, R. M., Baker, H. V., Cho, R. J., et al., & Inflamm & Host Response to Injury Large Scale Collab. Res. P. (2005). A network-based analysis of systemic inflammation in humans. *Nature, 437*(7061), 1032–1037.

Casu, B., Guerrini, M., Guglieri, S., Naggi, A., Perez, M., Torri, G., et al. (2004). Undersulfated and glycol-split heparins endowed with antiangiogenic activity. *Journal of Medicinal Chemistry, 47*(4), 838–848.

Casu, B., Guerrini, M., Naggi, A., Perez, M., Torri, G., Ribatti, D., et al. (2002). Short heparin sequences spaced by glycol-split uronate residues are antagonists of fibroblast growth factor 2 and angiogenesis inhibitors. *Biochemistry, 41*(33), 10519–10528.

Cestac, P., Bagheri, H., Lapeyre-Mestre, M., Sie, P., Fouladi, A., Maupas, E., et al. (2003). Utilisation and safety of low molecular weight heparins: Prospective observational study in medical inpatients. *Drug Safety, 26*(3), 197–207.

Cosgrove, S., Chotirmall, S. H., Greene, C. M., & McElvaney, N. G. (2011). Pulmonary proteases in the cystic fibrosis lung induce interleukin 8 expression from bronchial epithelial cells via a heme/meprin/epidermal growth factor receptor/Toll-like receptor pathway. *Journal of Biological Chemistry, 286*(9), 7692–7704.

Diamant, Z., Timmers, M. C., van der Veen, H., Page, C. P., van der Meer, F. J., & Sterk, P. J. (1996). Effect of inhaled heparin on allergen-induced early and late asthmatic responses in patients with atopic asthma. *American Journal of Respiratory and Critical Care Medicine, 153*(6 Pt 1), 1790–1795.

Downey, D. G., Bell, S. C., & Elborn, J. S. (2009). Neutrophils in cystic fibrosis. *Thorax, 64*(1), 81–88.

Eck, M. J., & Sprang, S. R. (1989). The structure of tumor necrosis factor-alpha at 2.6 A resolution. Implications for receptor binding. *Journal of Biological Chemistry, 264*(29), 17595–17605.

Fernando, H., Nagle, G. T., & Rajarathnam, K. (2007). Thermodynamic characterization of interleukin-8 monomer binding to CXCR1 receptor N-terminal domain. *FEBS Journal, 274*(1), 241–251.

Fryer, A., Huang, Y. C., Rao, G., Jacoby, D., Mancilla, E., Whorton, R., et al. (1997). Selective O-desulfation produces nonanticoagulant heparin that retains pharmacological activity in the lung. J*ournal of Pharmacology and Experimental Therapeutics, 282*(1), 208–219.

Goger, B., Halden, Y., Rek, A., Mosl, R., Pye, D., Gallagher, J., et al. (2002). Different affinities of glycosaminoglycan oligosaccharides for monomeric and dimeric interleukin-8: A model for chemokine regulation at inflammatory sites. *Biochemistry, 41*(5), 1640–1646.

Guerrini, M., Beccati, D., Shriver, Z., Naggi, A., Viswanathan, K., Bisio, A., et al. (2008). Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. *Nature Biotechnology, 26*(6), 669–675.

Guimond, S. E., Turnbull, J. E., & Yates, E. A. (2006). Engineered bio-active polysaccharides from heparin. *Macromolecular Bioscience, 6*(8), 681–686.

Hostettler, N., Naggi, A., Torri, G., Ishai-Michaeli, R., Casu, B., Vlodavsky, I., et al. (2007). P-selectin- and heparanase-dependent antimetastatic activity of nonanticoagulant heparins. *FASEB Journal, 21*(13), 3562–3572.

Jayson, G. C., & Gallagher, J. T. (1997). Heparin oligosaccharides: Inhibitors of the biological activity of bFGF on Caco-2 cells. *British Journal of Cancer, 75*(1), 9–16.

Jones, E. Y., Stuart, D. I., & Walker, N. P. (1989). Structure of tumour necrosis factor. *Nature, 338*(6212), 225–228.

Joseph, T., Look, D., & Ferkol, T. (2005). NF-kappaB activation and sustained IL-8 gene expression in primary cultures of cystic fibrosis airway epithelial cells stimulated with Pseudomonas aeruginosa. *American Journal of Physiology—Lung Cellular and Molecular Physiology, 288*(3), L471–L479.

Kishimoto, T. K., Viswanathan, K., Ganguly, T., Elankumaran, S., Smith, S., Pelzer, K., et al. (2008). Contaminated heparin associated with adverse clinical events and activation of the contact system. *New England Journal of Medicine, 358*(23), 2457–2467.

Koenig, A., Norgard-Sumnicht, K., Linhardt, R., & Varki, A. (1998). Differential interactions of heparin and heparan sulfate glycosaminoglycans with the selectins. Implications for the use of unfractionated and low molecular weight heparins as therapeutic agents. *Journal of Clinical Investigation, 101*(4), 877–889.

Kuschert, G. S., Hoogewerf, A. J., Proudfoot, A. E., Chung, C. W., Cooke, R. M., Hubbard, R. E., et al. (1998). Identification of a glycosaminoglycan binding surface on human interleukin-8. *Biochemistry, 37*(32), 11193–11201.

Lever, R., & Page, C. P. (2002). Novel drug development opportunities for heparin. *Nature Reviews Drug Discovery, 1*(2), 140–148.

Mitsuhashi, H., Nonaka, T., Hamamura, I., Kishimoto, T., Muratani, E., & Fujii, K. (1999). Pharmacological activities of TEI-8362, a novel inhibitor of human neutrophil elastase. *British Journal of Pharmacology, 126*(5), 1147–1152.

Morishima, Y., Nomura, A., Uchida, Y., Noguchi, Y., Sakamoto, T., Ishii, Y., et al.(2001). Triggering the induction of myofibroblast and fibrogenesis by airway epithelial shedding. *American Journal of Respiratory Cell and Molecular Biology, 24*(1), 1–11.

Mulloy, B., Forster, M. J., Jones, C., & Davies, D. B. (1993). N.M.R. and molecular-modelling studies of the solution conformation of heparin. *Biochemical Journal, 293*(Pt 3), 849–858.

Naggi, A., Casu, B., Perez, M., Torri, G., Cassinelli, G., Penco, S., et al. (2005). Modulation of the heparanase-inhibiting activity of heparin through selective desulfation, graded N-acetylation, and glycol splitting. *Journal of Biological Chemistry, 280*(13), 12103–12113.

Narhi, L. O., Philo, J. S., Li, T., Zhang, M., Samal, B., & Arakawa, T. (1996). Induction of alpha-helix in the beta-sheet protein tumor necrosis factor-alpha: Thermal and trifluoroethanol-induced denaturation at neutral pH. *Biochemistry, 35*(35), 11447–11453.

O'Sullivan, B. P., Linden, M. D., Frelinger, A. L., 3rd, Barnard, M. R., Spencer-Manzon, M., Morris, J. E., et al. (2005). Platelet activation in cystic fibrosis. *Blood, 105*(12), 4635–4641.

Ori, A., Wilkinson, M. C., & Fernig, D. G. (2008). The heparanome and regulation of cell function: Structures, functions and challenges. *Frontiers in Bioscience, 13*, 4309–4338.

Ori, A., Wilkinson, M. C., & Fernig, D. G. (2011). A systems biology approach for the investigation of the heparin/heparan sulfate interactome. *Journal of Biological Chemistry, 286*(22), 19892–19904.

Quinlan, C., Bates, M., Cotter, M., Riordan, M., Waldron, M., & Awan, A. (2012). Tinzaparin is safe and effective in the management of hemodialysis catheter thrombosis. *ASAIO Journal, 58*(3), 288–290.

Redini, F., Tixier, J. M., Petitou, M., Choay, J., Robert, L., & Hornebeck, W. (1988). Inhibition of leucocyte elastase by heparin and its derivatives. *Biochemical Journal, 252*(2), 515–519.

Rudd, T. R., Guimond, S. E., Skidmore, M. A., Duchesne, L., Guerrini, M., Torri, G., et al. (2007). Influence of substitution pattern and cation binding on conformation and activity in heparin derivatives. *Glycobiology, 17*(9), 983–993.

Rudd, T. R., Skidmore, M. A., Guimond, S. E., Cosentino, C., Torri, G., Fernig, D. G., et al. (2009). Glycosaminoglycan origin and structure revealed by multivariate analysis of NMR and CD spectra. *Glycobiology, 19*(1), 52–67.

Rudd, T. R., Uniewicz, K. A., Ori, A., Guimond, S. E., Skidmore, M. A., Gaudesi, D., et al. (2010). Comparable stabilisation, structural changes and activities can be induced in FGF by a variety of HS and non-GAG analogues: Implications for sequence-activity relationships. *Organic and Biomolecular Chemistry, 8*(23), 5390–5397.

Suzuki, T., Wang, W., Lin, J. T., Shirato, K., Mitsuhashi, H., & Inoue, H. (1996). Aerosolized human neutrophil elastase induces airway constriction and hyperresponsiveness with protection by intravenous pretreatment with half-length secretory leukoprotease inhibitor. *American Journal of Respiratory and Critical Care Medicine, 153*(4 Pt 1), 1405–1411.

Tosi, M. F., Zakem, H., & Berger, M. (1990). Neutrophil elastase cleaves C3bi on opsonized pseudomonas as well as CR1 on neutrophils to create a functionally important opsonin receptor mismatch. *Journal of Clinical Investigation, 86*(1), 300–308.

Varki, N. M., & Varki, A. (2002). Heparin inhibition of selectin-mediated interactions during the hematogenous phase of carcinoma metastasis: Rationale for clinical studies in humans. *Seminars in Thrombosis and Hemostasis, 28*(1), 53–66.

Vlodavsky, I., Mohsen, M., Lider, O., Svahn, C. M., Ekre, H. P., Vigoda, M., et al. (1994). Inhibition of tumor metastasis by heparanase inhibiting species of heparin. *Invasion and Metastasis, 14*(1–6), 290–302.

Walsh, R. L., Dillon, T. J., Scicchitano, R., & McLennan, G. (1991). Heparin and heparan sulphate are inhibitors of human leucocyte elastase. *Clinical Science (London), 81*(3), 341–346.

Warner, R. L., Bhagavathula, N., Nerusu, K. C., Lateef, H., Younkin, E., Johnson, K. J., et al. (2004). Matrix metalloproteinases in acute inflammation: Induction of MMP-3 and MMP-9 in fibroblasts and epithelial cells following exposure to proinflammatory mediators in vitro. *Experimental and Molecular Pathology, 76*(3), 189–195.

Wilmott, R. W., Frenzke, M., Kociela, V., & Peng, L.(1994). Plasma interleukin-1 alpha and beta, tumor necrosis factor-alpha, and lipopolysaccharide concentrations during pulmonary exacerbations of cystic fibrosis. *Pediatric Pulmonology, 18*(1), 21–27.

Xu, R., Rudd, T. R., Hughes, A.J., Siligardi, G., Fernig, D. G., & Yates, E.A. (2013). Analysis of the fibroblast growth factor receptor (FGFR) signalling network with heparin as coreceptor: Evidence for the expansion of the core FGFR signalling network. *FEBS Journal, 280*(10), 2260–2270.

Yates, E. A., Santini, F., Guerrini, M., Naggi, A., Torri, G., & Casu, B. (1996). 1H and 13C NMR spectral assignments of the major sequences of twelve systematically modified heparin derivatives. *Carbohydrate Research, 294*, 15–27.

Yip, L. Y., Lim, Y. F., & Chan, H. N. (2011). Safety and potential anticoagulant effects of nebulised heparin in burns patients with inhalational injury at Singapore General Hospital Burns Centre. *Burns, 37*(7), 1154–1160.

Yoshimura, Y., Hiramatsu, Y., Sato, Y., Homma, S., Enomoto, Y., Jikuya, T., et al. (2003). ONO-6818, a novel, potent neutrophil elastase inhibitor, reduces inflammatory mediators during simulated extracorporeal circulation. *Annals of Thoracic Surgery, 76*(4), 1234–1239.