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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.

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).

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Another cytokine, TNF-alpha (TNF-α), 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 TNF-α.

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-α, 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 313 K 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**–**1**c) and glycol-split heparin (series **B**; **2**–**2**d) 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 $CaCl_2$ 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 μg/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 IL-8 primary (Millipore, Bedford, MA) (1:500, 50 μL, 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