Brief Articles

Heparin Derivatives as Inhibitors of BACE-1, the Alzheimer's β-Secretase, with Reduced Activity against Factor Xa and Other Proteases

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Heparan sulfate (HS) regulates processing of the amyloid precursor protein by the Alzheimer's β-secretase (BACE-1). An HS analogue, porcine intestinal mucosal heparin, was systematically modified at the principal positions of *O*-sulfation and *N*-sulfation/acetylation and tested for BACE-1 inhibitory and anti factor Xa activities. The derivative with the highest anti-BACE-1 to anti-Xa activity ratio contained *N*-acetyl and 2-*O*- and 6-*O*-sulfates and also exhibited attenuated activities against cathepsin-D and renin, two other structurally related aspartyl proteases.

Introduction

Heparan sulfate (HS) has been identified as an inhibitor of the Alzheimer's disease (AD) β-secretase (BACE-1, β-site amyloid precursor protein cleaving enzyme-1, β-secretase-1).[1] Cleavage of amyloid precursor protein (APP) by BACE-1 is a key step in generating the neurotoxic amyloid beta peptide (Aβ), the main component of amyloid plaques.[2] HS and its highly sulfated structural analogue heparin, are glycosaminoglycans consisting of 1,4-linked disaccharide units of α-L-iduronic or β-D-glucuronic acid and either *N*-acetyl or *N*-sulfo-α-D-glucosamine. The principal positions of *O*-sulfation are C-2 of iduronate and C-6 of glucosamine, as well as, more rarely, C-3 of glucosamine. Variable substitution during biosynthesis results in considerable sequence diversity.[3] Heparin, the widely used pharmaceutical that has a higher degree of sulfation and is more homogeneous than HS, acts as a potent anticoagulant by increasing the rate of formation of irreversible complexes between antithrombin III and the serine protease clotting factors X_a and IIa. Heparin has been extensively employed as an analogue of HS and is a good activator of many biological processes involving HS.[3−6] Attenuation of the anticoagulant activity of heparin is vital if its derivatives are to be developed for use as novel pharmaceuticals.

AD is a progressive neurodegenerative disorder of the central nervous system, characterized by cerebral deposition of neurofibrillary tangles and β-amyloid (Aβ). There are currently no effective therapies that target these underlying causes. Cleavage of APP by α-, β-, and γ-secretases generates a variety of peptides, of which $Aβ_{1-40}$ and $Aβ_{1-42}$, created by the sequential action of β- and γ-secretases, are amyloidogenic and neurotoxic.[7] Initial cleavage of APP by BACE-1, a membrane-bound aspartyl protease, is the first and rate-limiting step in the production of Aβ, and increased susceptibility of APP to BACE-1 cleavage has been found in several pathological mutations of APP.[2,8,9] Consequently, the regulation of BACE-1 activity has become a key pharmaceutical target.

HS has been identified as a constituent of amyloid plaques and its abilities to interact with amyloid proteins, peptides, and fibrils,[10−12] promote aggregation,[13] and enhance the stability of fibrils[14] have been well-documented. Soluble heparin and heparin analogues have also been shown to inhibit these processes both in vitro and in vivo.[15−19] Recently, a novel role for HS was revealed when its ability to directly regulate BACE-1 cleavage of APP was discovered. Bovine lung heparin (BLH), porcine mucosal HS, and derivatives were found to inhibit BACE-1 activity, and the putative mechanism is by blocking access to the enzyme active site,[1] without interfering with APP processing by α- or γ-secretases. In this context, it is noteworthy that crystallography studies of BACE-1 complexed with peptide inhibitors showed a large binding pocket running across the surface of the protein, making effective inhibition with conventional small molecules difficult.[20]

Previous studies of the interaction of HS and other glycosaminoglycans with amyloidogenic pathways have concentrated on the direct interaction of heparin and other highly sulfated compounds with the amyloid proteins and peptides, rather than the enzymes that produce them. To date, however, relatively few studies concerning the structural requirements of HS activity have been undertaken. The use of unmodified heparin as a therapeutic agent against BACE-1 would doubtless lead to a number of side-effects, most notably an increased risk of internal bleeding and impaired blood clotting mechanisms. This is likely to preclude the clinical use of standard heparin saccharides because this would impose significant limits on the effective doses that could be used. In this study we report the development of several HS analogues, some of which possess very significantly reduced anticoagulant activities while maintaining strong inhibitory activity against BACE-1. The availability and pharmaceutical use of BLH have declined recently following concerns over the transmissible spongiform encephalopathies (TSEs), prompting us to examine these activities with PIMH, which possesses higher levels of β-D-glucuronic acid compared to that of BLH but is both widely available and poses no known TSE risk. We employed a modified panel of PIMH derivatives in which patterns of *O*- and *N*-sulfation and *N*-acetylation were varied over the predominant disaccharide repeating structure, permitting a systematic study of activity.

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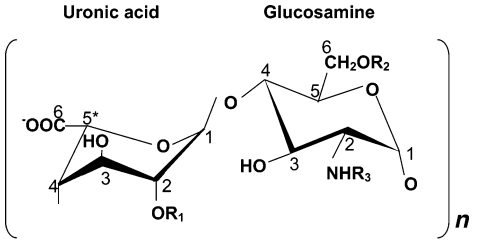
Table 1. Structures, IC_{50} Values Against BACE-1 and Anticoagulant Activities of Modified Heparins[a]



compound	R_1	R_2	R_3	IC_{50} (μg/mL)	R^2 of IC_{50}	ACA[b]	TR[c]
(1) PMIH	SO_3^-	SO_3^-	SO_3^-	0.028	0.998	100%	1
(2) *N*-acetyl	SO_3^-	SO_3^-	$COCH_3$	0.031	0.995	0.03%	3136
(3) UA-2-OH	H	SO_3^-	SO_3^-	0.053	0.995	0.4%	147
(4) UA-2-OH, *N*-acetyl	H	SO_3^-	$COCH_3$	0.091	0.999	0.03%	1092
(5) GlcN-6-OH	SO_3^-	H	SO_3^-	0.100	0.996	0.5%	61
(6) GlcN-6-OH, *N*-acetyl	SO_3^-	H	$COCH_3$	0.410	0.995	0.03%	237
(7) UA-2-OH, GlcN-6-OH	H	H	SO_3^-	0.786	0.994	0.03%	123
(8) UA-2-OH, GlcN-6-OH, *N*-acetyl	H	H	$COCH_3$	>100	n/a	0.03%	1
(9) per-sulfated #	SO_3^-	SO_3^-	SO_3^-	0.053	0.998	35.0%	2
(10) APA[d]				n/a	n/a	n/a	n/a

[a] Anticoagulant activities are expressed as a percentage of PIMH (defined as 100%). Substitution pattern is defined by R_1, R_2, and R_3, corresponding to position-2 of iduronate, -6 of glucosamine, and -2 of glucosamine, respectively. The therapeutic ratio was calculated from the IC_{50} against BACE-1/ anticoagulant activity. **UA**—uronic acid, either α-L-iduronic or β-D-glucuronic acid; **GlcN**—α-D-glucosamine. *The configuration shown at C_5 is α-L-iduronic acid. [b] ACA = anticoagulant activity. [c] TR = therapeutic ratio (BACE/Xa). [d] APA = 3-amino-1-propanesulfonic acid.

The panel was evaluated for the ability to inhibit BACE-1 cleavage of APP, potency as anticoagulants (anti factor Xa), and for their ability to inhibit other aspartyl protease family members.

BACE-1 Inhibitory Activities. The BACE-1 inhibitory properties of BLH with substitution patterns corresponding to (1), (2), (3), and (5) have been tested previously[1] using BLH as the starting material. In vitro cleavage of APP by BACE-1 was measured using a FRET peptide cleavage assay (Table 1). Following unmodified PIMH (1), the most effective inhibitor of BACE-1 was de-*N*-sulfated, re-*N*-acetylated PIMH (2). Ranked third and fourth most active were 2-*O* de-sulfated (3) and 2-*O* de-sulfated/*N*-acetylated PIMH (4), with similar IC_{50} values. This indicated that neither the *N*- nor the 2-*O* sulfates were absolute requirements for high level activity when accompanied by 6-*O* sulfation; (4), with a single 6-*O*-sulfate, was the most active monosulfated compound. Removal of the 6-*O*-sulfate (5) resulted in the least active disulfated compound. Removal of both the 2-*O*- and 6-*O*- sulfates (7) resulted in the least active monosulfated compound, while removal of all sulfates (8) almost abolished the anti-BACE-1 activity of PIMH. The activities of (4) and (5) indicated a significant role for the 6-*O*-sulfate in the inhibition of BACE-1. We also examined the effect of persulfated heparin (9), which was sulfated additionally at position-3 in both the iduronate and glucosamine residues. This exhibited a similar IC_{50} to unmodified PIMH. The lack of a direct correlation between sulfation level and BACE-1 inhibitory activity demonstrated clearly that activity was not simply related to charge density.

It is likely that the relationship between activity and substitution pattern is a complex one, which depends on conformational effects, because both iduronate ring[21] and glycosidic linkage geometries[22] are known to depend subtly on substitution patterns. We also examined the effect of 3-amino-1-propanesulfonic acid[16] (3APS, Alzehemed; (10)), a compound described as a "heparin mimetic", which is undergoing clinical trials for the treatment of AD and whose proposed mechanism of action is inhibition of Aβ aggregation. 3APS did not inhibit BACE-1, even at high concentrations (1000 μg/mL), indicating that its "heparin mimetic" activities do not extend to inhibition of BACE-1.

Anticoagulant Activities. Removal of the *N*-sulfate and replacement with an *N*-acetyl group had the greatest effect on reducing the ability of the chemically modified PIMH to interfere with the antithrombin III/factor Xa complex. The anti factor Xa activities of compounds possessing *N*-acetyl groups were at least 3000-fold lower than PIMH (Table 1). Removal of either the 2-*O* or the 6-*O* sulfate groups, on the other hand, reduced the antithrombin III/factor Xa activity by approximately 200-fold. It is well-established that the anti factor Xa activity of heparin/HS is due to the specific pentasaccharide sequence: -4) GlcNAc(6S) α(1-4) GlcA β(1-4) GlcNS(3,6S) α(1-4) IdoA-(2S) α(1-4)GlcNS(6S) α (1-. The presence of a 3-*O*-sulfate group within the central glucosamine residue is vital for anti factor Xa activity and its removal results in its virtual abolition,[23] while removal of either the *N*-sulfate from the central glucosamine or 2-*O*-sulfate from the iduronate residue have less dramatic, but nonetheless, deleterious effects.

The removal of *N*-sulfate groups and their replacement with *N*-acetyl in heparin derivatives (modification of (1) to (2)) explains the dramatic loss of activity exhibited by this compound, but de-*O* sulfation in iduronate, which occurs under highly basic conditions,[24] also resulted in a substantial reduction in anti factor Xa activity and has two potential causes. The first is the removal of the 2-*O*-sulfate group, but a second modification also occurs in rare glucosamine residues bearing both 3-*O*-sulfate and *N*-sulfate (e.g., in the pentasaccharide sequence AGA*IA), involving the formation of an *N*-sulfated aziridine group with loss of the 3-*O*-sulfate.[25] Other de-*O*-sulfated heparin derivatives such as (5) or (8), prepared under different conditions, do not contain this modification, and the reduction in their activities can only be attributed to the loss of the relevant groups within the pentasaccharide sequence. In particular, it is noteworthy that the 3-*O*-sulfate group is stable under mild de 6-*O*-sulfation conditions.[26]

Inhibition of Other Proteases Structurally Related to BACE-1. The closest structural relatives to BACE-1 are the aspartyl proteases pepsin, cathepsin D, and renin. These enzymes have functions in digestion, regulation of blood pressure, and lysosomal degradation of proteins, respectively.[27] An effective inhibitor of BACE-1 could potentially also interact with these, causing unwanted side effects if administered pharmaceutically. Activities of (1), (2), (3), and (4) against these proteases were also measured in FRET peptide cleavage assays. None of these compounds exerted an inhibitory effect on renin, even at concentrations up to 1000 μg/mL. Interestingly, unmodified PIMH showed some inhibitory activity against both pepsin and cathepsin D, with IC_{50} values of 0.23 μg/mL and 0.1 μg/mL, respectively. *N*-acetyl (2) and *N*-acetyl/ido-2-OH PIMH (4) showed a marked decrease in inhibitory activity against both pepsin and renin when compared to PIMH (1). The IC_{50} for *N*-acetyl PIMH (2) against pepsin was 3.27 μg/mL, which is 14-fold less potent than PIMH (1), whereas *N*-acetyl/ido-2-OH PIMH (4) did not inhibit pepsin at >1000 μg/mL. The IC_{50} values for (2) and (3) against cathepsin D were 0.27 μg/mL and 0.77 μg/mL, respectively. Thus, the modified forms of

PIMH exhibiting high levels of anti-BACE-1 activity do not significantly inhibit renin, pepsin, or cathepsin D and have much higher IC_{50} values than unmodified PIMH.

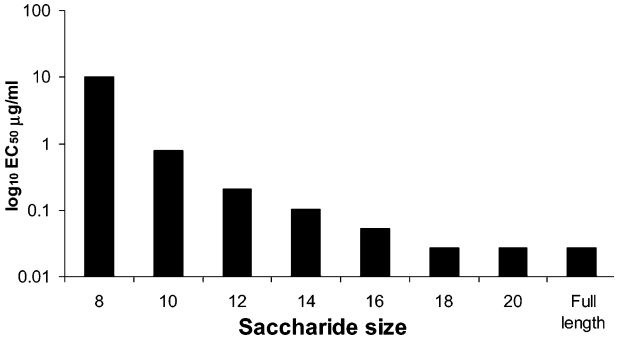
Inhibition of BACE-1 by Oligosaccharides. Full-length PIMH was enzymatically digested and fractionated by gel-filtration chromatography and the fragments were used to determine the effective size for inhibition of BACE-1 in the FRET peptide cleavage assay. A major shift in inhibitory activity (10-fold increase) was observed with deca- compared to octasaccharides, indicating the minimum requirement for high anti-BACE-1 activity. At 18 saccharide units, the activity was equivalent to full-length PIMH. These data are promising regarding in vivo administration because heparin saccharides as large as 3000 Da (equivalent to ~12 saccharides) can cross the blood−brain barrier (BBB).[28,29] Another possible side effect of standard heparin administration is heparin-induced thrombocytopenia, caused by the production of antibodies to heparin−platelet factor 4 complexes, but reduction in molecular weight and sulfation level has been shown to ameliorate this.[30] The retention of anti-BACE-1 activity in oligosaccharides with reduced levels of sulfation shows significant promise for pharmaceutical use by improving the potential uptake into the brain and through the reduction of anticoagulation and other unwanted side effects. It is clear that *N*-acetyl PIMH (2), which maintains potent BACE-1 inhibitory activity with virtually no anticoagulant activity, is an excellent lead for developing compounds to treat AD.

Experimental Section

1. Preparation of Modified Heparins. Chemically modified heparin compounds (1)−(9) were prepared by the following combinations of reactions (a) to (g) below: (1). PIMH starting material (Celsus Labs, Cincinnati, OH); (2). *N*-acetyl heparin (d) and (f); (3). Ido 2-de-*O*-sulfated heparin (a); (4). Ido 2-de-*O*-sulfated, *N*-acetylated heparin (a), (d), and (f); (5). 6-*O*-desulfated heparin (b) and (e); (6). 6-*O*-desulfated, *N*-acetylated heparin (b) and (f); (7). 6-*O*-desulfated, 2-*O*-desulfated heparin (c) and (e); (8). 6-*O*-desulfated, 2-*O*-desulfated, and *N*-acetylated heparin (c) and (f); (9). Per-sulfated heparin (g) and (e). Compounds were purified by size-exclusion and ion exchange chromatography and characterized by ^{1}H and ^{13}C NMR, disaccharide composition analysis by strong-anion exchange HPLC, and gel filtration on TSK-2000GSW$_{XL}$ (see Supporting Information). The data indicated the expected structure and compositional analysis, with equivalent size profiles and purity of ≥95%. Data compounds were desalted, lyophilized, and resuspended in the appropriate buffer prior to assay. **Chemical reactions.** (a) Selective removal of iduronate 2-*O*-sulfate: Selective removal of iduronate 2-*O*-sulfate was achieved as described by Jaseja and Perlin.[24] Note that there is concomitant modification in the small number of *N*- and 3-*O*-sulfated glucosamine units.[25] (b) Selective removal of glucosamine 6-O-sulfate: Selective removal of glucosamine 6-*O*-sulfate was carried out according to a modification of the method described.[31] (c) Complete removal of *O*- and *N*-sulfates: Complete removal of *O*- and *N*-sulfates was achieved using solvolytic de-sulfation by the method described.[31] (d) Selective de *N*-sulfation: Selective de *N*-sulfation was carried out employing controlled solvolytic de-sulfation under kinetic control as described.[32] (e) Re *N*-sulfation: Re *N*-sulfation was achieved by use of trimethylamine−sulfur trioxide complex as described.[33] (f) Re *N*-acetylation: Re *N*-acetylation employed acetic anhydride in saturated sodium bicarbonate.[34] (g) Complete *O*-sulfation: Complete *O*-sulfation of all available hydroxyl groups was carried out using excess sulfur trioxide−pyridine complex on the tetrabutylammonium salt of heparin in pyridine as described,[22] followed by re-*N*-sulfation,[33] taking precautions to avoid formation of an unusual *N*-sulfoaziridine modification.[35]

2. NMR Spectroscopy. The effectiveness of chemical treatments was monitored by ^{1}H and ^{13}C NMR at 500 and 125 MHz, respectively (D_2O, 27 ° C). Chemical shifts, δ/ppm (external standard), were in full agreement with well-defined model compounds.[34]

Chart 1. Determination of Size-Activity of Unmodified PIMH Fragments on BACE-1 Inhibition[a]



[a] R^2 values from the model fits were between 0.993 and 0.999.

3. Preparation of Sized Oligosaccharides. Porcine mucosal heparin and chemically modified *N*-acetyl heparin were digested with 100 mU of heparatinase II (Ibex Technologies, Inc., Montreal, Canada), per 100 mg in 100 mM sodium acetate, 0.1 mM calcium acetate, pH 7.0. The digested fragments were separated by gel filtration chromatography (Superdex-30, Amersham Pharmacia, UK, 2000 mm × 30 mm, 100 mM ammonium bicarbonate) and identified by reference to size-defined authentic standards.

4. Determination of BACE-1 Inhibition by in Vitro Peptide Cleavage Assay. The ability of the compounds to inhibit BACE-1 cleavage of APP was assessed using a fluorescent resonance energy transfer (FRET) peptide cleavage assay employing the FRET peptide 5-FAM-Glu-Val-Asn-Leu-Asp-Ala-Phe-Lys(QXL520)-OH, containing the Swedish amino acid variant (Anaspec, Inc., CA; Chart 1). When intact, the amino terminal fluorophore is quenched, but upon enzymatic cleavage, the fluorophore is released from quencher and fluoresces (520 nm). Assays were performed in triplicate in 96-well black plates (20 mM sodium acetate, 0.1% Triton-X-100, pH 4.5; 2.5 μM peptide per well and 4.0×10^{-3} units/well of recombinant human BACE-1 (Sigma)). The appropriate controls for enzyme activity and background fluorescence were employed and plates were incubated (1 h, 25 °C, stopped with 2.5 M sodium acetate). Inhibitors were added in a concentration range from 1000 to 0.0001 μg/mL. Fluorescence 480ex/520em was measured on a Polarstar plate reader (BMG LabTechnologies, U.K.) and data were analyzed by plotting log concentration of inhibitor against percent inhibition and fitting a four-parameter sigmoidal curve using BioDataFit 1.02 (Chang Bioscience, U.S.A.).

5. Anticoagulant Activity. Anti Factor Xa activity was measured against a porcine mucosal heparin (PIMH) standard of known activity (Sigma, UK) using a diagnostic grade Coatest Heparin test kit (Chromogenix, MA), adapted to a 96-well plate format, reading A_{405} (Polarstar plate reader (BMG LabTechnologies, U.K.)).

6. Activity against Other Proteases. The ability of compounds to inhibit the structurally related proteases pepsin and cathepsin D (Sigma, U.K.) was measured by FRET cleavage assay (5 pmol enzyme/well, EnzChek Protease Assay kit (Molecular Probes, U.K.) according to manufacturer's instructions). Activity against human recombinant renin (Cayman Chemical, MI), was measured by FRET peptide cleavage assay (0.08 pMoles enzyme/well, using the Renin Substrate 1 (Molecular Probes, Invitrogen, U.K.) according to manufacturer's instructions). IC_{50} values were calculated as described above.

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Supporting Information Available: Information on the purity and NMR characterization of the polysaccharides used in these

studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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