Inhibitors and activity-based probes for β-D-glucuronidases, heparanases and β-L-arabinofuranosidases

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General Introduction

Glycoside hydrolases (glycosidases/GHs) are widely expressed in all kingdoms of life and display the ability to catalyse the hydrolytic cleavage of glycosidic bonds occurring in oligo/polysaccharides and glyco-conjugates with exquisite enhancement of rate constants compared to the spontaneous hydrolysis of glycosides.[1] GHs represent the largest and best-studied class of Carbohydrate-Active enZymes (CAZymes)[2] responsible for the degradation of carbohydrates with varied configurational and functional group patterns, and they play many roles in human health and (patho)physiology. Often, abnormal levels of glycosidase activity are markedly linked to human pathologies. Illustrative examples of this are low levels (caused by genetic mutations) of glucocerebrosidase (GBA) in Gaucher disease[3] and overexpression of heparanase (HPSE) in several cancer tissues.[4-8] To date, several glycosidases feature as 'druggable' proteins of interest for their ability to bind biologically active molecules with great affinity at a site of binding which is typically embedded into the protein structure – hence not exposed to solvents. These characteristics make glycosidases established pharmacological targets among carbohydrate-processing proteins,[9] differently from other physiologically-significant carbohydrate-binding proteins such as lectins – conventionally linked to low druggability index in reason of the hydrophilicity and solvent exposure of their binding pockets.[9,10] These observations underpin the relevance of several glycosidases as pharmacological targets. Heparanase, specifically, is a representative example of GH targeted for therapeutic interventions. This enzyme is extensively studied for its roles in a wide array of human diseases, including carcinogenesis and cancer progression,[11] amyloidosis,[12,13] acute and chronic inflammation[14] and viral infection.[15] To date, the biomedical relevance of HPSE mostly pertains cancer treatment. In fact, heparanase overexpression correlates with aggressive cancer phenotypes characterised by increased tumour size, tumour angiogenesis, enhanced metastasis and poor clinical survival outcome.[11,16-18] These correlations make HPSE a prime target for cancer therapy.

The involvement of HPSE in cancer biology calls forth the need for inhibitors and probes able to selectively and specifically inactivate and detect the target enzyme. These tools have been instrumental in elucidating the catalytic domain, mechanism and itinerary of several target glycosidases, including heparanase, by means of *ab initio* molecular dynamics and X-ray crystallographic analysis of (covalent) ligand-enzyme crystal complexes.[19-23] Libraries of heparanase inhibitors may inform about structure-activity relationships (SARs), and thereby serve as initial scaffolds for the development of commodities aimed at treating heparanase-related diseases. Furthermore, mechanism-based, covalent and irreversible inhibitors can be

employed as starting scaffolds for the design of activity-based probes (ABPs), which enable profiling of heparanase in complex biological milieu for diagnosis and patient stratification. This dissertation reports on the synthesis and biochemical evaluation of covalent mechanism-based inhibitors for heparanase, as well as the development of broad-spectrum ABPs for retaining *exo*- and *endo*-β-D-glucuronidases alike, including HPSE. The Thesis further builds on the concept of ABP-based profiling of GHs and discusses the development of inhibitors and probes targeting retaining β-L-arabinofuranosidases, a group of non-canonical glycosidases possessing a contentious enzymatic mechanism. As these enzymes are mostly expressed by microorganismal members of the human gut microbiome, the enzymology of retaining β-L-arabinofuranosidases was addressed as part of the research activity conducted within Sweet Crosstalk Marie Sklodowska-Curie Action Innovative Training Network (MSCA-ITN). The novel chemical tools developed and presented in this thesis were decisive for unraveling β-L-arabinofuranosidases' mechanism of action, and might serve in the future as probing tools for studies of the human gut microbiome.

This chapter illustrates the value of heparanase in translational biomedicine. The enzymology and main biochemistry of heparanase underlying its role in cancer are covered and an overview of the activity-based protein profiling (ABPP) methodology is provided. The chapter ends with an outline of the content of the experimental chapters.

1.1 Heparanase: biochemistry and enzymology

HPSE is a mammalian *endo*-acting β-D-glucuronidase that acts on heparan sulfate (HS) polysaccharides. The enzyme is nearly ubiquitously expressed in human cells, with high expression levels reported in thrombocytes,[24] stromal cells,[25] mast cells,[26] placental trophoblasts,[27] osteoblasts[28] and immune cells.[26] HS polysaccharides are found in heparan sulfate proteoglycans (HSPGs),[29] which are primary constituents of the vascular extracellular matrix (ECM) and of the basement membrane.

HSPGs typically confer mechanical and structural integrity to the outer layer of endothelial cells. HPSE is initially expressed as latent pre-proenzyme (pre-proHPSE 68 kDa), which is converted into the inactive proenzyme proHPSE (65 kDa) by cleavage of an *N*-terminal signalling peptide intracellularly. The proenzyme is translocated to the Golgi apparatus, and then secreted into the extracellular matrix, where it interacts with membrane-bound proteins – among which syndecans – to undergo endocytosis and subsequent delivery to lysosomes. In the acidic microenvironment of the lysosomal compartments, proteolytic cleavage of a 6 kDa linker

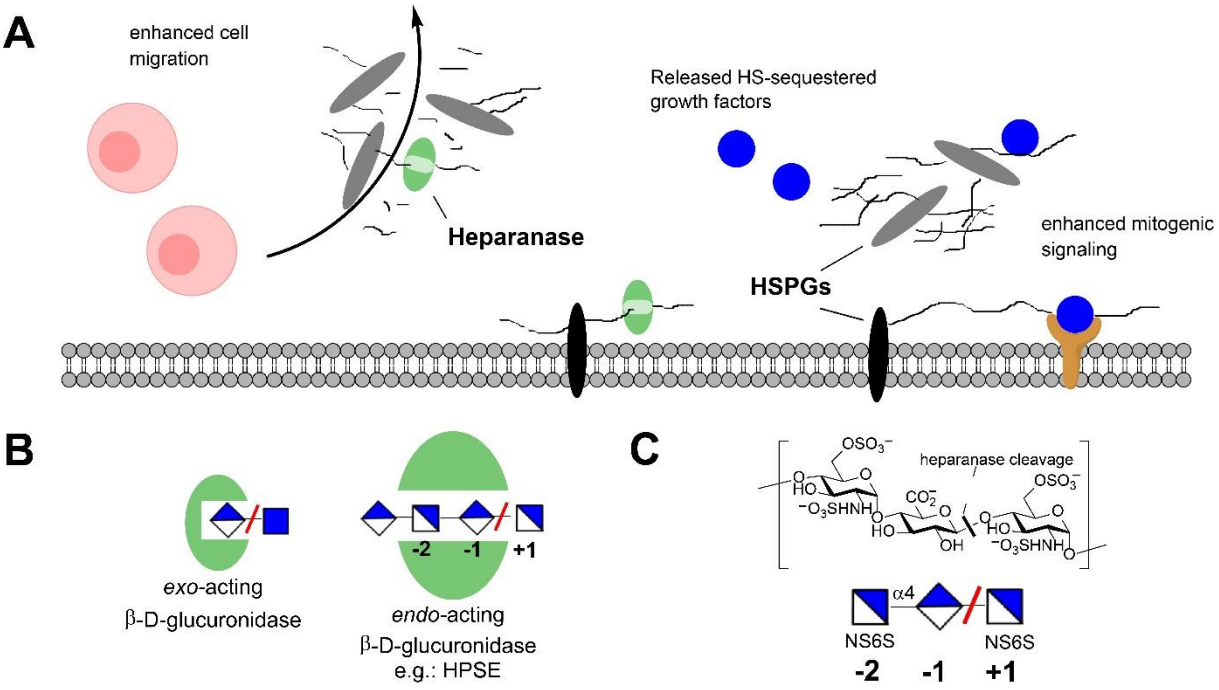


Figure 1.1. (A) Schematic representation of the modes of extracellular matrix degradation mediated by heparanase (HPSE). Degradation of HSPGs results in enhanced release of HS-sequestered growth factors, enhanced mitogenic signaling and cell migration. (B) Difference between *exo*-acting β-D-glucuronidase and *endo*-acting heparanase, showing the site of cleavage between GlcA (at -1 subsite) and GlcNS (at +1 subsite). (C) Site of cleavage by heparanase (HPSE) within the structure of heparan sulfate, showing one of the consensus minimal recognition moieties. Figure based on de Boer *et al*.[30]

region in proHPSE is catalysed by Cathepsin L protease,[31,32] thus releasing the enzymatically-active heterodimeric form of HPSE.[33] Upon maturation, active human HPSE primarily accumulates in lysosomes,[34] whereas distinct intracellular compartments (such as the nucleus[35] and autophagosomes[36]) are additional sites for HPSE subcellular localisation. The mature enzyme affects exosome production and secretion[37] for intercellular communication; and is transported to the extracellular space. In the latter, active HPSE exerts its hydrolytic activity onto ECM-embedded HSPGs, which are often bound to signaling entities such as growth factors[38] and cytokines[39] in the extracellular space (Scheme 1.1).[40] In this way, HPSE enzymatic activity contributes to the remodeling of the ECM and to the regulation of bioavailable HS-binding factors, thereby directly affecting cell adhesion, migration and proliferation. When overexpressed, HPSE enzymatic functions cause excessive HS turnover, breakdown of the ECM and release of HS-sequestered factors. These events lead in turn to an abnormal signaling cascade and to a compromised extracellular matrix, all of which contribute to the formation of a microenvironment promoting angiogenesis[41] and extravasation of cytokines[42] and cancer

cells.[11] Therefore, targeting extracellular HPSE holds potential as therapeutic strategy against tumour angiogenesis and metastasis.[43]

Being the prototypic HPSE binder, heparan sulfate is a polyanionic linear glycan made up of repeating units of glucosamine (GlcNAc/GlcNS) connected to hexuronic acids (GlcA/IdoA) via an α-1,4-glycosidic linkage.[44] The structure of HS features an heterogenous sulfation pattern, which is used as template to direct HS-binding factors to the corresponding sites of binding. The heterogeneity of HS is also of great importance for HPSE processing, because the enzyme acts on selected sites of cleavage after recognising distinct sulfation motifs in the vicinity of the target site. Knowledge of the recognition preferences, catalytic itinerary and structural properties of HPSE is essential for the rational design of HPSE inactivators via canonical structure-based approaches. However, biochemical characterisation of HPSE has been long hampered by the difficulties involved in the isolation of the native enzyme, and in the expression and purification of its recombinant form.[33,45-49] As per the enzymological properties of HPSE, the enzyme cleaves its native substrate at internal positions of the polysaccharidic HS chain, specifically at the β-glycosidic linkage of glucuronic acid units. Therefore HPSE is classified as *endo*-acting glycosidase (Figure 1.1B and 1.2).

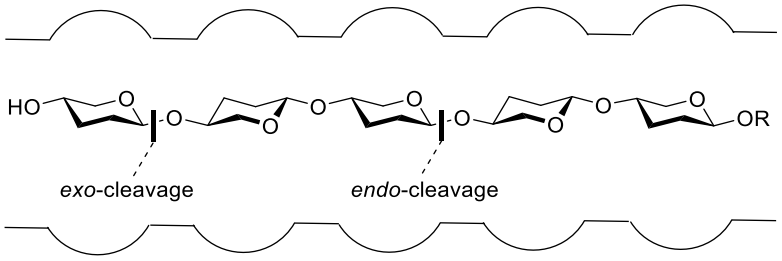
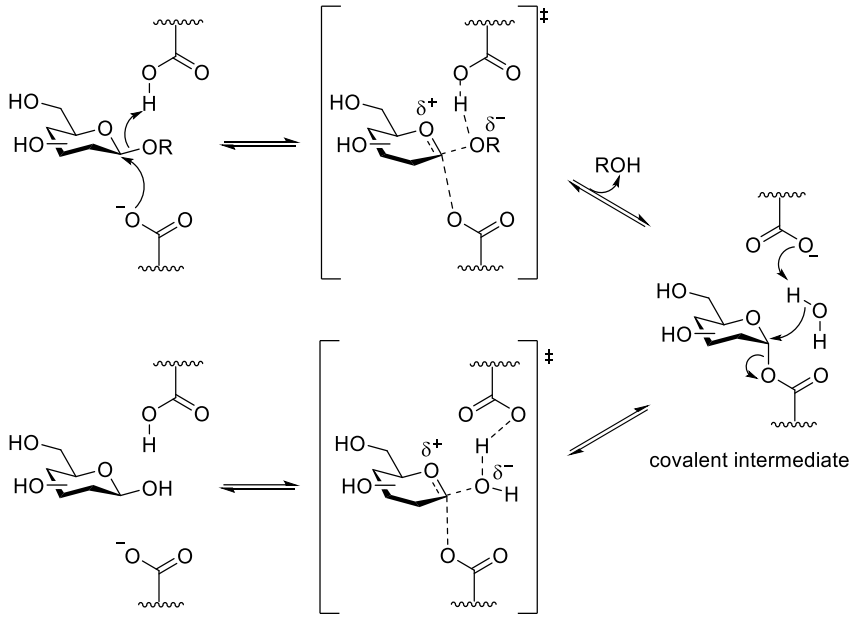


Figure 1.2. *Exo*- and *endo*-glycosidase cleavage sites in a polysaccharide chain.

Despite the initial lack of consensus regarding the preferential recognition moiety in HS, sulfation is deemed important for HPSE recognition and cleavage, and nowadays the trisaccharide sequence GlcN(S/Ac)6S-GlcA-GlcNS6S is accepted as one of the minimal preferred recognition motifs (Figure 1.1C),[50-53] and it is thought that the nature of the recognition motif varies slightly in function of nearby glycosidic units present in HS chain.[53] The stereochemical outcome of HPSE-catalysed hydrolysis involves retention of configuration at the anomeric centre, as a result of the Koshland double-displacement mechanism followed by the enzyme (Scheme 1.1). The mechanism of action of most retaining GHs was first presented by Koshland in 1953.[54] Originally, this mechanistic model didn't describe the nature of the catalytic residues, which were not determined at the time. From this standpoint, the

Koshland double-displacement model might account for additional retaining GHs using an atypical catalytic nucleophile, as in the case of sialidases possessing a tyrosine as catalytic nucleophile.[55-56] Nonetheless, Koshland double-displacement model doesn't cover the mode of action of non-canonical retaining GHs requiring cofactors (for instance, NAD[57] or L-ascorbate[58]) or the participation of a neighboring group (for instance, a 2-hydroxy[59] or a 2-*N*-acetyl[60,61] group). Given these atypical mechanistic modalities, the majority of retaining glycosidases employ a pair of carboxylic acid/carboxylate resulting from key residues, aspartic acid/aspartate or glutamic acid/glutamate, and evolve through a transition state with significant oxocarbenium-like character. In retaining glycosidases, the catalytic itinerary intercepts an oxocarbenium-like transition state in each of the two S_N2-type displacement steps.



Scheme 1.1. General mechanism of hydrolysis of a β-linked D-glycoside employed by retaining glycosidases as described by the Koshland double-displacement mechanism.[54]

The first step involves attack by a catalytic nucleophile with concomitant protonation of the leaving aglycon by the catalytic acid/base, which originates a covalent substrate-enzyme intermediate with inversion of anomeric configuration in an S_N2-type fashion. During the second step, a water molecule is deprotonated by the catalytic acid/base and then displaces the anomeric-linked carboxylate while inverting the stereochemistry of the anomeric center. This final step releases the enzyme and the hydrolysed product with net retention of anomeric configuration.

In the CAZy system, in where CAZymes are classified by amino acid sequence similarity,

HPSE falls under GH family 79, a group of retaining β-D-glucuronidases characterised by a high degree of amino acid sequence similarity yet differing in their substrate specificities. Enzymes belonging to GH79 display either *exo*- or *endo*-activity, indicating that they hydrolyse their substrates at the non-reducing terminus of a glycan substrate (i.e., *exo*-acting) or at internal positions of the polysaccharide chain (i.e., *endo*-acting) as illustrated in Figure 1.2. The variability of the site of enzymatic hydrolysis has been ascribed to the occurrence of a so-called '*exo*-pocket loop', a short variable amino acid sequence occluding the catalytic cleft of *exo*-acting GH79 members – such as *A.capsulatum* GH79 (*Ac*GH79)[62] and *Burkolderia pseudomallei* heparanase (*Bp*Hep)[63] – and thereby contouring a binding site preferentially for monosaccharidic substrates.' Insight into the structural differences across GH79 family further highlights the need to design inhibitory scaffolds primed for HPSE selectivity over *exo*-acting retaining β-D-glucuronidases. This represents a major research topic that the present Thesis aims to address.

1.2 Heparanase inhibitors in translational medicine

From the original discovery of a partially purified platelet enzyme able to cleave heparin and heparin-like oligosaccharides,[64] HPSE has emerged as prospective pharmacological target for the treatment of HPSE-mediated human pathologies. Because of the biological functions of the ECM and of the ECM-embedded HSPGs, the degradative activity exerted by HPSE on HSPGs and on the ECM underpins a wide array of physiological processes related to vital cell functions, including cell migration, proliferation and invasion, and cell-cell interactions. As a result, abnormal changes in the ECM structure and organisation imparted by HPSE[65-67] are connected to several human pathologies, most prominently malignancies, diabetic nephropathy, chronic inflammation, amyloidosis, viral infection and distinct autoimmune disorders. To date, an extensive body of information concerning the involvement of HPSE in cancers is available, and most biomedical studies on HPSE are directed to the development of therapies for HPSE-mediated cancers.[68] This section covers the main therapeutic modalities for HPSE-mediated malignancies and the inherent challenges in HPSE drug development.

A strong foundation for HPSE-targeted cancer therapy lies in the significant pro-angiogenic and pro-metastatic properties displayed by the enzyme in multiple cancer types. In several human malignant tissues, HPSE overexpression strongly correlates with increased metastasis and tumour angiogenesis, one of the hallmarks of cancer, and preclinical studies have demonstrated that angiogenesis and metastasis can be inhibited by HPSE inactivation.[69,70] HPSE mediates angiogenesis by releasing HS-bound growth factors, most importantly vascular

endothelial growth factor (VEGF) and fibroblast growth factor (FGF), upon enzymatic cleavage of HS chains.[71] Dysregulated release of growth factors in turn activates VEGF and FGF signaling cascades, leading to a significant increase in neovascularisation, characteristic of tumour phenotype. In adjunct to the pro-angiogenic quality, HPSE holds pro-metastatic effect which is strictly related to the interaction of HS with cell adhesion molecules, mostly integrins and selectins, facilitating cell-cell interactions at the basis of metastatic spread.[72] Importantly, both pro-angiogenic and pro-metastatic properties of HPSE have been found strongly dependent on the extracellular localisation and secretion of the enzyme, as demonstrated in a seminal preclinical study conducted on nonmetastatic mouse lymphoma cells inoculated with secreted or intracellular HPSE.[73] The findings of this study laid the groundwork for the identification of HPSE as pharmacological target in cancer treatment.

Today, it is acknowledged that HPSE contributes to cancer progression through pathways which are in some cases reliant on its non-enzymatic and scaffolding functions (rather than its enzymatic activity),[74-76] yet the molecular mechanisms by which non-enzymatic HPSE properties are implicated in tumour biology have not been fully elucidated. Therefore, to neutralise the aberrant biological activity of HPSE in tumour, inhibition of HPSE enzymatic activity has become the primary and most intensively studied therapeutic modality to date.[77] The field of HPSE drug design is dominated by chemically-diverse structures sought as HPSE inactivators, including modified heparins, sulfated oligo-/polysaccharides, antibodies, glycomimetics, small molecules and oligonucleotides. The majority of these have been designed based on structural similarity with endogenous HPSE ligands, and only a scarce number have been developed via computer-aided structure-based approaches. This rainbow of prospective HPSE inactivators is perhaps representative of the difficulty in identifying the structural determinants for effective HPSE engagement without employing the native ligand. Modified heparins and polysulfated oligosaccharides feature as ‘best-in-class’ candidates in reason of their elevated binding affinity for the desired HPSE target, accompanied by enhanced anti-metastatic and anti-angiogenic activities. Remarkably, all HPSE inhibitors that have progressed to clinical studies thus far (SST0001,[78] M402,[79] PI-88,[80] PG545[81]) are polyanionic oligo-/polysaccharidic competitive inhibitors (Figure 1.3), however, none of these have met the requirements for clinically-approved therapeutic efficacy.

This might be ascribed in part to the physico-chemical and pleiotropic properties of these compounds. In particular, their high polyanionic character is likely to contribute to the fast clearance observed *in vivo*.

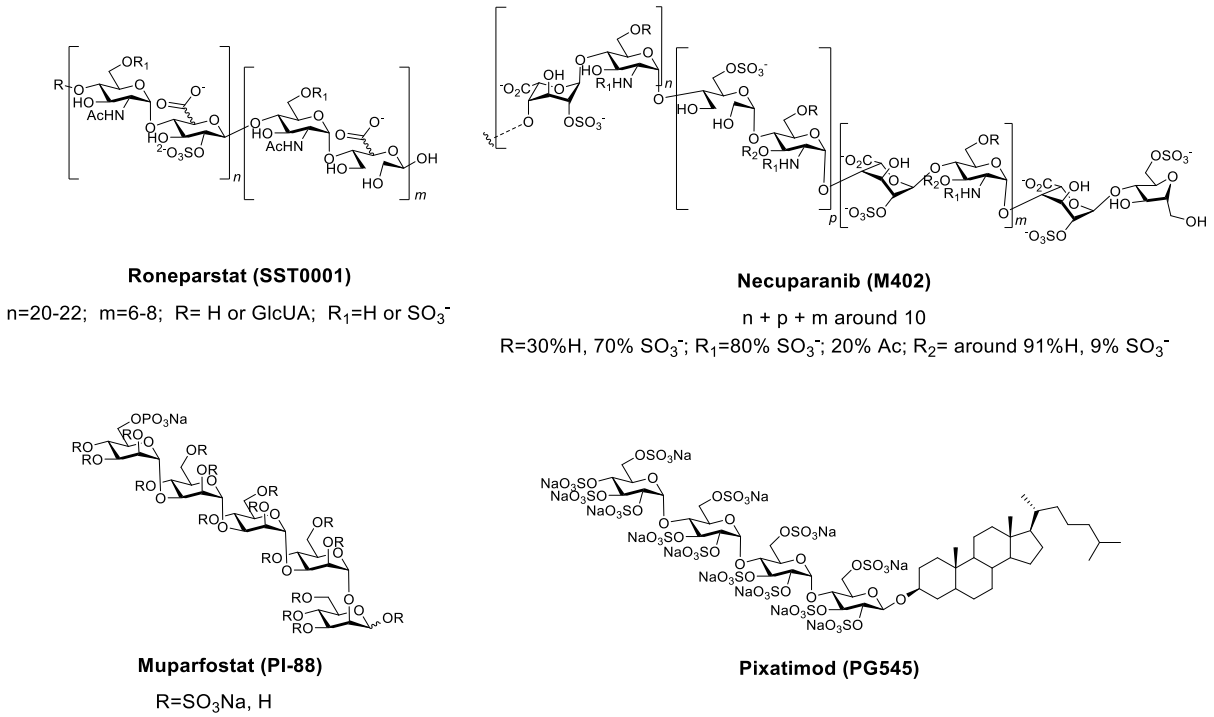


Figure 1.3. Chemical structures of HPSE inhibitors which have undergone clinical trials for the treatment of human malignancies.

In addition, the competitive mode of inhibition might not be suitable for sites of disease possessing elevated concentration of HS substrates, which might compete with reversible HPSE inhibitors depending on the inhibition kinetic profiles of distinct drug candidates. Because of the competitive inhibitory modality, the inhibitory efficacy of clinical candidates against HPSE activity exploits a high number of affinity-based non-covalent interactions with the binding subsites of the enzymatic pocket. The required high-affinity multi-subsite engagement of HPSE is attained with polysaccharidic structures rather than monosaccharide-type competitive inhibitors. Nonetheless, distinct monosaccharide-type 1-*N*-iminosugars, such as siastatin B (**1**) and its trifluoroacetamide analogues (**2-4**, Figure 1.4),[82-84] have displayed competitive HPSE inhibition and anti-metastatic effects. Such structures might be valuable in the generation of oligo-saccharide-like iminosugars as HPSE competitive inhibitors.

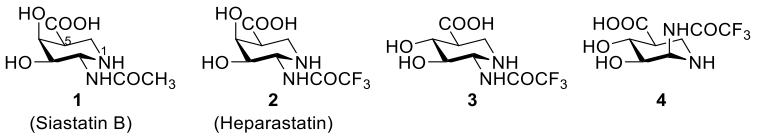
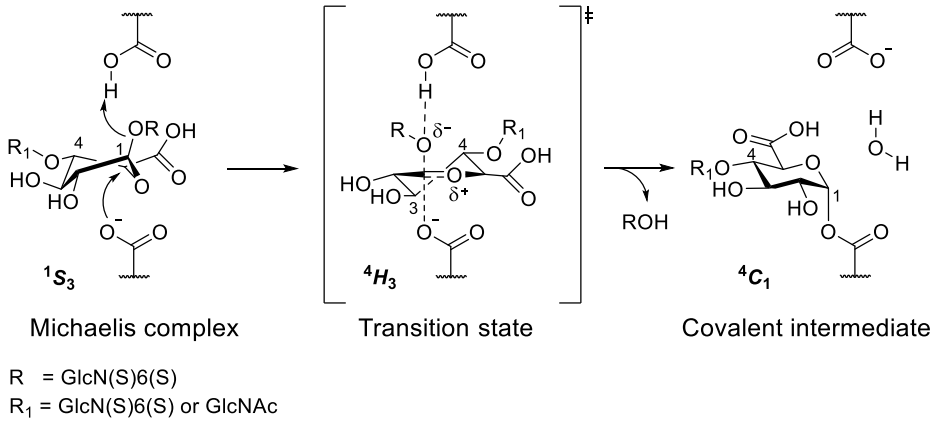


Figure 1.4. Chemical structures of monosaccharidic 1-*N*-iminosugars **1-4** possessing competitive inhibitory potency against HPSE and anti-metastatic activity.

1.3 Covalent, mechanism-based inhibitors of heparanase

As opposed to competitive inhibitors, rational design of covalent inhibitors is a viable strategy which does not necessitate an extensive network of high-affinity interactions for effective inhibition of *endo*-acting glycosidase targets as HPSE. Recently, mechanism-based, covalent and irreversible drug designs have come to the fore despite the initial concerns regarding their stability and reactivity.[85] Covalent targeting ensures prolonged target engagement, which might result in favourable pharmacodynamic properties and exquisite potency. Notable blockbuster covalent drugs on the market for cancer treatment are the Bruton's tyrosine kinase (BTK) inhibitor Ibrutinib (AbbVie)[86] and the epidermal growth factor receptor (EGFR) inhibitor Osimertinib (AstraZeneca).[87] For retaining glycosidases, incorporation of an electrophilic reactive moiety, canonically a strained three-membered ring epoxide or aziridine, yields covalent trapping of the nucleophilic catalytic residue in GHs, thereby compensating for their initial moderate or transient binding.[88,89] Glycosidases significantly distort the conformation of saccharidic substrates during catalysis, hence studying the conformational changes imparted to the native substrates are informative for designs of Michaelis complex and transition state (TS) mimetics as covalent inhibitors.[19,20] Heparanase, specifically, skews the chair ($^4C_1$) conformation of the glucuronic acid (GlcA), the energetically-preferred one in solution, to form a Michaelis complex featuring the substrate in a skew boat ($^1S_3$) conformation,[90] so that the aglycon is oriented pseudo-axially for effective nucleophilic attack by the catalytic nucleophile (Scheme 1.2).



Scheme 1.2. Conformational itinerary adopted by heparanase during the first half of the hydrolytic reaction of heparan sulfate catalysed by HPSE. The scheme illustrates the processing of the glucuronic acid unit in heparan sulfate.

During the transition state, further distorsion of the glucuronic ring into a higher energy half-chair ($^4H_3$) conformation allows to arrange atoms C5, O5, C1, and C2 in a coplanar orientation,

in which a transient oxocarbenium double bond can be formed between atoms O5 and C1. The transition state evolves then into a glycosyl-enzyme covalent intermediate in which the $^4C_1$ conformation of the glycon is restored. The second half of the enzymatic reaction entails the same distinct conformations of the first half, yet in reverse order. Thereby, HPSE processes its substrates via a $^1S_3 \rightarrow [^4H_3]^{\ddagger} \rightarrow {}^4C_1$ itinerary, just as retaining β-glucosidases[91] and *exo*-acting β-D-glucuronidases.

Specific and potent covalent mechanism-based inhibitors and probes for distinct GH targets can be attained through systematic chemical modification of functional groups and stereochemistry present in cyclophellitol, a naturally-occurring covalent inhibitor of retaining β-glucosidases.[21] In line with this strategy, *glucurono*-configured cyclophellitol **5**[92] proved to be an excellent *in vitro* and *in situ* inhibitor of retaining *exo*-acting β-D-glucuronidases.

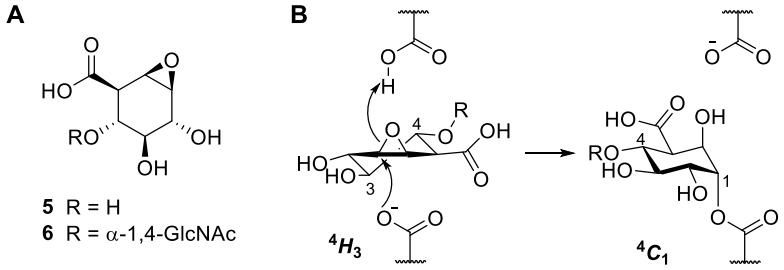


Figure 1.5. (A) Chemical structure of glucuronic cyclophellitol **5** and of HPSE mechanism-based inhibitor **6**. (B) Mode of action by mechanism-based inhibitors **5** and **6** against HPSE, with details of the conformational itinerary.

Subsequent extension of the glucuronic cyclophellitol into a pseudo-disaccharidic structure yielded a panel of covalent inhibitors amongst which compound **6** displayed significant reduction of cancer aggression *in cellulo* and amelioration of murine metastasis.[30] The preclinical efficacy of pseudodisaccharide **6** however is counteracted by its moderate drug-like physico-chemical properties. To date, achieving enhanced inhibitory efficacy and sufficient drug-likeness in a single HPSE-inactivating structure represents a major challenge in the field of HPSE drug design, and part of the research described in this Thesis addresses this aspect.

1.3 Assessment of heparanase activity for drug development

A major caveat in HPSE drug development is represented by the lack of a unified robust *in vitro* assay for measurements of HPSE enzymatic activity and inhibitor efficacy, which prompts careful comparison of reported half maximal inhibitory concentration (IC_{50}) values.[93,94] However, the current state of HPSE inhibitor assessment might change in the future as novel highly sensitive and robust fluorogenic substrates appear in literature. An example from this

category is a recently published difluorocoumarin-based fluorogenic scaffold.[95] Over the years, multiple assays were developed based on the use of cell-based or cell-free formats which directly or indirectly quantify the loss of HS substrates. Immobilisation of fluorescently/radioactively labeled HS substrates on a solid phase has been most intensively employed in HPSE-targeted research for several decades. This method involves detection of released labeled HS material and separation from the original intact substrate by gel-filtration analysis.[96,97,98] Beyond being cumbersome and time-consuming, methodologies based on labeled HS are subjected to significant variability due to the intrinsic complexity and heterogeneity of HS substrates, which makes the preparation of their labeled forms difficult to standardise. Other primary assessment modalities feature the detection of loss in HS substrates conjugated to an enrichment reporter (for example, biotin) for affinity-based measurements,[99] or use of antibodies against growth factors possessing high binding affinity for HS.[100] Recently, colorimetric[101] and fluorescence assays[102] which exploit the reaction of Fondaparinux, a commercially-available homogenous substrate of HPSE, with a water-soluble chromogenic dye or fluorescent marker have found more extensive application.

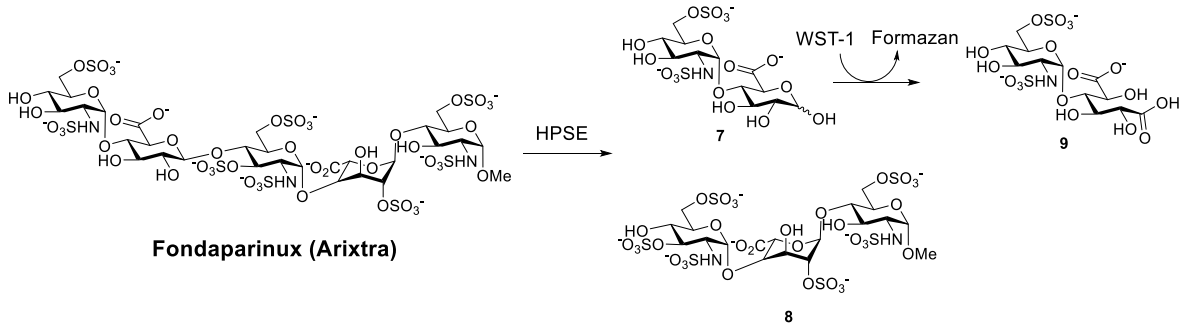


Figure 1.6. Schematic representation of the set of reactions which conventional Fondaparinux-based assays of HPSE enzymatic activity rely upon.[101]

Typically, a chromogenic dye (such as tetrazolium salt 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate termed WST-1) reacts with the disaccharide product (**7**) of the heparanase-catalysed Fondaparinux cleavage (Figure 1.6), thus allowing time-dependent quantification of the released reducing product **7** through absorbance measurement. By making use of an homogeneous substrate with one individual point of cleavage, this method allows to derive the inhibition kinetics against purified HPSE and to screen potential inhibitors in a simplified and more robust way. For instance, this method[101] has led to the successful identification of clinical candidate PG545,[103] and is amenable to high-throughput screening against recombinant HPSE. Nonetheless, the broad reactivity of the employed dyes towards non-reducing sugars present in complex biological samples makes

colorimetric and fluorometric Fondaparinux-based assays unsuitable for tests in more complex native biological settings.

To further complement information obtained from primary cell-free experiments, secondary cell-based assays of HPSE activity are frequently used. These experiments allow to read out proliferation and cell viability in tumour cell lines,[104] angiogenesis in tumour cells embedded in Matrigel,[41] or cell adhesion on cellware dishes mimicking the composition of the basement membrane. The scarce versatility and variability of several assays for HPSE as described above underscore the need for a streamlined and direct measurement of HPSE enzymatic activity. For retaining glycosidases, this is canonically accomplished by employing commercially available glycoside substrates conjugated to a fluorogenic aglycon, the hydrolysis of which is detected by fluorometric measurements. Recently, an increasing number of fluorogenic substrates with higher HPSE selectivity over *exo*-acting retaining β-D-glucuronidases has appeared in literature,[105] and in some instances these substrates have been instrumental to defining the conformational itinerary of HPSE.[90] Despite the suboptimal turnover rates of most published fluorogenic substrates today, derivatisation of fluorinated coumarins with HS-mimicking disaccharides has recently enabled access to a set of fluorinated coumarin-derived fluorogenic substrates.[95,106] Among these, a difluorocoumarin-based compound[95] displayed significantly higher HPSE affinity compared to Fondaparinux and elevated HPSE selectivity over other GHs. These distinctive features confer higher robustness and sensitivity to HPSE inhibition assays based on the use of this difluorocoumarin-derived substrate compared with precedent HPSE-targeted fluorogenic substrates. The proven effectiveness of this difluorocoumarin-based substrate in semi-high-throughput screening of HPSE inhibitors makes this scaffold a valuable research tool over conventional Fondaparinux-based assay formats for future research in the field of HPSE drug discovery.

An alternative solution for this current gap in HPSE-focused research is use of activity-based probes (ABPs) possessing elevated labeling efficacy towards HPSE. In HPSE-targeted research, ABPP methodology has been first established by the serendipitous discovery of HPSE in fibroblasts as target of cyclophellitol-inspired ABPs designed for labeling of retaining β-D-glucuronidases.[92] Recently, the panel of HPSE-selective ABPs has been expanded by employing disaccharide-type structures as more specific recognition motifs.[30] This design was proven to exclude the labeling of *exo*-acting forms. However, concomitant labeling of HPSE and *exo*-acting β-D-glucuronidases in complex biological milieu is of great value in assessing

the inhibitory selectivity and stability of covalent small-molecule HPSE inhibitors (Section 1.4). It is in this direction that part of this Thesis aims to contribute (Chapter 2 and 3).

1.4 Activity-based protein profiling

First described by Benjamin F. Cravatt in 1999,[107] activity-based protein profiling (ABPP) has become an established technology for the study of the functional state of enzymes from distinct classes in complex biological systems.[108,109] The technique entails the design of an activity-based probe (ABP) which covalently binds an enzyme or a group of enzymes from a distinct class, thus leading to the irreversible inactivation of the target(s).

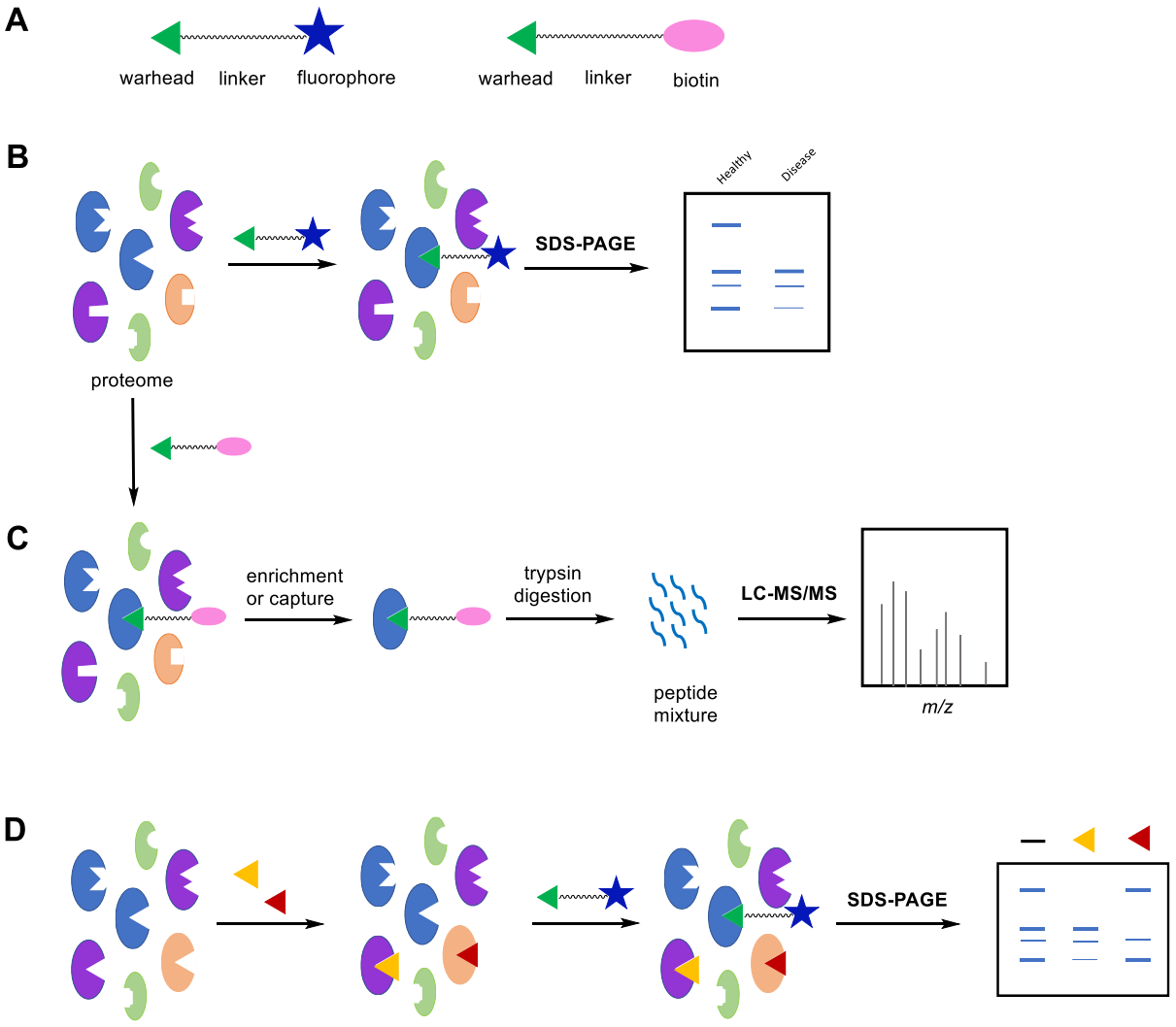


Figure 1.7. Activity-based protein profiling (ABPP) formats. (A) General structure of an activity-based probe (ABP) equipped with a fluorophore or with a biotin reporter; (B) Comparative ABPP experiment with fluorescent ABPs can be used for detection and analysis of enzyme activities by SDS-PAGE; (C) ABP-enabled enrichment in a proteome leading to identification of the target enzymes by proteomics; (D) Competitve ABPP (cABPP) enables discovery of enzymatic inhibitors.

ABPs feature a modular structure comprising three fundamental components: a reactive moiety (or warhead), at times annexed to a recognition motif, a reporter group and a linker (Figure 1.7A). The reactive moiety is designed to react with a catalytically active amino acid in the enzymatic site of the targeted enzyme or enzyme class to form a covalent, stable bond. As reporter group, fluorescent dyes (such as differently functionalised BODIPY, cyanine or rhodamine fluorophores) or enrichment agents (for instance biotin or a bio-orthogonal group) are commonly placed to visualise and enrich ABP-labeled proteins for subsequent studies, including immunoblotting or proteomic analysis. The linker, installed on the warhead in certain cases, consists of a linear chain of variable lipophilicity or hydrophilicity and ensures sufficient spacing between the reactive group and the reporter. For retaining glycosidases, specifically, covalent irreversible cyclophellitol-type inhibitors are proven suitable warheads, and chemical manipulation of their functional groups and of their configurational pattern often enables to tune the selectivity for a desired GH target. Installation of a reporter group via a linker on an aziridine[110,111], or at times on a distinct position of the recognition motif,[112] gives access to ABPs tailored for retaining glycosidases.[109,113-116]

Because of the versatility of ABPP experiments, this technique has found application in different formats. ABPP has emerged as powerful technology for biomedical research,[117-118] and has been integrated into different stages of the drug discovery and development process.[119] In one of its original formats, ABPP is used for the identification and biochemical characterisation of enzymes with potential pharmacological value. Generally, ABPs do not label precursor enzymes or enzymes with absent or malfunctioning activity, hence quantitated levels of ABP-modified proteins can be correlated with the expression levels of active enzymes. In this way, ABPP allows to discriminate the enzymatic activities of distinct phenotypes, for instance for the comparative analysis of healthy and disease cohorts. Comparative ABPP experiments are suitable with either a fluorescent ABP or an affinity-based reporter group, conventionally biotin. In the case of a fluorescent reporter, for instance, ABP-labeled proteins can be detected by in-gel fluorescence scanning after incubation of a proteome with a fluorescent ABP, followed by protein denaturation and resolution by SDS-PAGE (Figure 1.7B). Equipping an ABP with a biotin affinity tag grants access to alternative ABPP formats (Figure 1.7C). These workflows rely on the strong affinity interaction between biotin and its binding counterpart streptavidin, which is typically conjugated to a secondary reporter (such as horseradish peroxidase, HRP)[120] or immobilised on beads (such as streptavidin agarose beads). In early studies of Bertozzi and Vocadlo,[120] labeled proteins were identified on blot by use of a

streptavidin-HRP conjugate after protein denaturation and resolution by SDS-PAGE. Although Western blotting is an effective technique for target identification, it primarily suffers from background noise due to endogenously biotinylated proteins and consequently reduced sensitivity. The limitations of Western-blotting are circumvented in ABP-based pulldown experiments (Figure 1.7C). In this workflow, ABP-labeled proteins are enriched by affinity-based pulldown with streptavidin magnetic beads (Figure 1.7C), thus decreasing nonspecific labelling. Following on-bead digestion by a proteolytic enzyme (typically trypsin), the enriched proteins are degraded into peptides and the resulting peptide mixture is analysed by LC-MS/MS, from which protein sequences can be obtained with high sensitivity. Matching of the obtained sequences against distinct protein sequence databases allows for identification of the target protein.

Next to the identification of functional proteins, discovery of covalent enzymatic inhibitors is another primary application of ABPP. Methodologies named competitive ABPP (cABPP)[121] enable to screen compounds against enzymes in native proteomic mixtures, without prior purification or knowledge of the identity of a labeled enzyme (Figure 1.7D). This is a major advantage compared to conventional inhibitor screening methods. In cABPP experiments, ABP labeling is preceded by pre-incubation of a recombinant protein or biological sample with a covalent inhibitor. Upon denaturation and resolution by SDS-PAGE, quantification of the decrease in probe labeling intensity is used as read-out of inhibitory potency. In the case that the ABP employed hits multiple enzymes in the same sample, the selectivity of covalent inhibitors can be determined. In recent years, cABPP has emerged as a powerful tool to assess target engagement and selectivity profiles of potential drug candidates in the early stages of drug discovery research.[122] Noteworthily, competitive ABPP often makes use of fluorescence polarisation measurements to screen the inhibitory effects of enzymatic inhibitors on labeled proteins (FluoPol-ABPP).[123-125] As this ABPP modality is amenable to HTS platforms, FluoPol-ABPP has found application in the early discovery of drug hits. This, together with the other ABPP platforms discussed previously, make ABPP an essential technology to drive forward drug discovery and development programs.

1.5 Outline of this thesis

Currently, cyclitol-based inhibitors and probes have appeared as chemical tools to modulate and functionally interrogate HPSE enzymatic activity. The work presented in this Thesis contributed in part to expanding the panel of viable scaffolds amenable to glucuronic cyclophellitol lead optimisation. The first two chapters of this Thesis pertain the design and

development of irreversible HPSE cyclophellitol-type inhibitors with reduced hydrophilicity and expected enhancement in metabolic stability compared to precedent cyclophellitol-based inhibitors for the target enzyme. **Chapter 2** discusses the design and synthesis of a set of mechanism-based inhibitors of heparanase derived from canonical cyclophellitol, and their inhibitory activity and selectivity are biochemically evaluated against recombinant retaining β-D-glucuronidases and on complex biological samples. **Chapter 3** describes the synthesis of two mechanism-based heparanase inhibitors, namely a cyclophellitol-inspired carba-disaccharide with enhanced potency and expected enhanced hydrolytic stability together with its cyclophellitol-based pseudo-disaccharide analogue. The construction of the stabilised carbadisaccharidic inhibitor involves regioselective epoxide ring opening of a perbenzoylated carba-*manno*-1,2-epoxide in order to emulate conventional α-1,4-glycosidation, and is followed by further chemical elaboration, giving access to the desired cyclitols. **Chapter 4** extends the application of ABPs and inhibitors to the elucidation of the mechanism employed by rare microorganismal β-L-arabinofuranosidases. This Chapter reports on the synthesis of a panel of carba-β-L-arabinofuranosyl aziridines and epoxides as inhibitors and activity-based probes, as well as the biochemical evaluation of their activity towards recombinant retaining β-L-arabinofuranosidases. The research activity presented in this chapter was conducted within the Sweet Crosstalk MSCA-ITN. **Chapter 5** concludes this Thesis with a summary of each chapter and some directions for future work based on the here presented results.

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