Summary

This thesis describes the characterization and application of activity-based probes (ABPs) labeling lysosomal glycosidases in mechanism-based manner. Lysosomal glycosidases are acid hydrolases that fragment glycoconjugates in lysosomes. Several inherited diseases in man concern deficiencies of lysosomal glycosidase. The corresponding lysosomal storage disorders (LSDs) show characteristic lysosomal accumulation of undegraded substrates. The **General introduction** provides an overview of lysosomal glycosidases and the associated LSDs. Described are the present therapeutic options and diagnostic methods for these LSDs. Attention is paid to the current knowledge on the life cycle of lysosomal glycosidases and their catalytic mechanisms. Understanding on the latter has allowed generation of activity-based probes (ABPs) irreversibly labeling lysosomal glycosidases in mechanism-based manner. Present ABPs for lysosomal glycosidases are based on cyclophellitol and cyclophellitol aziridine scaffolds. This thesis describes how these probes can be employed as versatile research tools to study the disease-relevant enzymes.

Chapter 1 describes developed protocols for gel-based and microscopy-based detection of lysosomal glycosidases, in particular the β-glucosidase GBA, labeled with ABPs *in vitro* and *in vivo*. Cyclophellitol-type ABPs with an electrophilic epoxide group and a BODIPY fluorophore allow specific and sensitive visualization of active GBA molecules following their irreversible labeling. Labeling can be performed *in vitro* using enzyme preparations or cell and tissue extracts. Due to the amphiphilic nature of the ABP *in situ* labeling and detection of active GBA in cells and organisms is also feasible. The described protocols for ABP labeling of GBA can be extended to other cyclophellitol- and cyclophellitol aziridine-based ABPs labeling distinct glycosidases.

Chapter 2 investigates the *in vivo* target engagement of conduritol B epoxide (CBE) and cyclophellitol (CP) in cells and animals using a gel-based competitive activity-based protein profiling (cABPP) approach. CBE and CP are mechanism-based irreversible GBA inhibitors and have been used to generate Gaucher disease (GD) models in cells and animals. These inhibitors

may have additional *in vivo* glycosidase targets beyond GBA, depending on the applied dose and treatment duration. For the candidate off-target glycosidases a suite of glycosidase ABPs is available which allow the investigation of potential interaction of CBE and CP with these enzymes. Investigation of CBE-treated cells and zebrafish larvae revealed that at higher concentrations CBE also interacts *in vivo* with non-lysosomal glucosylceramidase (GBA2) and lysosomal α-glucosidase (GAA). A tight, but acceptable window for selective inhibition of GBA in the brain of mice is observed. On the other hand, CP was found to inactivate with equal affinity GBA and GBA2 and therefore is not suitable to generate genuine GD-like models.

Chapter 3 reports on novel irreversible inhibitors of GBA that are superior in selectivity towards the enzyme. This class of inhibitors carries a bulky hydrophobic substituent at C8, such as adamantyl and biphenyl-moieties. By gel-based ABPP experiments, it is shown that the novel compounds are far more potent and selective irreversible inhibitors of GBA as compared to CBE *in vitro*, in cultured cells, and in zebrafish larvae. The novel inhibitor with an adamantyl moiety was found to be brain-permeable and to allow generation of an authentic neuropathic GD model in zebrafish by exposure of animals to the compounds via food uptake.

Crucial for optimal application of novel glycosidase ABPs in LSD research is a careful biochemical characterization, which includes mechanism of action, inhibition kinetics, *in vitro* and *in vivo* potency, and identification of off-target glycosidases.

Chapter 4 describes the characterization of newly developed ABPs for the lysosomal glycosidases α-glucosidase (GAA) and β-glucuronidase (GUSB), enzymes deficient in Pompe disease and mucopolysaccharidosis type VII (MPSVII; Sly Syndrome), respectively. The ABPs label their respective target enzymes (GAA and GUSB) potently and in mechanism-based manner. The α-glucose configured ABP labels not only the lysosomal α-glucosidase GAA, but also the ER α-glucosidase II (GANAB). It was observed that at higher concentration GBA is a major off-target of both ABPs. By altering the labeling pH, and by pre-incubating samples with a specific GBA inhibitor, GAA and GUSB can be specifically visualized with the corresponding ABPs *in vitro* as well as in lysates of cells treated *in situ* with the ABPs. The diagnostic potential of the GAA ABP for Pompe disease is demonstrated with cultured fibroblasts from patients.

Chapter 5 documents the development and characterization of α-L-iduronide configured cyclophellitol aziridine ABPs for human α-L-iduronidase, whose inherited deficiency leads to

mucopolysaccharidosis type I (MPS I, Hurler syndrome). The new ABPs are shown to react irreversibly in mechanism-based manner with human recombinant α-L-iduronidase (rIDUA, Aldurazyme®). They exhibit lower potency and slower inhibitory kinetics compared to previous ABPs designed for other retaining glycosidases, possibly due to conformational constraints. The relative low affinity of the present ABP makes detection of endogenous IDUA in cells and tissues challenging. The fluorescent Cy5 ABP can efficiently label therapeutic iduronidase *ex vivo* and the enzyme's delivery to lysosomes—as intended in enzyme replacement therapy (ERT)—was successfully monitored by fluorescence microscopy of MPS I patient fibroblasts.

Chapter 6 reports on the biochemical evaluation of α- and β-mannose configured cyclophellitol aziridine ABPs for the retaining exo-α-mannosidases (Glycoside Hydrolase (GH) family 38) and exo-β-mannosidase (GH family 2)—enzymes implicated in diverse pathologies including cancer and LSDs. The ABPs label their respective target mannosidases in mechanism-based manner and are micromolar inhibitors. All five GH38 α-mannosidases are labeled by α-mannose configured ABPs in mouse testis homogenates. Each enzyme was labeled in lysates of cells upon overexpression. The unique molecular weight and pH optimum of each mannosidase allows their simultaneous activity-based profiling in complex biological samples, and should assist future screening for small molecule inhibitors/activators of these highly relevant enzymes.

β-Mannose configured ABPs label the GH2 β-mannosidase (MANBA) in mouse kidney extracts. Co-labeling of GBA occurs that can be prevented by pre-incubating samples with a GBA inhibitor.

Chapter 7 provides the biochemical characterization of β-galactose configured cyclophellitol aziridine ABPs, intended to label lysosomal β-galactosidase (GLB1) and galactocerebrosidase (GALC). The deficiency of GLB1 causes GM1 gangliosidosis (and Morquio B syndrome) whilst deficiency of GALC leads to Krabbe disease. The examined ABPs inhibit and label in mechanism-based manner recombinant and endogenous GLB1 and GALC. GLB1-like protein 1 and 2 (GLB1L and GLB1L2) are additionally labeled, two putative β-galactosidases with poorly understood physiological roles. The β-glucosidases GBA and GBA2 are major off-targets of β-galactose configured cyclophellitol aziridine ABPs. These new ABPs can assist in future fundamental and applied research on human β-galactosidases.

The thesis is concluded with a discussion summarizing the most important findings and highlighting the current and future applications of cyclophellitol and cyclophellitol aziridine-based ABPs in LSD research.