Picomolar inhibition of $\beta$-galactosidase (bovine liver) attributed to loop closure

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Abstract

In an effort to examine similarities in the active sites of glycosidases within the GH35 family, we performed a structure-activity-relationship study using our recently described library of galactonoamidines. The kinetic evaluation based on UV/Vis spectroscopy disclosed inhibition of $\beta$-galactosidase (bovine liver) in the picomolar concentration range indicating significantly higher inhibitor affinity than previously determined for $\beta$-galactosidase (A. oryzae). Possible alterations in the secondary protein structure or folding were excluded after further examination of the inhibitor binding using CD spectroscopy. Molecular dynamics studies suggested loop closing interactions as a rationale for the disparity of the active sites in the $\beta$-galactosidases under investigation.

Graphical Abstract

Keywords

inhibition; $\beta$-galactosidase; Amidine; carbohydrate
1. Introduction

Approximately 1% of all genes known to date code for glycoside hydrolases [3.2.1] documenting their ubiquity in Nature.[1] Glycoside hydrolases are divided into 14 clans based on structural similarities, and then further subdivided into 130 families based on reaction mechanisms, evolutionary relationships, and substrate specificity.[2] All β-galactosidases [3.2.1.23] belong to clan A and contain an (α/β)8 (TIM) barrel as catalytic domain. Two glutamic acid residues are located in β-strands four and seven of this TIM barrel and act as acid-base donor and nucleophile during catalytic turnover.[3]

β-Galactosidases are isolated from plants,[4] fungi,[5] bacteria,[6], and mammalian tissue, [7] and are found in four families of glycoside hydrolases (GH), namely GH-1, GH-2, GH-35, and GH-42.[8] Currently, about 1700 glycoside hydrolases are assigned to GH-35 including β-galactosidases,[9] exo-β-glucosaminidases, exo-β-1,4-galactanases and β-1,3-galactosidases. Among those, about 700 β-galactosidases are known,[9] but only 10 of them are structurally characterized using X-ray diffraction.[10] In the absence of structural data, information on stabilizing forces in the active site of an enzyme has been deduced from spectroscopic data.[11] The characterization of stabilizing forces between inhibitors and β-galactosidases may lead to the discovery of new therapeutics for diseases associated with these enzymes, such as lysosomal disorders,[12] diabetes,[13] and several neurological disorders.[14]

In this context, we set out to elaborate the differences and similarities during catalytic glycoside hydrolysis of β-galactosidases from A. oryzae (4IUG)[5] and bovine liver, using UV/vis and CD spectroscopy. Both enzymes belong to GH-35 and cleave exo-β-D-galactopyranosyl residues from oligosaccharides or other glycoconjugates in a retaining fashion.[15] Our spectroscopic results disclose distinct differences between the two β-galactosidases. Molecular dynamic studies suggest contributions of a loop closure for the β-galactosidase from bovine liver as stabilizing interaction within its active site.

2. Results and discussion

Our previous structure-activity relationship (SAR) studies using a library of galactonoamidines 1a-y (Chart 1) characterized all synthesized compounds as competitive inhibitors and revealed nanomolar inhibition of the catalytic activity toward β-galactosidase (A. oryzae).[16–18] The combined results indicated hydrophobic interactions with the aglycon,[16–18] and H-bonds with the glycon as a driving force for the interactions between inhibitors and amino acids within the active site. Similar results were expected for the inhibition of β-galactosidase from bovine liver that is classified in the same glycoside hydrolase family based on sequence similarity and mechanism. However, the extended SAR studies revealed distinct differences between the two β-galactosidases indicating altered support of inhibitor binding that caught our attention for an in-depth investigation. In absence of structural data, we hypothesized that a combination of π-π stacking, hydrophobic and possibly loop closure interactions, cause the noted dramatic difference in the inhibition of galactonoamidines toward the selected β-galactosidases. The details of the study are summarized and discussed below.
2.1. Structure-activity relationship

While glyconoamidines exist in tautomeric forms with endo- or exocyclic double bonds in a solution of organic solvents depending on their structure, the compounds are protonated under assay conditions in aqueous solution.[19] The resulting positive charge is delocalized (Chart 1).

Notably, a small number of galactonoamidines with aromatic and aliphatic aglycons (1d, l, m, p–r) showed comparable efficacy toward both enzymes rendering them efficient inhibitors of either hydrolase (Figure 1a). The efficacy of all other inhibitors 1 is remarkably higher toward β-galactosidase (bovine liver) than for β-galactosidase (A. oryzae) (Table 1, Figure 1b).[16]

The results suggest different stabilization of the corresponding inhibitor binding in the active sites of the two monomeric β-galactosidases under investigation but do not disclose the source of this discrimination. All studied galactonoamidines 1a–y were characterized as competitive inhibitors toward both β-galactosidases using Lineweaver-Burk, Dixon and Eddie-Hofstee plots, and representative examples using 1a are depicted (Figures 2a & b). All kinetic data were analyzed by non-linear regression applying the Michaelis-Menten model. Apparent catalytic rate constants (k_cat and k′_cat) and affinity constants (K_M and K′_M) were determined in absence and presence of galactonoamidines 1a–y. The inhibition constants (K_i) (Tables 1 & 2) were deduced from these data by standard methods.[20]

Analysis of the inhibition constants reveals that the affinity of any inhibitor 1 is about two orders of magnitude higher toward β-galactosidase (bovine liver) than toward β-galactosidase (A. oryzae). The resulting inhibition constants are in the picomolar instead of nanomolar concentration range. Consequently, the active site of the β-galactosidase (bovine liver) must support the inhibitor binding substantially better than that of β-galactosidase (A. oryzae). A synergy of H-bond interactions, π-π stacking, and hydrophobic interactions alone is unlikely to serve as a rationale to sufficiently explain this observation. Additional interactions, such as distorted protein folding upon inhibitor binding, or loop closure-supported catalysis may be present, which has been previously noted for other proteins with TIM barrels.[21–24] Further experiments using CD spectroscopy were therefore conducted to illuminate the inhibitor binding in the active sites of the β-galactosidases.

2.2. Effect of inhibitor binding on protein folding evaluated by circular dichroism spectroscopy

A possible influence of the inhibitor binding on protein structure and folding of both glycosidases was explored using CD spectroscopy with selected inhibitors. Amidine 1a was employed as the lead compound as it showed overall the lowest inhibition constant (K_i = 50 ± 10 pM) reflecting the highest binding affinity upon interacting with β-galactosidase (bovine liver). Amidine 1v was selected as an example for an inhibitor with modified glycon, and amidine 1x was chosen as a control compound without aglycon (for structures of 1v and 1x, see Table 2). The CD spectra of all assemblies between β-galactosidase (bovine liver) and inhibitors reveal only minor changes in the observed molar ellipticity (λ_max = 225 ± 3 nm) (Figure 3).
This observation indicates an unaltered three-dimensional structure of the enzyme and unaffected folding pattern upon coordination of the selected galactonoamidines. Similar conclusions were drawn by others for related studies.[25, 26] Consequently, alterations in the protein folding are unlikely to cause the observed strong inhibitor binding. The CD spectra obtained from interactions between the selected inhibitors and β-galactosidase (A. oryzae) are likewise unaffected.

Unfortunately, our attempts to derive and predict three-dimensional structures from the measured CD data by taking advantage of previously developed programs, K2D3[27] and BESTsel,[28] were futile. This conclusion was reached after comparing the identified amounts of α-helices and β-sheets of the published structure for β-galactosidase (A. oryzae) [5] to computed data derived from the measured CD data. The high amount of β-sheets determined by X-ray diffraction is not calculated correctly. Similar problems were noted by other researchers on similar occasion previously.[28] Consequently, the corresponding CD data were not analyzed further. Instead, molecular dynamic studies were performed in an attempt to provide a rationale for the drastically different affinity of the galactonoamidines upon binding to the selected enzymes.

### 2.3. Effect of inhibitor binding on protein folding evaluated by molecular dynamics studies

While the structure of β-galactosidase (A. oryzae) (4IUG)[5] is available in the Protein Database, the three-dimensional structure of β-galactosidase (bovine liver) is still unknown. Therefore, we constructed a model of this enzyme based on homology-based comparative modeling using the Robetta server.[29] Predicting a protein structure from an amino acid sequence is a grand challenge. While the Rosetta suite of programs used by the Robetta server is considered the state-of-the-art,[30] we are well aware of the limitations of our approach. In this specific case, however, we do benefit from the high degree of homology between β-galactosidase (bovine liver, Q58D55) and β-galactosidase (Homo sapiens, P16278), for which the solid state structure has been determined by X-ray diffraction (3wf3). In addition, we noted almost identical structures around the active site for the five highest-ranked models returned by the Robetta server. For example, the maximum root mean square error (RMSE) is 0.13 Å for all residues within a 20 Å distance from the active site for the five homology models. Such a good structural agreement is probably due to a very good sequence homology with β-galactosidase (H. sapiens) near the active site, as to be expected for enzymes classified in the same GH family.[3]

To examine the quality of the predictions, each model was submitted to the Quality Modeling Energy Analysis (QMEAN) server.[31] An estimate of the model reliability was produced as QMEAN6 score by a combination of 6 linear terms into a composite score (0–1).[32] The structure selected for further analysis had a composite QMEAN6 score of 0.98 indicating a model with a high level of confidence. A BLAST comparison between our model for β-galactosidase (bovine liver) and β-galactosidase (H. sapiens) reveals 518 identical and 73 very similar amino acid residues indicating in a sequence similarity of 90.5 %.

Initially, we performed docking predictions using AutoDock Vina with the inhibitors 1a, 1v and 1x in the active sites of the β-galactosidases from bovine liver and A. oryzae. However,
the predicted free energies of the docked conformations did not differ significantly and thus did not disclose a rational for the experimentally observed superior binding of the inhibitors in the active site of β-galactosidase (bovine liver). Therefore, molecular dynamic studies based on GROMACS were used instead to characterize the inhibitor binding.[33] Interactions with catalytic amino acid residues, π-π stacking promoting aromatic amino acids, and the relative number of H-bond interactions are highlighted. Similar studies to clarify interactions between enzymes and carbohydrate analogs were reported by others recently.[34, 35]

The modeling study suggests that amidine 1a interacts with both catalytic residues of β-galactosidase (bovine liver), namely the proton donor Glu-187 and the nucleophile Glu-267. The modeling suggests that amidine 1v does not stay in the active site, and interacts with Glu-267 only due to steric hindrance. Amidine 1x interacts with both catalytic residues as well, but to some lesser extent as discussed below. Other researchers have previously speculated that two binding pockets exist within the active site of β-galactosidase (bovine liver) to support interactions with the glycon and the aglycon of inhibitors and substrates. [36] Our molecular dynamic studies support this hypothesis and disclose initial binding of the glycon of 1a in one of the binding pockets in the active site followed by binding of its aglycon with subsequent loop closure (Figure 4).

This loop closure occurs via a CH-π stacking interaction between Trp272 and Tyr484 after the induced fit of 1a in the active site (for a movie, see Supporting Information). Its binding is further supported by π-π stacking interactions of its aglycon with Trp272 and Tyr484 (Figure 4). Additionally, inhibitor 1a shows the highest number of H-bond interactions toward amino acids of both β-galactosidases under investigation (Figures 5a & 7a). The corresponding images display well-embedded inhibitor structures (Figures 6a & 8a).

However, the modeling study implies that the aglycon of 1a is nested within a closed loop of β-galactosidase (bovine liver) (Figure 6a), while the active site in β-galactosidase (A. oryzae) remains open (Figure 8a). Similar loop closing interactions were previously reported by others, e.g. for β-galactosidase (E. coli), [6, 37, 38] but were not noted for β-galactosidase (bovine liver) yet.

Although the initial docking study suggests 1v to bind within the active site during the initial docking studies in a similar fashion as the other selected inhibitors, 1v moves out of the active site during the energy minimization with the GROMOS model. As a result, inhibitor 1v stabilizes in a flipped and twisted orientation relative to the other inhibitors only partially within the active site showing π-π stacking interactions with Tyr269 (Figure 6b). This shift causes the inhibitor to interact with only one of the two catalytic residues, namely Glu-267, throughout the entire 30ns simulation. The experimentally determined inhibition constants (see above) indicate a 30,000-fold higher affinity for 1a over 1v. The result is echoed by the observations of the modeling study. Similar conclusions on inhibitor binding in presence of steric hindrance were also previously reported by us and others.[39–41] Additionally, the frequency of H-bond interactions between β-galactosidase (bovine liver) and galactonoamidine 1v is only slightly smaller than for 1a (Figure 5b). As 1x is a control compound lacking the aglycon of 1a and 1v, the corresponding aglycon binding site remains unoccupied (Figure 5C), and the loop closure is incomplete. The modeling study
furthermore reveals that the number of H-bond interactions is overall reduced once a control compound without aglycon is employed (1x, Figures 5c & 7c).

The inhibitor binding toward β-galactosidase (A. oryzae) appears to be driven slightly differently. In short, interactions of amidines 1a and 1x with both catalytic residues are noted; Glu-200 (proton donor) and Glu-298 (nucleophile). Steric hindrance implemented at C-2 of the glycon allows amidine 1v to interact only with the nucleophilic amino acid Glu-298. In contrast to above-described interactions within β-galactosidase (bovine liver), inhibitor 1v binds within the active site of β-galactosidase (A. oryzae) and assumes a different orientation than 1a (Figure 8b). At the same time, the number and frequency of H-bond interactions are significantly lower for amidine 1v than for its parent 1a (Figure 6b).

The combined modeling results provide a rationale for the experimentally observed picomolar inhibition of β-galactosidase (bovine liver) by galactonoamidines 1 compared to weaker nanomolar inhibition toward β-galactosidase (A. oryzae). While the amidines use H-bond interactions for stabilization of the glycon in both glycosidases, a combination of a closed loop, π-π-stacking, and hydrophobic interactions is apparent for galactonoamidine binding toward β-galactosidase (bovine liver).

3. Conclusions

Two β-galactosidases from the same glycoside hydrolase family (GH35) were evaluated upon their interactions with galactonoamidines to compare the efficiency of their active sites. Given the enzyme classification based on structure and mechanism similarities, comparable interactions and inhibition ability were initially expected. All compounds were found to be competitive inhibitors towards both glycosidases. However, SAR studies using UV/vis spectroscopy revealed inhibition in the picomolar concentration range toward β-galactosidase (bovine liver) that is two order of magnitude stronger than previously determined inhibition of the same inhibitors toward β-galactosidase (A. oryzae).

Galactonoamidine 1a was identified as the most potent inhibitor toward both β-galactosidases. CD spectroscopy revealed insignificant alterations of the protein structure upon inhibitor binding that does not account for the noted significant difference in inhibitor potency. Molecular modeling studies using 1a, 1v and 1x suggested loop closure and π-π stacking interactions in addition to hydrophobic interactions and H-bond formation as a rationale for the considerably higher affinity of the galactonoamidine inhibitors toward β-galactosidase (bovine liver). By contrast, only hydrophobic interactions and H-bond formation support the binding of galactonoamidines to β-galactosidase (A. oryzae).

4. Experimental

4.1. Instrumentation

UV/Vis data were recorded on a FilterMax F5 Multi-Mode Microplate Reader from Molecular Devices using 96-well, medium-binding microlon ELISA-plates from Greiner Bio-one. CD spectra were recorded on a Jasco J-1500 CD Spectrometer Model 150 with EXOS liquid cooling system from Koolance Inc. with the supplied Spectra Manager software Version 2.12.00 using a Fisher Scientific Suprasil quartz microcell with 500 μL.
capacity, 45 mm height, and an optical path length l of 10 mm. All pH values were obtained using a Beckman Φ 250 pH meter equipped with a refillable ROSS combination pH electrode from Orion with epoxy body and an 8 mm semi-micro tip. Nanopure water at a resistance of 18.2 mΩ was obtained from a ThermoScientific Barnstead E-pure™ water purification system.

4.2. Materials and Methods

4.2.1. Chemicals—β-galactosidases from bovine liver and Aspergillus oryzae were obtained from Sigma-Aldrich and stored at −18 °C until use. Metal-free sodium hydroxide at 99.999 % purity and HEPES buffer were purchased from Sigma-Aldrich and used as received. All buffer solutions were prepared by standard methods at ambient temperature accounting for temperature differences at their intended use. All galactonoamidines were synthesized as described previously.[16]

4.2.2. UV/Vis spectroscopy

4.2.2.1. Assays using β-galactosidase (bovine liver)

4.2.2.1.1. Enzyme stock solution: The commercially obtained lyophilized powder of β-galactosidase (bovine liver) was dissolved in 5 mL HEPES buffer and stored in 50 μL aliquots at −80 °C until use. A BCA assay revealed a protein content of 93.5591 ± 4.18745 μg/mL that translates into a 139.64 ± 6.25 μM protein concentration of the solution (M = 67,000 g mol⁻¹).[7, 42]

For determination of IC₅₀ values, a 50 μL aliquot of the frozen enzyme solution was thawed, diluted into 5 mL with 5 mM HEPES buffer and stored on ice until use. For determination of inhibition constants, a 20 μL aliquot of the frozen protein solution was used and diluted in identical fashion.

4.2.2.1.2. Substrate stock solution: Substrate stock solutions for the determination of IC₅₀ values were prepared by dissolving 9.15–9.62 mg (0.030–0.032 mmol) of 4-nitrophenyl-β-D-galactopyranoside in 5 mL of 5 mM HEPES buffer immediately prior to use. Stock solutions for the determination of inhibition constants were prepared likewise using 9.98–10.27 mg (0.033–0.034 mmol) of the same substrate in 10 mL of buffer solution.

4.2.2.1.3. Inhibitor stock solution: Appropriate aliquots of a 500 μM aqueous solution of the galactonoamidines 1a–y were diluted with nanopure water yielding 0.005, 0.05, 0.125, 0.25, 0.5, 2.5, 10, 20, 30, 50, 100, and 200 μM inhibitor stock solutions that were stored at ambient temperature until use. For the determination of IC₅₀ values, 11 of those solutions with different inhibitor concentrations were used in 10 μL aliquots. For the determination of Kᵢ values, 2 or more solutions with concentrations between 0.5 and 1.5 nM (1a, 1c–d, 1f–j, 1l–m, 1o–r), 1 and 2 nM (1b, 1k, 1n, 1s–t), 5 and 6 nM (1u), 2,500 and 10,000 nM (1v–w), 10,000 and 20,000 nM (1x), 5,000–50,000 nM (1y) were used,

4.2.2.1.4. As say t o determine IC₅₀ values: Typically, a 10 μL aliquot for each of 11 different inhibitor stock solutions was mixed with 30 μL substrate stock solution and 20 μL of the buffer solution in a 96-well plate. After equilibration at 30 °C over 30 min, a 40 μL
aliquot of the enzyme stock solution was added. The resulting solutions were thoroughly mixed, and the change in absorbance followed at 405 nm over 240 min in 10 min intervals. In a control experiment, the aliquot of the inhibitor solution was replaced by the same amount of buffer solution. All experiments were done as duplicates.

4.2.2.1.5. Data analysis to determine IC$_{50}$ values: The final absorbance read for the enzymatic substrate hydrolysis in presence and absence of inhibitor were transferred into percentages of overall inhibition, and plotted against the inhibitor concentration in logarithmic scale.

4.2.2.1.6. As say to determine K$_i$ values: Typically, a 0–70 μL aliquot of the substrate stock solution was pipetted in 96-well plates in 10 μL increments, a constant 10 μL aliquot of the inhibitor stock solution was added, and the nominal volume of all solutions increased to 80 μL with appropriate amounts of buffer solution. The resulting solutions were equilibrated at 30 °C for 30 min prior to addition of a constant 20 μL aliquot of enzyme stock solution. All solutions were thoroughly mixed, and the change of absorbance was read immediately at 405 nm for 45 min in 27s intervals. All experiments were performed in duplicate or more.

4.2.2.1.7. Data analysis to determine K$_i$ values: The measured change in absorbance was plotted over time in presence and absence of inhibitor to determine the initial rate of the substrate hydrolysis and transformed into concentration by using the apparent extinction coefficient for 4-nitrophenolate at pH 7.5 in 5 mM HEPES buffer and a total volume of 100 μL ($e_{mol} = 13,300 ± 60 M^{-1} cm^{-1}$). The obtained initial rates were corrected for enzyme concentration and graphed as a function of the respective substrate concentration. The resulting hyperbolic data were fitted by non-linear regression to determine apparent Michaelis-Menten constants (K$_m$ and K’$_m$) and rate constants (k$_{cat}$ and k’$_{cat}$) in absence and presence of inhibitors, respectively. All experiments were conducted in duplicate, and the data were averaged. Kinetic parameters for at least two different inhibitor concentrations were determined, and Lineweaver-Burk plots ([E]/rate vs 1/[S]) constructed to visualize and examine the inhibition type. The inhibition constant (K$_i$) for competitive inhibition was then calculated from equation (1):

$$K'_m = K_m \times (1 + ([I]/K_i)) \quad (1)$$

4.2.2.2. Assays using β-galactosidase (A. oryzae): The experimental procedure describing the cleavage of 2-chloro-4-nitrophenyl β-D-galactopyranoside by β-galactosidase (A. oryzae) and the inhibition of this reaction in the presence of galactonoamidines 1a–y is described in detail elsewhere.[16, 17, 43] The developed assays were used for selected inhibitors to determine kinetic parameters, inhibition constants, and IC$_{50}$ values as described.
4.2.3. CD spectroscopy

4.2.3.1. Enzyme stock solution: Typically, an aliquot of the above-described β-galactosidase (bovine liver) solution was thawed and diluted to 5 mL with 5 mM HEPES buffer yielding an enzyme stock solution that was kept on ice until use.

4.2.3.2. Inhibitor stock solutions: Solutions of 1a, 1v, and 1y were prepared by dissolving 3–5 mg of the freeze-dried compound in 10 mL of nanopure water in volumetric flasks. Subsequent serial dilutions of 30–50 μL aliquots afforded 100 μM stock solutions of the respective inhibitors that were stored at ambient temperature prior to use.

4.2.3.3. Assay to measure ellipticity: Typically, 250μL of the enzyme stock solution was mixed with the same volume of 100 mM inhibitor stock solution. After equilibration, the ellipticity of the resulting solution was measured between 190–275 nm with a resolution of 0.5 nm, and the data from 5 accumulation scans were averaged. The resulting data were adjusted for contributions of unbound inhibitor and buffer effects by background corrections prior to data analysis.

4.2.3.4. Data analysis: The measured specific rotation of the circularly polarized light θ [mdeg] was transformed into molar ellipticity [θ] in [deg M⁻¹ m⁻¹] by using equation (1),

$$\text{[θ]} = \theta \times 100/(c \times l) \quad (1)$$

the molar extinction coefficient Δε in [deg M⁻¹ m⁻¹] by using equation (2),

$$\Delta \varepsilon = [\theta]/3298.2 \quad (2)$$

and the mean residue molar ellipticity [θ]_MR [deg M⁻¹ m⁻¹ residue⁻¹] by using equation (3),

$$[\theta]_\text{MR} = [\theta]/N; \quad (3)$$

θ represents the ellipticity in degree, c the molar protein concentration, l the optical pathlength [cm] and N the number of residues in the protein (N=653[44] and 1005[45], respectively). The resulting rotation data were smoothed by a 7-point Savitzky-Golay filter and plotted against the wavelength λ [nm].

4.2.4. Molecular Dynamics—The structure of β-galactosidase (bovine liver) is not known yet, and was thus predicted through comparative modeling using the Robetta server and the known structure of β-galactosidase (H. sapiens).[29] Out of the five highest-ranked models returned, one was selected for further analysis based on its QMEAN6 score of 0.98, which indicates a very high quality prediction.[31] The structure of β-galactosidase (A. oryzae, 4IUG) was obtained from the Protein Data Bank (http://www.rcsb.org/pdb/).[10] All
water molecules, solvents, and substrates were removed from the structure file prior to processing simulations.

4.2.4.1. Docking studies of glycosidases with coordinated galactonoamidine inhibitors: Mol files of the galactonoamidine inhibitors were initially converted into pdb files using PYMOL,[46] and then transferred into pdbqt files that allow all non-ring bonds to rotate during docking with the Python Molecular Viewer, vers. 1.5.6.[47]Likewise, the coordinates of the enzymes were transferred from pdb into pdbqt files after addition of Kollman charges using AutoDock Tools.[48] A 30 × 30 × 30 Å³ grid box was set at the center of the active site of β-galactosidase (A. oryzae), while a 15 × 15 × 15 Å³ grid box was used for β-galactosidase (bovine liver). Docking calculations were performed in the presence of the selected inhibitors using AutoDock Vina at default parameters.[49] Nine conformations were visualized for each of the docked amidines with PYMOL.[46] The conformation with the lowest free energy estimate for each of the inhibitor-enzyme assemblies was chosen for subsequent molecular dynamic studies and exported as pdb file; β-galactosidase (bovine liver) with 1a (−6.7 kcal/mol), 1v (−5.9 kcal/mol), and 1x (−5.3 kcal/mol); β-galactosidase (A. oryzae) with 1a (−7.1 kcal/mol), 1v (−6.9 kcal/mol), and 1x (−5.8 kcal/mol).

4.2.4.2. Molecular dynamic studies of glycosidases with coordinated galactonoamidine inhibitors: All molecular dynamic simulations were performed with GROMACS 4.6.7.[50] The GROMACS coordinate (“.gro”) and topology (“.top”) files of the enzymes were produced with pdb2gmx and the GROMOS96 43A1 force field.[33] The inhibitor conformation with the lowest free energy, as predicted by AutoDock Vina, was exported as a pdb file and submitted to the PRODRG server to obtain the coordinate (“.gro”) and topology (“.itp”) files.[51] It has been recognized that the partial charges produced by PRODRG lead to artifacts when used with the GROMOS force field.[52] The partial charges of the inhibitors were thus edited based on a best practice approach suggested by Lemkul.[52] The enzyme-inhibitor assembly was simulated using the combined gro and topology files based on the configuration predicted by Autodock Vina.[53] The complex derived from β-galactosidase (bovine liver) and the selected inhibitors were solvated with 41945–41956 water molecules in a 1367 nm³ cubic box using the single point charge (SPC) model.[54] Likewise, the corresponding complexes from the inhibitors and β-galactosidase (A. oryzae) were solvated with 75208–75214 SPC water molecules in a slightly larger 2389 nm³ cubic box. Subsequently, random water molecules were replaced by sodium ions to neutralize the system, i.e. for β-galactosidase (bovine liver): 1 Na⁺ and for β-galactosidase (A. oryzae): 28 Na⁺. The solvated enzyme-inhibitor complexes were then minimized using the steepest-decent method to remove possible steric clashes.[33] Afterward, a NVT simulation at 303K over 100 ps and a NPT simulation at 1 bar over 500 ps were performed using the stochastic rescaling thermostat.[55] Last, a 31 ns NPT simulation was performed using the Nosé-Hoover thermostat [56] with a relaxation time of 2.0 ps and the Parrinello-Rahman barostat [57] with a relaxation time of 5.0 ps. The final 30 ns of the NPT simulation were treated as production trajectories for data analyses.
All molecular dynamic studies were carried out using a 2 fs time step size. The Coulomb interactions were treated with the particle-mesh Ewald method with a real space cutoff of 12 Å and a fourth-order spline with a Fourier grid spacing of 1.3 Å.[58, 59] Lennard-Jones interactions were also cut off at 12 Å.[60] All bond lengths were constraint using a fourth order LINCS algorithm.[61] The number of H-bonds was determined using the H-bond plugin implemented in the Visual Molecular Dynamics (VMD) program with a cut-off distance for donor-acceptor interactions of ~ 3.0 Å and a cut-off angle for donor-H atom-acceptor assemblies of 20°.[62]

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Efficacy of the inhibition of β-galactosidases from bovine liver (solid blue circle) and A. oryzae (open cyan triangle) during substrate hydrolysis in presence of galactonoamidines (a) 1d; and (b) 1h; I in nM.
Figure 2.
Lineweaver-Burk plots depicting competitive inhibition of the hydrolysis of model substrates by 1a for (a) β-galactosidase (bovine liver) in presence of 0 nM (wine), 0.05nM (red) and 0.075 nM (orange) inhibitor concentrations; and (b) β-galactosidase (A. oryzae) at 0 nM (wine), 0.05nM (purple) and 0.075 nM (magenta) inhibitor concentrations.
Figure 3.
CD spectra of β-galactosidase (bovine liver) (a) without inhibitor (black, $\lambda_{\text{max}} = 224$ nm); in presence of (b) 1a (red, $\lambda_{\text{max}} = 225$ nm), (c) 1v (blue, $\lambda_{\text{max}} = 225$ nm); and (d) 1x (green, $\lambda_{\text{max}} = 227$ nm).
Figure 4.
Snapshots of the induced fit of 1a in the active site of β-galactosidase (bovine liver) (a) before, (b) during and (c) after loop closure.
Figure 5.
Number of H-bonds in correlation to their occurrence for \( \beta \)-galactosidase (bovine liver) in presence of (a) 1a, (b) 1v, and (c) 1x as observed by molecular dynamics simulations over 30ns.
Figure 6.
Active site of β-galactosidase (bovine liver) with coordinated galactonoamidines (a) 1a, (b) 1v, and (c) 1x extracted from the molecular dynamic studies.
Figure 7.
Number of H-bonds in correlation to their occurrence for β-galactosidase (*A. oryzae*) in presence of (a) 1a, (b) 1v, and (c) 1x as observed by molecular dynamics simulations over 30ns.
Figure 8.
Active site of β-galactosidase (A. oryzae) with coordinated galactonoamidines (a) 1a, (b) 1v, and (c) 1x extracted from the molecular dynamic studies.
Chart 1.
Galactonoamidines 1a–y at assay conditions

R = H
R = aliphatic linear, cyclic
R = aryl
Table 1

Inhibition of the hydrolysis of model substrates by β-galactosidases from bovine liver (5 mM HEPES buffer, pH 7.50; $k_{cat} = 4.24 \pm 0.26$ [min$^{-1}$]; $K_M = 0.51 \pm 0.01$ mM) and A. oryzae (50 mM acetate buffer, pH 5.00; $k_{cat} = 2400 \pm 140$ [min$^{-1}$]; $K_M = 0.70 \pm 0.01$ mM) in the presence of galactonoamidines 1a–t.

![Chart 1: Structures of galactonoamidines 1a-t](image)

| Compound | Aglycon R | β-galactosidase (Bovine Liver) | β-galactosidase (A. oryzae)[16] |
|----------|-----------|-------------------------------|-------------------------------|
|          | $K_i \pm \Delta K_i$ [nM] | IC$_{50}$ [nM] | $K_i \pm \Delta K_i$ [nM] | IC$_{50}$ [nM] |
| **Aromatic aglycon, parent structure** | | | | |
| 1a | 0.05 ± 0.01 | 18.9 | 21.1 ± 1.2 | 49 |
| **Aromatic aglycon with electron-donating substituents** | | | | |
| 1b | 0.20 ± 0.02 | 118 | 23.6 ± 2.4 | 623 |
| 1c | 0.11 ± 0.01 | 16.2 | 29.4 ± 5.2 | 131 |
| 1d | 0.07 ± 0.01 | 57.6 | 8.0 ± 0.5 | 36 |
| Compound | Aglycon R | $\beta$-galactosidase (Bovine Liver) | $\beta$-galactosidase (A. oryzae)[16] |
|----------|-----------|-----------------------------------|-----------------------------------|
| 1e       | ![Image](image1.png) | $K_i \pm \Delta K_i$ [nM] | $IC_{50}$ [nM] |
| 1f       | ![Image](image2.png) | $0.23 \pm 0.01$ | 43.1 | $16.1 \pm 0.3$ | 117 |
| 1g       | ![Image](image3.png) | $0.13 \pm 0.02$ | 9.4 | $20.3 \pm 2.3$ | 115 |
| 1h       | ![Image](image4.png) | $0.19 \pm 0.02$ | 9.1 | $10.1 \pm 2.5$ | 331 |
| 1i       | ![Image](image5.png) | $0.16 \pm 0.01$ | 15.2 | $15.7 \pm 0.1$ | 122 |
| 1j       | ![Image](image6.png) | $0.12 \pm 0.01$ | 26.2 | $17.1 \pm 3.4$ | 150 |

**Aromatic aglycon with electron-withdrawing substituents**

**Cyclic aliphatic aglycon with methylene spacer**
| Compound | Aglycon R | β-galactosidase (Bovine Liver) | β-galactosidase (A. oryzae) [16] |
|----------|-----------|-------------------------------|-------------------------------|
| 1k       | ![Image](1k.png) | $0.39 \pm 0.07$ | $> 1400$ | $9.5 \pm 1.1$ | $36$ |
| 1l       | ![Image](1l.png) | $0.08 \pm 0.01$ | $11.6$ | $11.3 \pm 0.9$ | $22$ |
| 1m       | ![Image](1m.png) | $0.16 \pm 0.03$ | $45.4$ | $6.3 \pm 0.6$ | $36$ |
| Linear aliphatic aglycon with aromatic end group or branching |
| 1n       | ![Image](1n.png) | $0.32 \pm 0.02$ | $1030$ | $149 \pm 2$ | $226$ |
| 1o       | ![Image](1o.png) | $0.11 \pm 0.01$ | $24.1$ | $10.8 \pm 0.7$ | $107$ |
| 1p       | ![Image](1p.png) | $0.24 \pm 0.03$ | $42.7$ | $48.4 \pm 3.3$ | $33.5$ |
| 1q       | ![Image](1q.png) | $0.18 \pm 0.02$ | $21.6$ | $7.8 \pm 1.4$ | $36$ |
| Compound | Aglycon R | $K_i \pm \Delta K_i$ [nM] | $IC_{50}$ [nM] | $K_i \pm \Delta K_i$ [nM] | $IC_{50}$ [nM] |
|----------|-----------|-----------------------|----------------|-----------------------|----------------|
| 1r       | ![Structure 1r](image1) | 0.06 ± 0.01            | 41.3           | 8.6 ± 1.2              | 36             |
|          |           |                       |                |                       |                |
|          |           | Cyclic aliphatic without spacer |
| 1s       | ![Structure 1s](image2) | 0.32 ± 0.02            | $> 1000$       | 31.9 ± 1.8             | 369            |
|          |           |                       |                |                       |                |
| 1t       | ![Structure 1t](image3) | 2.34 ± 0.30            | $> 1000$       | 602 ± 52               | 4870           |

**Chart 1.** Structures of galactonoamidines 1a-t
Inhibition of the hydrolysis of model substrates by \( \beta \)-galactosidases from bovine liver (5 mM HEPES buffer, pH 7.50) and \textit{A. oryzae} (50 mM acetate buffer, pH 5.00) in the presence of galactonoamidines \textbf{1d, 1u–y}; \( R = -\text{CH}_2-\text{C}_6\text{H}_4-\text{CH}_3 \)

| Compound | Glycon structure | \( \beta \)-galactosidase (Bovine Liver) | \( \beta \)-galactosidase (\textit{A. oryzae}) |
|----------|-----------------|--------------------------------------|--------------------------------------|
| 1d       |                 | \( K_i \pm \Delta K_i [nM] \) | \( IC_{50} [nM] \) | \( K_i \pm \Delta K_i [nM] \) | \( IC_{50} [nM] \) |
| 1u       |                 | \( 0.60 \pm 0.07 \) | 850 | \( 2,400 \pm 300 \) | 33,300 |
| 1v       |                 | \( 1,500 \pm 220 \) | 10,800 | \( 3,400 \pm 300 \) | 41,400 |
| 1w       |                 | \( 840 \pm 60 \) | 9,200 | \( 1,400 \pm 200 \) | 40,700 |
| 1x       |                 | \( 2,000 \pm 200 \) | \( > 50,000 \) | \( 150 \pm 20 \) | 830 |
| Compound | Glycon structure | β-galactosidase (Bovine Liver) | β-galactosidase (A. oryzae) |
|----------|-----------------|-------------------------------|-----------------------------|
| 1y       | ![Glycon Structure](image) | $K_i \pm \Delta K_i$ [nM] | $IC_{50}$ [nM] |
|          |                 | 2,700 ± 260                  | 8,700                       |
|          |                 |                               | 615 ± 30                    |
|          |                 |                               | > 50,000                    |