Supporting Information

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SI Materials and Methods

Molecular Biology. Recombinant protein was made in insect cells using genes for wild-type and E367K human α-NAGAL (including the native signal sequence and C-terminal His_6 tags) that were cloned into pIB/V5-HIS TOPO-TA vectors using TOPO cloning as described (1). The N201Q (a glycosylation mutant for crystallography), S160C, R329W, and E367K (Schindler/Kanzaki mutants) were created by site-directed mutagenesis (Phusion; NEB). For cellular studies, α-NAGAL gene was cloned into the p3xFLAG-CMV-14 vector (Sigma), creating a C-terminal fusion of the FLAG epitope.

Insect Cell Culture. Tn5 (High Five) insect cells were transfected with the α-NAGAL/pIB/V5-HIS plasmid in T25 flasks. Following transfection, cells were cultured in the presence of 100 μg/mL blasticidin to establish a stably transfected polyclonal cell line. After selection, 1-L cultures were grown in 3-L baffled polycarbonate Fernbach shake flasks (Corning). Cultures grown in serum-free medium [either SFX-Insect (HyClone) or a homemade equivalent (2)] produced 1–2 mg protein/L.

Protein Purification. Proteins were purified as previously described (1). Briefly, spent culture medium was concentrated and buffer exchanged into a Ni-column loading buffer with a tangential-flow filter. Initial purification on a Ni^{2+}-Sepharose-FF column (GE Lifesciences) was followed by a SOURCE15Q anion-exchange column (GE Lifesciences) at pH 6.0. Purified α-NAGAL fractions were pooled and concentrated to 10–20 mg/mL.

Enzyme Inhibition. DGJNAc was synthesized as described (3), and DGJ was purchased (Toronto Research Chemicals). To determine the K_i of DGJ and DGJNAc for α-NAGAL, we measured the IC_{50} as a function of enzyme concentration, as DGJNAc is a tight-binding inhibitor. Two millimolar colorimetric substrate *para*-nitrophenyl-α-*N*-acetylgalactosamine (*p*NP-α-GalNAc) (Toronto Research Chemicals) in assay buffer (100 mM phosphate, 100 mM citrate buffer, pH 4.5) was added to an equal volume of purified α-NAGAL (0.0125–0.2 mg/mL) and inhibitor in assay buffer with 0.1 mg/mL BSA. Inhibitor concentrations ranged from 50 pM to 100 μM for DGJNAc, 50 pM to 1 mM for DGJ, and 10 μM to 100 mM for GalNAc and Gal. After incubation for 10–40 min at 37 °C, samples were diluted 30-fold into 200 mM sodium borate, pH 9.8, and absorbance measurements were taken at 400 nm. Semilog sigmoid dose–response curves were fit in KaleidaGraph to determine IC_{50} values according to the following:

$$\text{normalized activity} = A_{min} + \frac{A_{max} - A_{min}}{1 + 10^{b(\log[I] - \log IC_{50})}},$$

where [I] is the inhibitor concentration, A_{min} and A_{max} are the minimum and maximum activities, and b is the Hill coefficient describing the slope. IC_{50} values for tight-binding competitive inhibitors were modeled as follows (4):

$$IC_{50} = K_i \cdot (1 + [S]/K_M) + [E]_T/2,$$

where [S] and $[E]_T$ are the substrate and enzyme concentrations, and K_M is the Michaelis constant of 0.70 μM for the substrate (1). K_i values were extracted from four-parameter global fits of sigmoid dose–response curves in pro Fit (Quantum Soft) according to the following:

$$\text{normalized activity} = A_{min} + \frac{(A_{max} - A_{min})}{1 + \left(\frac{[I]}{K_i(1 + [S]/K_M) + [E]_T/2}\right)^b}$$

with the symbols as above.

For the specificity assays, insect cell-expressed α-NAGAL, α-GAL A, or GLB1 at 0.025 mg/mL was incubated with 3.25 mM *p*NP-α-Gal (α-GAL A assays), *p*NP-α-GalNAc (α-NAGAL assays), or *p*NP-β-Gal (GLB1 assay), and tested for inhibition with 20 μM DGJ or DGJNAc. Assays were quenched with 200 mM sodium borate, pH 9.8, and absorbance was read at 400 nm.

Proteolysis. We applied a limited proteolysis assay (5, 6) to wild-type and E367K α-NAGAL, where the amount of α-NAGAL resistant to thermolysin digestion reflected the stabilizing effect of the pharmacological chaperones. The 1.5 mg/mL purified α-NAGAL was preincubated with 0–1000 μM DGJNAc or DGJ, and then diluted 10-fold into 4 M urea, 0.1 M Tris·HCl, pH 7.2. Following overnight incubation at room temperature, 0.5 mg/mL thermolysin (in 10 mM $CaCl_2$, 50 mM NaCl, 20 mM Tris·HCl, pH 8.3) was diluted eightfold into the α-NAGAL–compound mixtures. Digestions ran for 10 min at 37 °C and were quenched with 0.5 M EDTA and boiling before SDS/PAGE. Gels were stained with GelCodeBlue (Pierce), imaged, and quantified in GeneTools (SynGene).

X-ray Crystallography. Crystals were grown [in 8–16% (wt/vol) PEG 3350, 70 mM citric acid, and 30 mM Bis-Tris propane, pH 3.4], separated, and harvested as previously described (1), but larger crystals grew at pH 3.4. Crystals were transferred to harvest buffer (16% PEG 3350, 70 mM citric acid, and 30 mM Bis-Tris propane, pH 3.4). Crystals were then transferred to harvest buffer containing ligand and/or cryoprotectant [5 mM DGJNAc and 23% (vol/vol) glycerol, 50 mM DGJ and 23% glycerol, or 20% (wt/vol) D-glucose] for 10 min before cooling in liquid nitrogen. For the iminosugar soaks, 1000–1350 frames of 0.2° phi-sliced diffraction data per crystal were collected at beam lines X6A and X25 at Brookhaven National Laboratory. Diffraction data were processed with HKL2000 (7) and phased by Fourier synthesis using protein atoms from Protein Databank (PDB) code 3H53 (1). Three structures were built in Coot (8), and refined in Refmac (9) with matched 5% R_{free} sets. Given the high-resolution diffraction data, NCS restraints were removed in later rounds of refinement. Distances between atoms were measured in Coot (8), and statistical tests on the distances were calculated in Excel (Microsoft). Maximal coordinate errors were calculated by Procheck (9).

Mammalian Cell Experiments. Forty-eight-well plates (pretreated with 0.01 mg/mL poly-lysine) were seeded with 10^5 HEK 293T cells/well in 0.25 mL of transfection mix [0.1 μg α-NAGAL/p3xFLAG plasmid, 0.5 μL of Lipofectamine 2000 (Invitrogen), in Dulbecco's modified Eagle medium (DMEM) (Mediatech) with 5% (vol/vol) FBS (HyClone)]. A volume of 0.25 mL of growth medium (DMEM with 5% FBS) with 0, 0.2, or 1 mM DGJNAc or DGJ was added to each well. Growth medium was removed after 24 h, cells were washed in ice-cold PBS, and 100 μL of lysis buffer [PBS, 1% Triton X-100, 5 mM EDTA, 1× HALT protease inhibitor mixture (Pierce)] was added to each well. Cells were lysed by vortexing. Identical volumes of lysate were separated by 12% reducing SDS/PAGE, transferred to PVDF membranes, and analyzed by Western blotting using FLAG M2 monoclonal primary antibodies (Sigma) and alkaline phosphatase-conjugated secondary anti-

bodies detected by colorimetric nitroblue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate (NBT/BCIP) substrate (Pierce). Experiments were performed in triplicate, and α-NAGAL band intensities were normalized to expression of α-NAGAL in the absence of compound using GeneTools (SynGene). Western blots with antibodies against GAPDH (Millipore) were used as loading controls.

For generation of stable HEK 293T cell lines, transfected cells were cultured in the presence of 400 μg/mL G418 sulfate for 2 wk, establishing stably integrated, polyclonal cell lines.

Density Gradient Ultracentrifugation. The stably integrated HEK 293T cells were grown in DMEM with 5% FBS, alone or supplemented with 100 μM DGJ or DGJNAc. The cells were incubated under normal growth conditions at 37 °C with 5% CO_2 in a humidified incubator for 24 h (Fig. 4*C*) or 96 h (Fig. 4*D*) before homogenization.

For optimal separation of the mature and immature α-NAGAL forms (as in Fig. 4*C*), a gradient of 7.5–25% (wt/vol) iodixanol (OptiPrep; Axis-Shield Density Gradient Media) was used. For optimal separation of the lysosomal and ER markers (as in Fig. 4*D*), a finer gradient of 11–22% iodixanol was used.

293T cells were homogenized in 10 mM Hepes, pH 7.5, 1 mM EDTA, and 250 mM sucrose using a ball-bearing homogenizer. Homogenate was spun at 1000 × *g* to remove nuclei. The post-nuclear supernatant (PNS) was mixed with 30% iodixanol to reach the desired iodixanol concentration. Heavy gradient solution (mixed with PNS) and light solution were layered, and a continuous gradient was formed with a Gradient Station (BioComp Instruments). Gradients were centrifuged for 16 h at 178,000 × *g* in a Beckman SW41 rotor and separated into 12 fractions using the Gradient Station.

Samples were electrophoresed on 10% gels and blotted with antibodies against Lamp2 (Santa Cruz Biotech), Calnexin (Santa Cruz Biotech), FLAG-M2 (Sigma), GAPDH (Millipore), or α-NAGAL (made against our recombinant protein). Deglycosylation was performed according to manufacturer's instructions with Endo H_f and PNGase F (NEB). GAPDH was used as a loading control for samples input into the gradients in Fig. 4*D*.

Figures and Calculations. Molecular figures were drawn in POVScript+ (10). Ramachandran statistics were calculated in RAMPAGE (11). Distance calculations on the coordinates treat each monomer in the asymmetric unit as an independent variable. Student *t* tests on paired distances between protein and ligand were calculated in KaleidaGraph. Free energy calculations used differences in binding affinity at 298K according to $\Delta\Delta G = -RT \ln (K_1/K_2)$ and assume reversibility of the thermodynamic cycle.

1. Clark NE, Garman SC (2009) The 1.9 Å structure of human α-*N*-acetylgalactosaminidase: The molecular basis of Schindler and Kanzaki diseases. *J Mol Biol* 393(2):435–447.
2. Maiorella BL, et al. (1993) Effect of culture conditions on IgM antibody structure, pharmacokinetics and activity. *Biotechnology (N Y)* 11(3):387–392.
3. Best D, et al. (2010) Synthesis of 2-acetamido-1,2-dideoxy-D-galacto-nojirimycin [DGJNAc] from D-glucuronolactone: The first sub-micromolar inhibitor of α-*N*-acetylgalactosaminidases. *Tetrahedron Lett* 51(17):2222–2224.
4. Copeland RA (2000) *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis* (Wiley-VCH, New York), 2nd Ed, p 397.
5. Guce AI, Clark NE, Rogich JJ, Garman SC (2011) The molecular basis of pharmacological chaperoning in human α-galactosidase. *Chem Biol* 18(12):1521–1526.
6. Park CW, Marqusee S (2005) Pulse proteolysis: A simple method for quantitative determination of protein stability and ligand binding. *Nat Methods* 2(3):207–212.
7. Otwinowski Z, Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods in Enzymology: Macromolecular Crystallography, Part A*, eds Carter CW, Sweet RM (Academic, New York), Vol 276, pp 307–326.
8. Emsley P, Cowtan K (2004) Coot: Model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60(Pt 12 Pt 1):2126-2132.
9. Collaborative Computational Project, Number 4 (1994) The CCP4 suite: Programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* 50(Pt 5):760–763.
10. Fenn TD, Ringe D, Petsko GA (2003) POVScript+: A program for model and data visualization using persistence of vision ray-tracing. *J Appl Cryst* 36:944–947.
11. Lovell SC, et al. (2003) Structure validation by Cα geometry: φ, ψ, and Cβ deviation. *Proteins* 50(3):437–450.

Table S1. Crystallographic statistics

	PDB code		
	4DO4	4DO5	4DO6
Soak	DGJNAc	DGJ	Glucose
Beam line	NSLS X25	NSLS X6A	NSLS X25
Wavelength, Å	1.100	1.000	1.100
Space group	*C*2	*C*2	*C*2
Cell lengths, Å	154.1, 114.5, 68.6	154.4, 114.5, 68.6	153.3, 114.3, 68.6
Cell β angle, °	95.7	95.6	96.4
Resolution, Å (last shell)	50.0–1.40 (1.42–1.40)	50.0–1.50 (1.55–1.50)	50.0–1.60 (1.63–1.60)
Observations overall	1,160,205 (43,181)	722,297 (39,291)	868,516 (20,530)
Unique observations (last shell)	228,468 (10,879)	178,912 (13,588)	145,750 (4,888)
Completeness, % (last shell)	98.9 (94.9)	96.1 (73.2)	94.7 (63.8)
Multiplicity (last shell)	5.1 (4.0)	4.0 (2.9)	6.0 (4.2)
R_{sym}, % (last shell)	5.8 (84.2)	4.6 (64.4)	6.0 (65.3)
$\langle I/\sigma_I \rangle$ (last shell)	26.3 (1.5)	22.6 (1.5)	29.6 (1.7)
Refinement			
R_{work}/R_{free} (%)	14.1/17.8	15.9/17.8	15.4/17.0
No. of atoms: total	7,720	7,750	7,429
Protein	6,359	6,385	6,346
Carbohydrate	227	206	275
Water	998	1,027	789
Other	136	132	19
Average B factors, Å^2	29.5	29.4	30.8
Protein	26.5	26.2	28.1
Carbohydrate	81.5	71.0	67.8
Water	46.9	41.2	41.0
Ramachandran plot, %			
Favored	97.1	96.8	97.1
Allowed	2.7	2.9	2.7
Outlier	0.3	0.3	0.3
RMS deviations			
Bonds, Å	0.012	0.010	0.008
Angles, °	1.46	1.36	1.24