Supporting Text

Preparation of Glycosylated Enzymes. α-L-rhamnosidase from *Penicillium decumbens*, (naringinase, EC 3.2.1.40) is a commercially available (Sigma-Aldrich) enzyme preparation composed of α-rhamnosidase and β-glucosidase activities. Naringinase was purified to yield pure wild type α-rhamnosidase activity by dialysis (12,000-14,000 molecular weight cut off, Medicell International Ltd., London, 5 liters × 5), BioGel P100 size exclusion chromatography (BioRad, eluant pH 4.8/0.1 M NaCl) and DEAE-Sepharose ion-exchange chromatography (Amersham Pharmacia Bioscience; eluant, pH 6.0/20 mM L-Histidine 0-0.35 mM NaCl gradient) to give N-WT. N-WT was deglycosylated using endoglycosidase-H (endo-H, 32 U / 100 mg N-WT) in pH 6.0, 0.1 M orthophosphate buffer, 37^{o}C, purified by dialysis (50,000 molecular weight cutoff SpectraPor DispoDialyser, Spectrum Laboratories, Rancho Dominguez, CA) to give N-DG.

Treatment of N-WT with HF for 3h at 0˚C (1) allowed exhaustive chemical deglycosylation and confirmed previous results for the major deglycosylation product.(2) Proteins were glycosylated using the 2-imino-2-methoxyethyl 1-thioglycoside method (3,4). For example, sodium methoxide (240 μl in 1M MeOH) was added to a solution of cyanomethyl tetra-*O*-acetyl-1-thiogalactopyranoside (270 mg, 0.67 mmol) in dry methanol (24 ml), stirred for 36 h; then the solvent removed *in vacuo* to give β-D-galactose (Gal)-2-imino-2-methoxyethyl 1-thioglycoside (IME) (170 mg) as a white gum. N-WT (50 mg) and Gal-IME (170 mg) were dissolved in pH 8.5, 0.25 M sodium

tetraborate solution and stirred at room temperature for 24 h. The solution was dialysed (deionised water 5 liters × 5), purified by size exclusion chromatography (Sephadex G25 PD10, Amersham Pharmacia Bioscience, eluant water) and lyophilized to yield white powder. Glycosylated enzymes were characterized by gel electrophoresis (10% SDS/PAGE, pH 8.8, Tris buffer, Vertical Slab Gel Kit, Atto Corporation, Tokyo, Japan) and MALDI MS (Ciphergen Biosystems PBS II, Ciphergen Biosystems, Fremont, US; sinapinic acid matrix 10 mg/ml 3:2 water/acetonitrile, 0.2% TFA). MS Data: **N-WT** 76148; **N-WT-Gal** 79443 (N-WT+3295 ~+14 Gal); **N-DG** 69341; **N-DG-Gal** 71892 (N-DG +2551 ~+11 Gal); **N-DG-Man** 72857 (N-DG +3516 ~+15 Man); **N-DG-dGal**, 72886 (N-DG-dGal +3545 ~+10 Gal). Michaelis-Menten parameters were determined according to the initial rates method ($[E]_0 = 3.1\times10^{-7}$ M, *para*-nitrophenyl α-L-rhamnopyranoside (*p*NP-Rha) 0.25 – 3.5 mM, pH 7.0, 0.1 M orthophosphate) and nonlinear regression using Grafit 4.0.13 (Erithacus Software, Horley, UK). Kinetic data: k_{cat} / s^{-1}; K_M / M; k_{cat}/K_M / $s^{-1}M^{-1}$ **N-WT**: 2.54 ± 0.140; 0.026 ± 0.002; 97.08; **N-WT-Gal**, 0.96 ± 0.032, 0.037 ± 0.004; 25.93; **N-WT-Man**, 2.34 ± 0.091; 0.046 ± 0.002; 51.08; **N-WT-dGal**, 1.35 ± 0.184; 0.064 ± 0.003; 21.13; **N-DG**, 0.37 ± 0.041; 0.019 ± 0.003; 19.50; **N-DG-Gal**, 0.26 ± 0.012; 0.013 ± 0.001; 19.72; **N-DG-Man**, 0.29 ± 0.009; 0.015 ± 0.002; 19.14; **N-DG-dGal**, 0.87 ± 0.079; 0.012 ± 0.001; 72.48.

Synthesis of Chemical Glycosylation Reagents

Acetobromogalactose and Acetobromomannose. Prepared according to published procedures by peracetylation (5) and anomeric bromination (6).

2-*S*-(2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl)-2-thiopseudourea Hydrobromide. Acetobromogalactose (5.0 g, 12.2 mmol) and thiourea (1.2 g, 15.8 mmol) were suspended in dry acetone (50 ml) in a 250 ml round-bottom flask fitted with a magnetic stirrer and reflux condenser, under an inert atmosphere. The mixture was refluxed for 2 h, reduced in volume (to 30 ml) *in vacuo* and chilled for 3 h. The resulting white amorphous solid was filtered, washed with cold acetone and dried (4.06 g, 82%). mp = 174.7–176.5°C (lit. value = 171.0-171.5°C); $[\alpha]_D^{22}$ = +10.6 ($CHCl_3$, c = 1.0); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.99 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.15 (s, 3H, Ac), 2.20 (s, 3H, Ac), 4.147-4.179 (m, 2H, H-6, H-6'), 4.44 (t, 1H, *J* 6.6Hz, H-5), 5.19 (dd, 1H, *J* 3.28, 9.85Hz, H-3), 5.39 (m, 1H, *J* 9.8Hz, H-2), 5.50 (d, 1H, *J* 3.28Hz, H-4), 5.55 (br s, 1H, H-1), 8.28 (s, NH_2), 9.47 (s, NH_2); ^{13}C NMR (62.9 MHz, $CDCl_3$) δ = 20.4, 20.6, 20.8, 20.9 (4 × $\underline{C}H_3CO$-), 61.4 (C-6), 66.2, 67.0, 71.3, 75.8, 81.9 (C-1), 169.0 (?), 169.6, 169.9, 170.0, 170.2 (4 × $\underline{C}$=O); IR (KBr) 3400 (-(C=NH)-NH_2 stretch), 1758 (-(C=O)-OMe stretch), 1644 (-(C=NH)-NH_2 stretch), 1228, 1057 ((-(C=O)-OMe stretch) cm^{-1}; MS *m/z* (ES): found 407, $[M+H]^+$ requires 407; Elemental Analysis: Theoretical: C 36.97%; H 4.76%; N 5.75% Actual: C 36.79%; H 4.53%; N 5.68%.

Cyanomethyl 2,3,4-tri-*O*-acetyl-β-D-galactopyranoside. 2-*S*-(2,3,4-tri-*O*-acetyl-*β*-D-galactopyranosyl)-2-thiopseudourea hydrobromide (3.7 g, 7.6 mmol) was stirred with dry acetone (20 ml) and water (20 ml). Sodium bisulfite (2.0 g, 38 mmol), potassium carbonate (1.6 g, 11.5 mmol) and chloroacetonitrile (2.5 ml, 40 mmol) were then added in order, and the mixture stirred at room temperature for 3 h. This solution was poured over

ice water (50 ml) and stirred vigorously for 3 h at 4°C. Following extraction with chloroform (4 × 40 ml), the organic layers were combined and washed with sodium chloride solution (2 × 60 ml, 1 M) and dried over anhydrous magnesium sulfate. The organic solution was filtered and all solvent removed *in vacuo* and purified by flash-column chromatography (3:1 EtOAc/hexane) yielding white crystals (2.05 g, 67%). mp = 97.6-99.2°C (lit. value = 95-97°C); $[\alpha]_D^{22}$ = -56.96 ($CHCl_3$, c = 0.95); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.99 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.16 (s, 3H, Ac), 3.34 (d, *J* 16.9 Hz, 1H, SCH_2'), 3.63 (d, *J* 16.9 Hz, 1H, SCH_2), 4.00 (td, *J* 1.01, 7.07 Hz, 1H, H-5'), 4.15 (m, 2H, H-6, H-6'), 4.69 (d, *J* 9.85 Hz, 1H, H-1'), 5.09 (dd, *J* 3.28, 9.85 Hz, 1H, H-3'), 5.24 (t, *J* 10.0 Hz, 1H, H-2'), 5.46 (dd, *J* 1.01, 3.28, 1H, H-4'); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 14.5 (S<u>C</u>H_2), 20.5, 20.6, 20.6, 20.7 (4 × <u>C</u>H_3CO-), 61.3 (C-6), 66.7, 67.1, 71.4, 75.0, 82.2 (C-1), 115.8 (<u>C</u>≡N), 169.8, 169.9, 170.1, 170.4 (4 × <u>C</u>=O); IR (KBr) 2250 (C≡N stretch), 1742 (-(C=O)-OMe stretch), 1221, 1051 (-(C=O)-OMe stretch); HRMS *m/z* (ES): found 426.0831, $[M+Na]^+$ requires 426.0835; Elemental Analysis: Theoretical: C 47.64%; H 5.25%; N 3.57% Actual: C 47.61%; H 5.27%; N 3.46%.

2-*S*-(2,3,4-tri-*O*-acetyl-α-D-mannopyranosyl)-2-thiopseudourea Hydrobromide. Acetobromomannose (2.68 g, 6.51 mmol) and thiourea (0.66 g, 8.60 mol) were suspended in acetone (25 ml, dry) and refluxed under an inert atmosphere for 3.5 h. The resultant solution was reduced in volume (to 15 ml) *in vacuo* and chilled for 3 h. The resulting white amorphous solid was filtered, washed with cold acetone and dried (1.3 g,

51%). mp = 124.5-128.1°C (lit. value = 125-128°C); $[\alpha]_D^{22}$ = +104.03 ($CHCl_3$, c = 1.1); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.01 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.20 (s, 3H, Ac), 4.20 (m, 1H, *J* 12 Hz, H-6'), 4.25 (m, 1H, *J* 2.6 Hz, H-6), 4.41 (m, 1H, H-5), 5.10 (dd, 1H, *J* 3.4, 9.8 Hz, H-3), 5.32 (m, 1H, *J* 9.8 Hz, H-4), 5.43 (d, 1H, *J* 1.8 Hz, H-2), 6.29 (d, 1H, *J* 1.8 Hz, H-1), 8.231 (s, 2H, -NH_2), 9.73 (s, 2H, -NH_2); ^{13}C NMR (62.9 MHz, $CDCl_3$) δ = 20.7, 20.7, 20.8, 20.9 (4 × $\underline{C}H_3CO$-), 65.0 (C-6), 68.2, 69.0, 69.1, 71.4, 82.3 (C-1), 168.4, 169.3, 169.6, 170.3 (4 × $\underline{C}$=O); IR (KBr) 3400 (-(C=NH)-NH_2 stretch), 1759 (-(C=O)-OMe stretch), 1644 (-(C=NH)-NH_2 stretch), 1228, 1057 ((-(C=O)-OMe stretch); MS *m/z* (ES): found 407, $[M+H]^+$ requires 407; Elemental Analysis: Theoretical: C 36.97%; H 4.76%; N 5.75% Actual: C 36.85%; H 4.72%; N 5.48%.

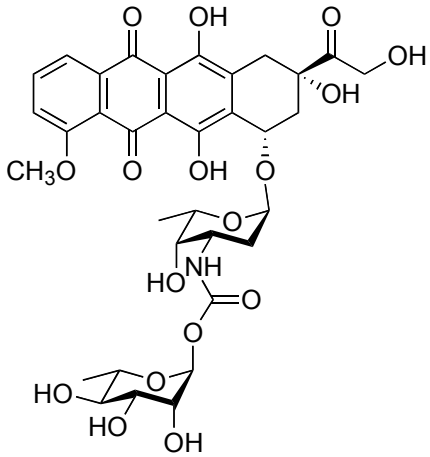
Cyanomethyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside. 2-*S*-(2,3,4-tri-*O*-acetyl-*α*-D-mannopyranosyl)-2-thiopseudourea hydrobromide (5.413 g, 13.83 mmol) was dissolved in water (30 ml) and acetone (30 ml), and sodium bisulfite (2.4 g, 45.6 mmol), potassium carbonate (2.33 g, 16.92 mmol) and chloroacetonitrile (7.2 ml, 115.2 mmol) were added in order and the resultant mixture was stirred for 6 h. The mixture was poured into ice water (50 ml) and stirred for 1 h, and a white precipitate formed. Extraction by chloroform (3 × 80 ml), washing with sodium chloride solution (3 × 40 ml, 1M) and flash-column chromatography (3:1 EtOAc/hexane) yielded a white crystalline solid (3.54 g, 64%). mp = 131.2-133.4°C (lit. value = 130-131°C); $[\alpha]_D^{22}$ = +111.7 ($CHCl_3$, c = 0.52); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 2.00 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.11 (s, 3H, Ac), 2.18 (s, 3H, Ac), 3.31 (d, *J* 17.2 Hz, 1H, SCH_2'), 3.47 (d, *J* 17.2 Hz, 1H, SCH_2), 4.16 (dd, *J*

2.02, 12.1 Hz, 1H, H-6'), 4.31 (m, *J* 2.02, 5.3 Hz, 1H, H-5'), 4.33 (m, *J* 5.1 Hz, 1H, H-6), 5.19 (dd, *J* 3.53, 10.1 Hz, 1H, H-3'), 5.34 (dd, *J* 9.9, 10.1 Hz, 1H, H-4'), 5.37 (dd, *J* 1.5, 3.5 Hz, 1H, H-2'), 5.46 (d, *J* 1.5 Hz, 1H, H-1'); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 15.8 ($S\underline{C}H_2$), 20.6, 20.7, 20.7, 20.8 (4 × $\underline{C}H_3CO$-), 62.0 (C-6), 65.8, 69.2, 69.5, 69.9, 82.4 (C-1), 115.6 ($\underline{C}\equiv N$), 169.6, 169.7, 169.8, 170.5 (4 × $\underline{C}$=O); IR (KBr) 2248 (C≡N stretch), 1745 (-(C=O)-OMe stretch), 1227, 1078 (-(C=O)-OMe stretch); HRMS *m/z* (ES): found 426.0830, $[M+Na]^+$ requires 426.0835; Elemental Analysis: Theoretical: C 47.65%; H 5.25%; N 3.47% Actual: C 47.39%; H 5.22%; N 3.36%.

Bis(2-{[2-(1-thio-β-D-galactopyranosyl)ethanoyl]amino}ethyl)(2-aminoethyl)propanoyl-thiocyanomethyl (dGal-IME). Bis(2-{[2-(1-thio-β-D-galactopyranosyl)ethanoyl]amino}ethyl)(2-aminoethyl)amine (98.9 mg, 0.16 mmol), γ-thiobutyrolactone (0.14 ml, 1.6 mmol) and chloroacetonitrile (0.20 ml, 3.2 mmol) were added to an aqueous solution of sodium hydrogen carbonate (1 ml, 0.5 M) and methanol (1 ml) in a round bottom flask fitted with reflux condenser, magnetic stirrer bar, and inert atmosphere. The mixture was heated for 24 h at 50°C. The mixture was neutralized with 2 M HCl, concentrated *in vacuo* and purified by flash chromatography ($CHCl_3$/MeOH/H_2O/Et_3N 60:35:7:1) to yield a pale-yellow amorphous solid. The product was loaded onto Dowex 50W2-200 (H^+) in water/methanol (1:1) and eluted with 10% NH_3 solution, yielding a pale-yellow amorphous solid/oil after freeze drying (54.7 mg, 45%). $[\alpha]_D^{22}$ = -25.5 (D_2O, c = 0.9); ^{1}H NMR (400 MHz, D_2O) δ = 1.98 (tt, *J* 7.2, 7.3Hz, 2H, $C\underline{H}_2CH_2S$), 2.40 (t, *J* 7.3 Hz, 2H, $C\underline{H}_2CH_2CH_2S$), 2.63-2.67 (m, 6H, NCH_2), 2.79 (t, *J* 7.2 Hz, 2H, $CH_2CH_2C\underline{H}_2S$), 3.15 (t, *J* 6.5 Hz, 2H, $NCH_2C\underline{H}_2NHCO(CH_2)_3S$), 3.22 (t, *J*

6.7 Hz, 4H, $NCH_2C\underline{H}_2NHCOCH_2S$), 3.29 (m, *J* 6.0 Hz, 4H, $SC\underline{H}_2CO$), 3.40 (d, *J* 15.3 Hz, 2H, $SC\underline{H}_2CN$), 3.51 (dd, *J* 3.3, 6.5 Hz, 2H, H-2'), 3.60 (dd, *J* 2.7, 3.5 Hz, 2H, H-3'), 3.68-3.81 (m, 6H, H-5', H-6'), 3.89 (d, *J* 3.1 Hz, 2H, H-4'), 4.41 (d, *J* 9.5 Hz, 2H, H-1'); ^{13}C NMR (63 MHz, CD_3OD) δ = 11.5 ($S\underline{C}H_2CN$), 26.8 ($\underline{C}H_2SCH_2CN$), 34.8 ($COCH_2\underline{C}H_2$), 35.0 ($CO\underline{C}H_2CH_2$), 36.2 ($S\underline{C}H_2CO$), 39.8 ($\underline{C}H_2NHCO(CH_2)_3S$), 40.1 ($SCH_2CONH\underline{C}H_2$), 48.2 ($\underline{C}H_2CH_2NHCO(CH_2)_3S$), 55.4 ($SCH_2CONHCH_2\underline{C}H_2$), 63.8 (C-6'), 71.7 (C-4'), 72.1 (C-2'), 76.9 (C-3'), 81.8 (C-5'), 88.0 (C-1'), 114.9 (C≡N), 173.8 (COCH2S), 176.0 ($CO(CH_2)_3S$); IR (KBr) 2236 (C≡N stretch); HRMS *m/z* (ES): found 760.2578, $[M+H]^+$ requires 760.2567. This reagent was activated methoxide immediately before use (see above).

Synthesis of DOX-Rha



N-(α-L-Rhamnopyranosyloxycarbonyl) Doxorubicin. *N*-(2,3,4-Tri-*O*-acetyl-α-L-rhamnopyranosyloxycarbonyl) doxorubicin (320 mg, 0.37 mmol) was dissolved in 50 ml of dry MeOH, 10 ml of dry DMF, and 2.5 ml of dry THF. The solution was cooled to 0°C before NaOMe (54 mg, 1 mmol) dissolved in 1 ml dry MeOH was added. The reaction was carefully followed by TLC, and quenched after 1 h 40 min with Dowex-H^+ when no starting material could be detected. The mixture was filtered, and concentrated *in vacuo* to afford compound **DOX-Rha** (265 mg, 94 %) as a red powder. m/z (ES^-) 732 (M-H, 100%). HRMS (ES^-) calculated for $C_{34}H_{38}NO_{17}$ (M-H) 732.2140, found 732.2139.

Preparation of Mannosylated Poly-L-lysine (PLL-Man). Cyanomethyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (266 mg, 0.66 mmol) was dissolved in dry methanol (60 ml), treated with methanolic sodium methoxide (4.5 ml, 0.1 M) and stirred under a nitrogen atmosphere for 24 h when all solvent was removed *in vacuo*. Poly-L-lysine (50 mg, MW = 24,000, Sigma) was dissolved in sodium tetraborate solution (5 ml, 0.25 M, pH 8.5), added to this residue and stirred at room temperature for 36 h. The resultant solution was dialyzed by using SpectraPor Cellulose Ester dialysis tubing (12,000-14,000 molecular weight cut off, Spectrum Laboratories, Rancho Dominguez, CA, 2 liters × 4 dialysate) and then freeze-dried yielding PLL-Man (14.6 Man per PLL chain as determined by NMR) as a white powder. ^{1}H NMR (500MHz, D_2O) δ = 1.28 (br m, -C<u>H</u>$_2$CH$_2$CH$_2$NH-), 1.49 (br m, -C<u>H</u>$_2$CH$_2$NH-), 1.62 (br m, -C<u>H</u>$_2$CH$_2$CH$_2$CH$_2$NH-), 2.78 (br m, -CH$_2$NH-), 3.50-3.97 (m, Man), 4.15 (br t, -NH-C<u>H</u>(-C(O)-)-CH$_2$-).

Enzyme Assays

Initial Rates Michaelis–Menten Kinetic Analysis. Michaelis–Menten parameters of the glycosylated enzymes ($[E]_0 = 3.1 \times 10^{-7}$ M) were determined by using a solution of *para*-nitrophenyl α-L-rhamnopyranoside ([S] = 0.25–3.5 mM) in pH 7.0, 0.1 M orthophosphate buffer gave kinetic parameters (7) according to the initial rates method.

Specific Activity Determination. Activity of glycosylated enzymes was determined under standard specific activity conditions.

Method. Change in absorbance at 405 nm over 1 min was determined when glycosylated enzyme (10 μl, 0.5 mg/ml) was added to a solution of *para*-nitrophenyl α-L-rhamnopyranoside (200 μl, 3.5 mM, pH 7.0, 0.1 M orthophosphate) at 37°C.

Protein Labeling

Preparation of Radioiodinated Enzymes. The glycosylated enzymes were ^{123}I-labeled using 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril-mediated tyrosine iodination(8). Example protocol: Na^{123}I solution (600 MBq in 27 μl NaOH solution, diluted to 200 μl with PBS, MDS Nordion SA, Fleurus, Belgium) was added to a solution of N-DG-Gal (1 ml, 10 mg/ml in PBS) in an iodination tube (precoated with 0.12 μmol of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Pierce) and agitated at room temperature for 20 min. The solution was transferred to a new vial; sodium metabisulfite (0.1 M in PBS, 10 μl) was added; the solution stood for 10 min and purified by size-exclusion chromatography (Sephadex G25 PD10, Supelco; eluant PBS) and dialysis (5,000 molecular weight cutoff, SpectraPor DispoDialyser Spectrum Laboratories, 1 liter × 3).

An investigation into the ^{123}I-labeling of N-WT was undertaken to determine the effect of reaction and purification conditions on label integrity. In addition, the effect of liver lysosome preparation on labeled N-WT was determined. This study showed that the purification technique removed all low-molecular-weight ^{123}I.

Preparation of Tritium-Labeled Enzymes (Example Protocol). A solution of N-WT (2.0 mg in 0.2 ml, pH 8.0, 0.1 M, sodium tetraborate buffer) was added to a solution of *N*-succinimidyl-[2,3-^{3}H]propionate (115 MBq, 3.48 TBq/mmol, Amersham Pharmacia Biosciences) in sodium tetraborate buffer (0.45 ml). After 2 h, the protein was purified by size-exclusion chromatography (Sephadex G25 PD10, eluant PBS). Radioactivity was determined using a Beckman LS600TA liquid scintillation counter (Beckman Coulter) with Starcint scintillant (Packard BioScience, Pangbourne, UK).

Biodistribution Studies. Studies were carried out using both male Wistar rats and male New Zealand White rabbits; the rabbits showed superior visualization and resolution in gamma scintigraphy. Representative examples of gamma scintigraphy images illustrating distribution of ^{123}I-labeled glycosylated enzymes within male New Zealand White rabbits are shown below. Extended retention of glycosylated enzyme is clear when animals are dosed with N-WT and N-DG-Gal and contrasts with retention levels when animals are dosed with N-DG and N-DG-Gal + AF.

Gamma Scintigraphy in New Zealand White Rabbits. Biodistribution studies were performed in male New Zealand White rabbits (Harlan, Bicester, UK). Hypnorm (Janssen/Roche Products,) sedated animals (n = 3-4, average mass 1 kg) were injected with ^{123}I-labelled enzyme (dose 2.5 mg/kg, ~3 MBq) solution in PBS via an ear vein. Where appropriate, blocker (100 mg/kg in PBS: AF for N-WT-Gal, N-WT-dGal, N-DG-Gal, N-DG-dGal; mannosylated poly-L-lysine (PLL-Man) for N-WT-Man, N-DG-Man) was dosed 1 min prior to dose of ^{123}I-labeled enzyme. Imaging was carried out on a Maxi

Gamma Camera 406 (GE Medical Systems, Slough, UK, and Park Medical Systems Ltd, Hemel Hempstead, UK) at 1, 10, 30, 60, 90, 120 min. Concurrent sampling of blood from the contralateral ear vein (~1 ml) was in accordance with Laboratory Animal Science Association (LASA) guidelines (9). After killing, organ radioactivity was determined by using a NaI-type PCA-P well counter (Oxford Instruments Inc., Witney, UK).

Microautoradiography. Male Wistar rats (~ 250 g, Charles River Laboratories) were anaesthetised (1:1:5 Hypnorm/Hypnovel/water, dose 0.8 ml/kg, Janssen/Roche Products) by an indwelling tail-vein needle and then injected with ^{3}H-labelled enzyme (dose 2.5 mg/kg, ~1.5 MBq, in 1 ml of PBS) by ear vein. Where appropriate, blocker (AF or PLL-Man) was dosed 1 min before dose. Superficial vein blood sample volumes (taken 0, 1, 5, 10, 15, 20 min) were in accordance with LASA guidelines. The animals were sacrificed by Euthatal (Rhone-Merieux, Harlow, UK) overdose, and livers and kidneys removed. Two-milimeter sections (n = 4) were taken from different lobes of each liver and frozen in TissueTek OCT (Sakura Finetek USA, Torrance, CA) onto a cork disk for use in confocal microscopy analysis, and remaining tissue stored in buffered formalin. Organ samples were prepared for microautoradiography by impregnating and embedding in wax, 4-μm-thick sections prepared on a Shandon rotary microtome AS325 (ThermoShandon, Pittsburgh) and mounted onto Vectabond-coated microscope slides. Residual wax was removed by dipping in xylene and industrial methylated spirits/water tanks, and the slides coated with Ilford K-type nuclear emulsion (Ilford). After appropriate exposure time, development was carried out according to manufacturers'

instructions (n = 3 grids/section), and tissue stained using Mayer's hematoxylin solution (Sigma-Aldrich).

Confocal Microscopy. Tissue samples embedded in Tissue Tek OCT were prepared for confocal microscopy by mounting Cryomicrotome AS620 (ThermoShandon)-prepared 7-μm sections on a microscope coverslip. The coverslip was loaded into a confocal microscopy cell and submerged in buffer (500 μl, 145 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes/10 mM glucose, pH 7.4). A phase image was recorded, a solution of Mendiaxon-α-L-rhamnopyranoside (MEND-Rha, 2 mM, 50 μl) was added and then fluorescence images (8-s interval, Leica TCS NT confocal microscope, ×10 fluotar lens, argon laser 360 nm excitation, 405 ± 40 nm emission, Leica Microsystems, Milton Keynes, U.K.).

Additional Biodistribution Methods in Rat Model. Male Wistar rats (~250 g) were weighed and tail-marked for identification purposes. The rats were kept in a hotbox at 38^{o}C for approximately 15 min prior to use to allow tail-vein dilation. The rats were anaesthetized using a solution of Hypnorm (2 ml, fentanyl/fluanisone), Hypnovel (2 ml, midazolam), and water (10 ml) for injection, by an indwelling needle implanted into the caudal vein. Initial anesthetic dose (0.2 ml) was topped-up by additional doses (0.1 ml) as required during the study. The rats were positioned on a heating table (36-40^{o}C) throughout study. The dose (10 mg/kg) was administered over 30 s by the previously implanted catheter in the tail vein. Saline solution (0.5 ml, 0.9%) was administered to flush in the dose. If appropriate, blocker (100 mg/kg, asialofetuin or PLL-Man in PBS)

was administered 1 min prior to the glycosylated enzyme dose in the same fashion as the dose. Rats were killed by dislocation of the neck 20 min after receiving the dose, and radioactivity in the liver was determined by using a NaI-type PCA-P well counter (Oxford Instruments, Witney, U.K.). Liver dose levels determined: N-DG, 14%; N-WT, 32%; N-DG-Gal, 37%; N-DG-Gal + AF, 12%; N-WT-Gal, 30%; N-WT-Gal + AF, 17%; N-WT-Man, 16%; N-WT-Man + pLL, 18%.

Blood Levels of ^{123}I-Labeled Enzyme. Blood samples were taken from the ear vein (10, 30, 60, and 120 min after receiving the dose) and centrifuged at 10,000 rpm for 10 min. Radioactivity in plasma (100 μl) was determined by using a NaI-type PCA-P well counter (Oxford Instruments, Witney, UK). Values were standardized for animal weight (dose size) and corrected for decay.

Organ Distribution After Killing in New Zealand White Rabbits. After killing, organs were collected and radioactivity was determined by using a NaI-type PCA-P well counter (Oxford Instruments, Witney, U.K.). Values were standardized for animal weight (dose size) and corrected for decay and were in accord with gamma scintigraphy data.

Activity Level of Delivered Enzyme

The level of activity of glycosylated enzyme once targeted to the liver was determined by measuring release of *para*-nitrophenol from *para*-nitrophenyl α-L-rhamnopyranoside under standard conditions. Relative activity across the sample range was determined by

comparing samples with activity in undosed liver sample as a standard. In calculating activity level per liver an average liver mass of 15 g has been assumed.

Method. Liver (1 g) from the experiment above was homogenized with PBS (1.5 ml), and the resulting supernatant centrifuged (13,000 rpm, 30 min). A portion of the resultant supernatant (20 μl) was added to a solution of *para*-nitrophenyl α-L-rhamnopyranoside (1 ml, 3.5 mM, pH 7.0, 0.1 M orthophosphate). Absorbance at 405 nm was determined after 5 min incubation. Liver from undosed animal was used as control and showed no activity.

Calculation of Prodrug Release Based on Biodistribution of Enzyme Activities.

Assumptions. New Zealand White Rabbit (1 kg; average blood volume, 65 ml, average hepatocyte volume, 10 ml), dosed at 10 min with 10 mg/kg of MEND-Rha (31 μmol).

Biodistribution and Activity Data Use for Calculation. N-DG-Gal activity = 0.2 unit/mg, t = 10 min, serum N-DG-Gal = 0.072 mg, hepatocyte rhamnosidase activity (as determined by homogenate and MAR distribution) = 0.2 units.

Calculation. Assuming all serum enzyme is fully active, 0.072 mg of N-DG-Gal generates rhamnosidase activity in Serum = 2.2×10^{-4} units/ml. Under these conditions and assuming constant release rate, 10 mg of MEND-Rha would be converted in the blood in 35.9 h, the measured level of rhamnosidase activity in hepatocytes = 2×10^{-2} units/ml. Under these conditions and assuming a constant release rate, 10 mg of MEND-Rha would be converted in the hepatocytes in 62 min. Under these conditions and

assuming an equal systemic concentration of prodrug this would create concentrations of active drug in the ratio = serum/hepatocytes = 1:[(2.2 × 10^{-4}/65)]/[2 × 10^{-2}/10] = 1 : 590.

HPLC Analysis of Targeted MEND Levels. Liver and kidney samples (n = 2) from dosed and undosed animals 30 min after glycosylated enzyme treatment,10 min after treatment with MEND-Rha prodrug (5 mg/kg) were homogenized and the homogenate analyzed by HPLC (Spherisorb S5 ODS-2 RP C-18 column, 200 x 4.6 mm, mobile phase H_2O / 3% CH_3COOH : CH_3CN / 3%CH_3COOH; 94:6 for 10 min, 94:6→40:60 over 30 min, MEND rt 29 min, fluorescence detection excitation 340 nm, emission 425 nm). Measured MEND levels: treated liver, 2.38 mg/kg; untreated liver, 0 mg/kg; treated kidney, 0.02 mg/kg; and untreated kidney 0 mg/kg.

Glycosylated Enzyme Stability Testing. Enzymes and prodrug were assayed with lysosomal extract from male Wistar rats. Liver lysosome preparation (10), (300 μl of protein content 1.5 mg/liter as determined by bicinchoninic acid method (11); proteolytic activity 15 nM min^{-1}.(mg protein)$^{-1}$, as determined by release of *para*-nitroaniline from *N*-benzoyl-Phe-Val-Arg-*para*-nitroanilide] was added to a solution of glycosylated enzyme (680 μl, pH 5.5, 0.2 M citrate-phosphate/1 mM EDTA/5 mM GSH plus 20 μl 10% Triton X100 solution) and incubated at 37^{o}C. At regular intervals, 100-μl aliquots were removed at regular intervals and rhamnosidase Michaelis–Menten parameters towards *para*-nitrophenyl α-L-rhamnopyranoside (*p*NP-Rha) determined. Specifically, solutions of *p*N-Rha in pH 7.0 0.1 M phosphate buffer (190 μl, 3.5, 3.0, 2.5, 2.0, 1.5, 1.0, 0.5 and 0.25 mM) were incubated at 37^{o}C in a multiwell plate for 5 min. The

glycosylated enzyme solution (10 μl) from the stability test was added to each well and absorbance at 405 nm was recorded every 6 s for 5 min (SpectraMax Plus spectrometer), with agitation before each reading. Michaelis-Menten parameters were determined according to the initial rates method and non-linear regression by using GRAFIT 4.0.13 (Erithacus Software, Horley, U.K.).

For trypsin stability testing a solution of glycosylated enzyme (0.5 mg) in phosphate buffer (0.1 M, pH 7, 900 μl) was incubated at 37˚C for 5 min. This solution was added to a pre-incubated (37˚C, 5 min) solution of bovine pancreatic trypsin (100 μl, 0.067 mg/ml) in phosphate buffer (0.1 M, pH 7), mixed thoroughly and maintained at 37˚C. Aliquots (100 μl) were removed at regular intervals and rhamnosidase Michaelis–Menten parameters towards *p*NP-Rha determined.

Tumor Disease Model. Thirty nine male MF1 nude mice (5 to 6 weeks old at the start of the study) were obtained from the Nottingham Academic Unit of Cancer Studies breeding colony. Under anaesthetic, Hypnorm (Roche)/Hypnovel (Jansen), a small incision was made, on the left flank of the animal, through the skin and the peritoneal wall. The spleen was gently exteriorised and cells, in a volume of up to 0.5 ml of HepG2 (human hepatocellular carcinoma) culture (ECACC; Porton Down, U.K.) injected into the tip of the spleen. With a syringe insert, the spleen was gently pushed back into the peritoneal cavity. The muscle wall was sutured, and the wound closed with metal wound clips. After recovery, the mice were allocated to their respective groups: (*i*) Doxorubicin (DOX)-Rha pro-drug *only* 10mg/kg i.v., three times weekly; (*ii*) enzyme *only* 2.5 mg/kg

intravenously, three times weekly; and (*iii*) enzyme (N-DG-Gal) then pro-drug (DOX-Rha) dosed 20 min after the enzyme preparation, intravenously, three times weekly. Treatment began on day 2 after the cells were injected and continued, three times weekly, until termination. At termination day 42, bromodeoxyuridine was administered to obtain the proliferative index of the liver tumour. Mice were terminally anaesthetized and serum samples obtained by cardiac puncture. Livers were weighed and formalin-fixed for histological quantification of total number of lesions per group and total "tumour burden" (The sum of each animal's tumour burden was calculated from approximate cell number multiplied by the number of lesions visible.)

Analysis of Variance/Statistical Analysis

Analysis of variance (*P* values) was determined by independent samples *t* test (two-tailed) using SPSS for WINDOWS 11.5.0 (SPSS, Chicago). N-DG-Gal +/- AF 10 min blood levels ($P = 0.004$); N-DG vs. N-DG-Gal ($P = 0.01$); N-WT vs. N-DG liver levels ($P = 0.027$); N-DG-Gal +/- AF liver levels ($P < 0.001$); N-DG-Man vs. N-DG liver levels ($P = 0.008$); N-WT vs. N-DG-Gal hepatocyte levels ($P < 0.001$); N-DG vs. N-DG-Gal hepatocyte levels ($P < 0.001$); N-WT vs. N-DG-Gal nonparenchymal levels ($P < 0.001$); N-DG vs. N-DG-Gal non-parenchymal levels ($P < 0.001$); N-DG-Gal +/- AF hepatocyte levels ($P < 0.001$); N-DG-Gal +/- AF nonparenchymal levels ($P < 0.001$); N-WT-dGal vs. N-WT-Gal hepatocyte level ($P = 0.006$)

1. Mort, A. J. & Lamport, D. T. A. (1979) *Anal. Biochem*. **81,** 289–309.
2. Young, N. M., Johnston, R. A. Z. & Richards J. C. (1989) *Carbohydr. Res.* **191,** 53–62.
3. Lee, Y. C., Stowell, C. P. & Krantz, M. J. (1976) *Biochemistry* **15,** 3956–3963.
4. Stowell, C. P. & Lee, Y. C. (1982) *Methods Enzymol*. **83,** 278-288.

5. Levene, P. A. (1924) *J. Biol. Chem*. **59,** 141–144.
6. Potter, A. L., Sowden, J. C., Hassid, W. Z. & Doudoroff, M. (1948) *J. Am. Chem. Soc*. **70,** 1751–1752.
7. Romero, C., Manjón, A., Bastida, J. & Iborra, J. L. (1985) *Anal. Biochem.* **149,** 566–571.
8. Fraker, P. J. & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun*. **80**, 849–857.
9. Laboratory Animal Science Association (1998) *Good Practice Guidelines: Collection of Blood Samples* (Laboratory Animal Science Association, Tamworth, U.K.)
10. Trouet, A. (1974) *Methods Enzymol.*, **31**, 323–329.
11. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem*. **150,** 76-85.