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ORIGINAL ARTICLE

Lectin-directed enzyme activated prodrug therapy (LEAPT): Synthesis and evaluation of rhamnose-capped prodrugs

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Abstract

The lectin-directed enzyme activated prodrug therapy (LEAPT) bipartite drug delivery system utilizes glycosylated enzyme, localized according to its sugar pattern, and capped prodrugs released by that enzyme. In this way, the sugar coat of a synthetic enzyme determines the site of release of a given drug. Here, prodrugs of doxorubicin and 5-fluorouracil capped by the nonmammalian L-rhamnosyl sugar unit have been efficiently synthesized and evaluated for use in the LEAPT system. Both are stable in blood, released by synthetically D-galactosylated rhamnosidase enzyme, and do not inhibit the uptake of the synthetic enzyme to its liver target. These results are consistent with their proposed mode of action and efficacy in models of liver cancer, and confirm modular flexibility in the drugs that may be used in LEAPT.

Keywords: Lectins, carbohydrates, doxorubicin, 5-fluorouracil, prodrug, enzyme release, l-rhamnose, d-galactose

Introduction

Carbohydrate-based ligand–receptor mechanisms are involved in a number of key cellular processes such as immune response (Varki, 1993), cell surface communication and signaling (Dwek, 1996), as well as inflammation (Weis and Drickamer, 1996; Zhang et al., 2010). Given the high specificity of interaction with carbohydrates and the broad range of cellular receptors that can potentially be specifically targeted by glycosylated molecules, a number of glycoprotein and glycopolymer-based systems have been developed to deliver drugs selectively to the desired active sites (Davis and Robinson, 2002; Singh et al., 2008). However, many of these systems are plagued by unwanted drug release at sites different from the desired site of action due to the very nature of the release mechanisms involved, for example endogenous lysosomal degradation. The challenge of achieving high site selectivity, which is for example of particular importance for the treatment of cancer cells while avoiding cytotoxic effects on non-cancerous ones, has been cleverly addressed by using bipartite systems such as antibody-directed enzyme prodrug therapy, using monoclonal antibody (mAb)–enzyme conjugates to release prodrugs at predetermined sites (Bagshawe, 1994). Similarly, catalytic antibodies have been suggested in a potential antibody-directed abzyme prodrug therapy (Wentworth et al., 1996). Both methods rely on the localization of the biocatalytic entity—an enzyme or a catalytic antibody—at a site determined by specific interactions with antigens presented on cells; a drug is subsequently released from its prodrug by this localized enzyme.

An approach that could exploit specific endogenous localization interactions could present clear advantages with regard to selectivity, mechanism, and avoiding the need to induce an effective, tolerated mAb as a part of a subsequent construct. We have previously described a novel catalytic, bipartite drug delivery system, lectin-directed enzyme activated prodrug therapy

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(LEAPT), which exploits the selectivity and specificity of carbohydrate–protein interactions to localize glycoconjugates in a switchable and sugar-dependent manner (Robinson et al., 2004). In the first phase of this strategy a glycosylated enzyme is targeted to specific cell types or tissues (Scheme 1). In the second phase, prodrugs capped with sugars are then administered. The glycosylated enzyme is then able to activate the prodrugs at the site of interest by cleaving the prodrug linkage. The colocalization of both prodrug and enzyme relies on their precise glycosylation. Selective enzyme targeting can be achieved by modification of the enzyme with carbohydrates that bind specific lectins on the surface of the targeted cells. Asialoglycoprotein receptors (ASGPRs) are expressed in abundance on the surface of hepatocytes (Lis and Sharon, 1998) and can specifically bind the sugars D-galactose (D-Gal) and L-rhamnose. Moreover, ASGPRs have the ability to trigger receptor-mediated endocytosis (RME) and transfer their ligands inside the cell. Therefore, we designed a system in which drugs of interest are capped with rhamnose and are released by a rhamnosidase enzyme, which is by itself gets glycosylated with Gal. For these potential prodrugs, the rhamnoside cap would serve (1) to decrease the cytotoxicity of the drug before its release, (2) to deliver the prodrug to specific cell types by RME—in this case hepatocytes after binding to ASGPR and subsequent endocytosis, and (3) to increase the sometimes poor solubility of drugs used in certain therapies. Galactosylation of the rhamnosidase would enable ASGPR-mediated internalization of this enzyme into the hepatocytes by RME. The non-mammalian sugar L-rhamnose (Rha)—and its associated glycosidase (L-rhamnosidase)—was chosen as a sugar cap to avoid prodrug activation at unwanted sites by endogenous mammalian glycosidases before reaching the ASGPR.

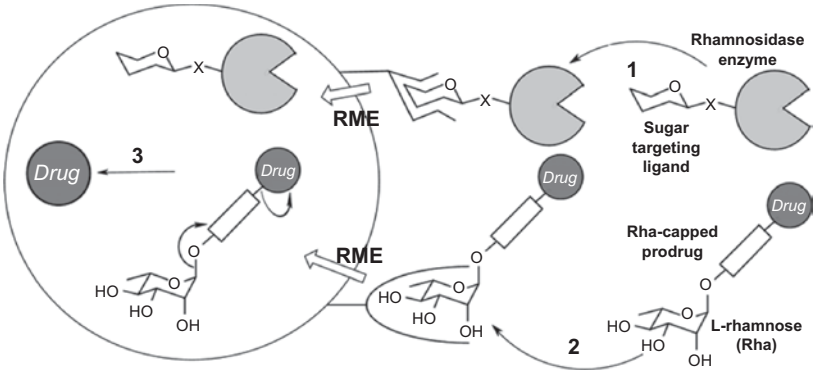
To validate this strategy and assess its therapeutic potential, we set out to prepare representative rhamnose-capped prodrugs, Rha-doxorubicin (Dox) and Rha-5-fluorouracil (5Fu), the two known cytotoxic compounds (Scheme 2).

Anthracycline doxorubicin, which intercalates in DNA and inhibits topoisomerase activity, has been used since the 1960s in the treatment of a wide range of malignancies such as breast cancer, Hodgkin's and non-Hodgkin's lymphoma, and acute leukemia (Blum and Carter, 1974; Launchbury and Habboubi, 1993). However, Dox is plagued by adverse effects and its cardiotoxicity, in particular, is well established (Lefrak et al., 1973), and like other anthracyclins, it is associated with cardiomyopathies that lead to congestive heart failures. 5Fu is an inhibitor of thymidilate synthase and is a major anticancer agent used in the treatment of gastrointestinal malignancies and breast cancer, and its combination with other drugs has had a great impact on the treatment of colorectal cancer (Longley et al., 2003), despite few low-response rates, for example in the first-line treatment of advanced colorectal cancer (Johnston and Kaye, 2001). The stability of the two prodrugs in blood was then evaluated to assess whether they are stable to circulating glycosidases. As the vital second component, rhamnosidase was modified with galactose (Gal) residues, and the biodistribution of this glycoengineered enzyme was determined *in vivo* by gamma scintigraphy.

Materials and methods

Synthesis—general

Proton nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DQX 400 (400 MHz) spectrometer, and the spectra were assigned using COSY. Carbon nuclear magnetic resonance spectra were recorded on a Bruker DQX 400 (100.6 MHz) spectrometer and were assigned using HMQC. Multiplicities were assigned using DEPT sequence. All chemical shifts are quoted on the δ scale in parts per million using residual solvent as the internal standard. Low-resolution mass spectra were recorded on a Micromass Platform 1 spectrometer using electrospray ionisation (ESI). High-resolution mass spectra were recorded by Dr. Neil J. Oldham in the Chemistry Research Laboratory on a Walters 2790 Micromass LCT ESI mass spectrometer using chemical ionization (NH_3, Cl) techniques as stated; m/z values are reported in dalton and are followed by their percentage abundance in parentheses. Infra red spectra were obtained with a Bruker Tensor 27 spectrophotometer, adsorption maxima being recorded in wave numbers (cm^{-1}) and classified as s (strong) and br (broad). Thin layer chromatography (TLC) was carried



Scheme 1. Concept of the LEAPT strategy (Robinson et al., 2004).

out on Merck TLC silica gel 60 F_{254} aluminium plates. Visualization of the plates was achieved using a UV lamp (λ_{max} = 254 or 365 nm), and/or ammonium molybdate (5% in 2 M H_2SO_4) or sulfuric acid (5% in EtOH). Flash column chromatography was carried out using Sorbsil C60 40/60 silica. DCM was distilled from calcium hydride. THF was distilled from sodium wire and benzophenone. Remaining anhydrous solvents were purchased from Fluka and Acros. "Petrol" refers to the fraction of petroleum ether boiling in the range 40–60°C.

Prodrug synthesis

1,2,3,4-Tetra-O-acetyl-L-rhamnose (1)

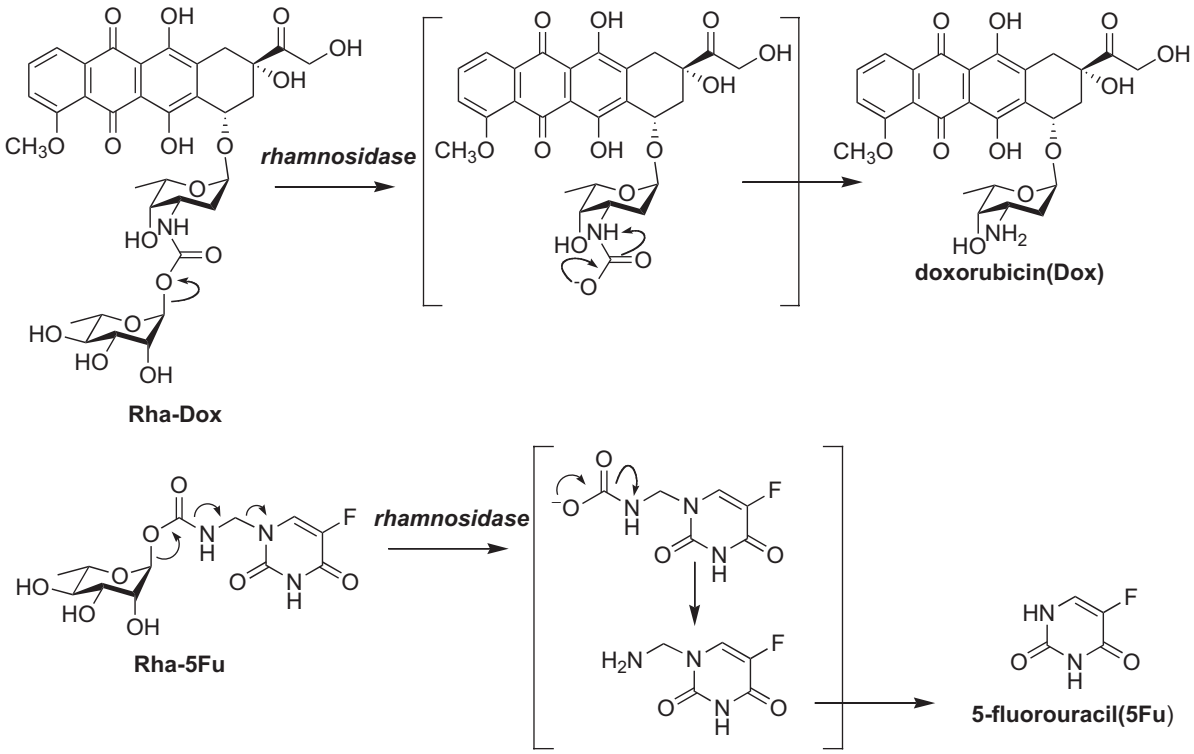
L-Rhamnose monohydrate (12 g, 0.66 mmol) was dissolved in acetic anhydride (40 ml) and pyridine (40 ml) and the mixture was stirred overnight at room temperature. The reaction mixture was diluted with AcOEt (300 ml), poured into ice water (500 ml), and stirred for 1 h. The aqueous fraction was separated and extracted twice with AcOEt (2 × 250 ml). The combined organic fractions were washed with 5% HCl (100 ml), saturated $NaHCO_3$ (2 × 100 ml), water (100 ml), and brine (100 ml); dried over $MgSO_4$; and concentrated *in vacuo*. Purification by flash column chromatography (AcOEt/petrol 1/2) afforded 1,2,3,4-tetra-*O*-acetyl-L-rhamnose 2 (22.5 g, quantitative) as a colorless syrup and a mixture of α- and β-anomers (α/β 1/3). For the α-anomer: R_f 0.49 (AcOEt/petrol 1/1); 1H NMR (400 MHz, $CDCl_3$): δ = 1.23 (3H, d, $J_{5,6}$ 6.3 Hz, H-6), 2.00, 2.06, 2.16, 2.21 (4 × 3H, 4 × s, 4 × $COCH_3$), 3.90–3.97 (1H, m, H-5), 5.12 (1H, at, J = 9.9 Hz, H-4), 5.24–5.25 (1H, m, H-2), 5.30 (1H, dd, $J_{2,3}$ 3.2 Hz, $J_{3,4}$ 9.9 Hz, H-3), 6.01 (1H, d, $J_{1,2}$ 2.0 Hz, H-1) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.2 (C-6), 20.7, 20.7, 20.8, 20.9 (4 × $COCH_3$), 68.7, 70.4 (C-2, C-5), 90.6 (C-1), 168.4, 169.8, 169.8, 170.1 (4 × 4 × $COCH_3$) ppm; m/z (ES^+) 355 (M–Na, 100%).

2,3,4-Tri-O-acetyl-L-rhamnose (3)

1,2,3,4-Tetra-*O*-acetyl-L-rhamnose **2** (20.15 g, 60.7 mmol) was dissolved in dry THF (160 ml) under argon. Benzylamine (10 ml, 91.5 mmol) was added and the mixture was stirred at room temperature for 18 h before addition of water (300 ml). After extraction with DCM (450 ml, then 2 × 150 ml), the combined organic fractions were washed with 10% HCl (100 ml), saturated $NaHCO_3$ (100 ml), and brine (100 ml), dried over $MgSO_4$, and concentrated *in vacuo*. Purification by flash column chromatography (AcOEt/petrol 2/3) afforded 2,3,4-tri-*O*-acetyl-L-rhamnose **3** (16.0 g, 91%) as a white powder and a mixture of α- and β-anomers. For the major anomer α: R_f 0.38 (AcOEt/petrol 1/1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.22 (3H, d, $J_{5,6}$ 6.3 Hz, H-6), 2.00, 2.06, 2.16 (3 × 3H, 3 × s, 3 × $COCH_3$), 4.95 (0.2 H, bs, OH), 4.10–4.16 (1H, m, H-5), 5.08 (1H, t, $J_{4,5}$ 9.9 Hz, H-4), 5.15–5.17 (1H, m, H-1), 5.27 (1H, dd, $J_{1,2}$ 1.8 Hz, $J_{2,3}$ 3.3 Hz, H-2), 5.37 (1H, dd, $J_{2,3}$ 3.3 Hz, $J_{3,4}$ 9.9 Hz, H-3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.4 (C-6), 20.7, 20.8, 20.9 (3 × $COCH_3$), 66.4 (C-5), 68.8 (C-3), 70.2 (C-2), 71.1 (C-4), 92.1 (C-1), 170.1, 170.2, 170.3 (3 × $COCH_3$) ppm.

(2,3,4-Tri-O-acetyl-α-L-rhamnopyranosyl) 4-nitrophenyl carbonate (4)

2,3,4-Tri-*O*-acetyl rhamnose **3** (15.90 g, 54.4 mmol) and 4-nitrophenyl chloroformate (11.6 g, 57.5 mmol) were



Scheme 2. Doxorubicin and 5-fluorouracil prodrugs and their postulated activation mechanisms by rhamnosidase.

dissolved in dry DCM (160 ml) under an atmosphere of argon. Pyridine (4.4 ml, 54.4 mmol) was added, and the mixture was stirred overnight at room temperature. The reaction mixture was concentrated *in vacuo*. The residue was purified by flash chromatography (AcOEt/petrol 2/3) to afford compound **4** (14.87 g, 60%) as an yellow powder. R_f 0.4, AcOEt/petrol 2/3); $[\alpha]^{21}{}_D = -67.7$ (*c* 1, $CHCl_3$); ν_{max} (KBr) 1790, 1753 (s, CO), 1619 (s, Ar), 1533 (s, NO_2), 1351 (s, NO_2) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 1.30$ (3H, d, $J_{5,6}$ 6.1 Hz, H-6), 2.04, 2.09, 2.20 (3 × 3H, 3 × s, 3 × $COCH_3$), 4.04–4.16 (1H, m, H-5), 5.19 (1H, at, *J* 10.1 Hz, H-4), 5.37 (1H, dd, $J_{2,3}$ 3.5 Hz, $J_{3,4}$ 10.1 Hz, H-3), 5.43 (1H, dd, $J_{1,2}$ 2.0 Hz, $J_{2,3}$ 3.5 Hz, H-2), 6.02 (1H, d, $J_{1,2}$ 1.8 Hz, H-1), 7.46 (2H, d, *J* 9.1 Hz, Ar-H_a), 8.31 (2H, d, *J* 9.1 Hz, Ar-H_b) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 17.4$ (C-6), 20.6, 20.7, 20.7 (3 × $COCH_3$), 68.2 (C-2), 68.4 (C-3), 69.4 (C-5), 70.0 (C-4), 95.2 (C-1), 121.7 (Ar_a), 125.4 (Ar_b), 145.7 (CNO_2), 150.4, 155.0 (Ar-O, OC(O)O), 169.7, 169.8, 170.0 ($COCH_3$) ppm.

N-(2,3,4-Tri-O-acetyl-α-L-rhamnopyranosyloxycarbonyl) glycine (5)

Compound **4** (2.23 g, 7.10 mmol) was dissolved in 75 ml of acetone. Water (40 ml), $NaHCO_3$ (1.81 g, 21.5 mmol) and glycine (1.62 g, 21.6 mmol) were added. The reaction mixture was stirred for 3 h at room temperature until no starting material could be detected by TLC. The reaction mixture was neutralized with 20% HCl to pH 3. After extraction with AcOEt (3 × 100 ml), the organic fractions were combined, washed with brine (100 ml), dried over $MgSO_4$, and concentrated *in vacuo*. Purification by flash column chromatography (AcOEt, 1% MeOH) afforded compound **5** as a white powder (1.54 g, 80%). $[\alpha]^{21}{}_D = -34.6$ (*c* 0.5, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 1.24$ (3H, d, $J_{5,6}$ 6.1 Hz, H-6), 2.03, 2.07, 2.18 (3 × 3H, 3 × s, 3 × $COCH_3$), 3.97 (1H, dd, $J_{4,5}$ 9.9 Hz, H-5), 4.08–4.14 (1H, m, CH_2), 5.13 (1H, pt, $J_{3,4}$ 10.1 Hz, H-4), 5.28–5.30 (1H, m, H-2), 5.34 (1H, dd, $J_{1,2}$ 2.0 Hz, H-3), 5.76 (1H, t, J_{NH-CH2} 5.3 Hz, NH), 5.96 (1H, d, $J_{1,2}$ 2.0 Hz, H-1) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.4 (C-6), 20.7, 20.8, 21.1 (3 × $COCH_3$), 42.4 (CH_2), 68.4, 68.7, 68.9 (C-2, C-3, C-5), 70.5 (C-4), 91.6 (C-1), 153.6 (OC(O)N), 169.9, 170.0, 170.8 (3 × $COCH_3$), 173.0 (CO_2H) ppm; *m*/*z* (ES^-) 390 (M–H, 100%); HRMS (ES^-) calculated for $C_{15}H_{20}NO_{11}$ (M-–H) 390.1036, found value 390.1055.

Acetoxy-N-(2,3,4-tri-O-acetyla-L-rhamnopyranosyloxycarbonyl) methylamine (6)

Compound **5** (500 mg, 1.28 mmol) was dissolved in a mixture of dry THF (30 ml) and toluene (6 ml) under argon. Pyridine (105 µl, 1.30 mmol) and lead tetra-acetate (715 mg, 1.61 mmol) were successively added and the mixture was refluxed (70°C) for 45 min. The reaction was cooled to room temperature and filtered over Celite®. The filtrate was concentrated *in vacuo*. The residue was purified by flash chromatography (AcOEt/petrol 1/1) to afford compound **6** (185 mg, 35%) as a white powder. $[\alpha]^{21}{}_D = -28.6$ (*c* 0.3, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 1.25$ (3H, d, $J_{5,6}$ 6.3 Hz, H-6), 2.00, 2.11, 2.17 (3 × 3H, 3 × s, 3 × $COCH_3$), 3.89–3.96 (1H, m, H-5), 5.11 (1H, dd, $J_{3,4}$ 10.1 Hz, $J_{4,5}$ 9.9 Hz, H-4), 5.19-5.25 (1H, m, H-3), 5.22 (2H, s, H8), 5.27 (1H, dd, $J_{1,2}$ 1.5 Hz, $J_{2,3}$ 3.3 Hz, H-2), 6.00 (1H, d, $J_{1,2}$ 1.5 Hz, H-1), 6.10 (1H, pt, J_{NH-CH2} 7.1 Hz, NH) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.4 (C-6), 20.6, 20.8, 20.8, 20.9 (4 × $COCH_3$), 66.2 (CH_2), 68.5, 68.6, 68.7 (C-2, C-3, C-5), 70.3 (C-4), 91.8 (C-1), 153.1 (OC(O)N), 169.8, 169.8, 170.1 (3 × $COCH_3$), 171.8 ($COCH_3$) ppm; *m*/*z* (ES^+) 423 (M+ NH_4^+, 30%), 428 (MNa^+, 100%); HRMS (ES^+) calculated for $C_{16}H_{23}NO_{11}Na$ (MNa^+) 428.1169, found 428.1165.

N-(2,3,4-tri-O-acetyl-α-L-rhamnopyranosyloxycarbonyl)-(5-fluoro-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-methylamine (7)

Compound **6** (169 mg, 0.417 mmol) was dissolved in dry DMF (10 ml). Triethylamine (60 µl, 0.43 mmol) and 5Fu (178 mg, 1.36 mmol) were successively added and the reaction mixture was stirred at room temperature for 16 h. The reaction mixture was concentrated *in vacuo*. The white residue was dissolved in water (50 ml) and AcOEt (50 ml). The organic fraction was separated and the aqueous fraction was extracted with AcOEt (2 × 50 ml). The combined organic fractions were washed with brine (20 ml), dried over $MgSO_4$, and concentrated *in vacuo*. Purification by flash column chromatography (AcOEt) afforded compound **7** (154 mg, 77%). $[\alpha]^{21}{}_D = -60.0$ (*c* 1, $CHCl_3$); ν_{max} (KBr) 3410 (b, NH), 1751 (s, CO), 1234 (s, CF) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 1.23$ (3H, d, $J_{5,6}$ 6.1 Hz, H-6), 1.99, 2.05, 2.16 (3 × 3H, 3 × s, 3 × $COCH_3$), 3.94–4.01 (1H, m, H-5), 4.99–5.14 (3H, m, H-8, H-4), 5.23–5.28 (2H, m, H-3, H-2), 5.96 (1H, bs, H-1), 7.35 (1H, t, J_{NH-CH2} 6.6 Hz, N*H*CH_2), 7.71 (1H, d, $J_{5,6}$ 5.1 Hz, H-6'), 10.25 (1H, d, *J* 4.5 Hz, CONHCO) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 17.4$ (C-6), 20.7, 20.7, 20.8 (3 × $COCH_3$), 55.2 (C-8), 68.6, 68.6, 68.7 (C-2, C-3, C-5), 70.3 (C-4), 91.9 (C-1), 129.3 (C-6'), 141.4 (C-5'), 150.2 (C-2'), 154.6 (OC(O)N), 157.5 (C-4'), 169.8, 169.9, 170.3 (3 × $COCH_3$) ppm; *m*/*z* (ES^-) 474 (M–H^+, 100%); HRMS (ES^-) calculated for $C_{18}H_{21}N_3O_{11}F$ (M–H^+) 474.1160, found 474.1181.

N-(α-L-rhamnopyranosyloxycarbonyl)-(5-fluoro-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-methylamine (8)

Compound **7** (124 mg, 0.26 mmol) was dissolved in dry MeOH (6 ml) under argon. The solution was cooled to 0°C before addition of sodium methoxide (22 mg, 0.42 mmol). After 1 h, the reaction was quenched by addition of Dowex-H^+. The mixture was filtered, concentrated, and purified by flash chromatography (AcOEt/MeOH 5/1) to afford compound **8** (91 mg, 99%) as a white powder. $[\alpha]^{21}{}_D = -27.4$ (c 0.5, MeOH); ν_{max} (KBr) 3415 (b,OH, NH), 1699, 1654 (s, CO, C=C), 1243 (s, CF) cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 1.26$(3H, d, $J_{5,6}$ 6.1 Hz, H-6), 3.42 (1H, pt, *J* 9.6 Hz, H-4), 3.60–3.70 (2H, m, H-3, H-5), 3.73–3.84 (1H, m, H-2), 5.02 (2H, s, H-8), 5.88 (1H, d, H-1) ppm; *m*/*z* (ES^-) 348 (MH^+, 30%), 384 (MCl^-, 100%); HRMS (ES^-) calculated for $C_{12}H_{15}N_3O_8F$ (M–H^+) 348.0843, found 348.0836.

N-(2,3,4-tri-O-acetyl-α-L-rhamnopyranosyloxycarbonyl) doxorubicin (10)

Compound **4** (1.57 g, 3.44 mmol) and doxorubicin hydrochloride **9** (2.00 g, 3.44 mmol) were dissolved in dry DMF (125 ml). Triethylamine (0.7 ml, 5.2 mmol) was added and the mixture was stirred overnight before being concentrated *in vacuo*. The residue was purified by flash chromatography (AcOEt, then AcOEt/MeOH 9/1) to afford compound **10** (2.77 g, 94%) as a red powder (R_f 0.18, AcOEt). $[\alpha]^{21}_D = +12.6$ (*c* 1, $CHCl_3$); *m/z* (ES^-) 858 (M–H, 50%); HRMS (ES^+) calculated for $C_{40}H_{49}N_2O_{20}$ (M + NH_4) 877.2879, found 877.2874.

N-α-L-rhamnopyranosyloxycarbonyl) doxorubicin (11)

Compound **10** (320 mg, 0.37 mmol) was dissolved in a mixture of 50 ml dry MeOH, 10 ml dry DMF, and 2.5 ml 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 and 40 min with Dowex-H^+ when no starting material could be detected. The mixture was filtered and concentrated *in vacuo* to afford compound **11** (265 mg, 94%) as a red powder. $[\alpha]^{21}_D = +19.6$ (c 0.15, MeOH); *ν* IR (KBr) *ν* ($CHCl_3$), 4300 (OH, NH), 1725 cm^{-1} (NHCO); ^1H NMR (400 MHz, H_2O): $\delta = 1.19$ (3H, d, H-6'), 1.32 (3H, d, H-6″), 1.78–192 (3H, m, H-2', $H\text{-}8_a$), 2.11 (1H, d, $H\text{-}8_b$), 2.39 (1H, d, $H\text{-}10_a$), 2.70 (1H, d, $H\text{-}10_b$), 3.60–3.72 (7H, m, H-3', H-3″, H-5″, H-4″,OCH_3), 3.73–3.89 (1H, m, H-2″), 4.08–4.14 (1H, m, H-5'), 4.55 (1H, bs, H-7), 5.29 (1H, bs, H-1'), 5.88 (1H, d, H-1″), 7.1 (1H d H-1), 7.10 (1H, d, H-1), 7.35 (1H, t, H-2) ppm; *m/z* (ES^-) 732 (M–H, 100%); HRMS (ES^-) calculated for $C_{34}H_{38}NO_{17}$ (M–H) 732.2140, found 732.2139.

Purification of α-L-rhamnosidase (N-WT)

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. Purification and deglycosylation of naringinase was carried out using methods described previously (Robinson et al., 2004). Naringinase was purified to yield pure wild-type α-rhamnosidase activity in four steps: (1) dialysis (12–14 kDa MWCO, Visking dialysis tubing); (2) BioGel P100 size exclusion chromatography (BioRad, eluant pH dionised water); (3) BioGel P100 size exclusion chromatography (BioRad, eluant pH 4.8, 0.1 M NaCl); (4) DEAE Sepharose ion exchange chromatography (Amersham Biosciences, eluant pH 6.0, 20 mM L-histidine 0–0.35 mM NaCl gradient). In Steps 3 and 4, the fractions containing solely α-L-rhamnosidase activity were combined, freeze-dried, and desalted by dialysis. α-L-Rhamnosidase activity was assessed using para-nitrophenyl α-L-rhamnopyranoside (para-NP α-L-Rha) as substrate by mixing equal volumes of fraction constituent with 3.5 mM para-NP α-L-Rha in orthophosphate buffer and determining release of para-nitrophenol at 405 nm.

Preparation of deglycosylated α-L-rhamnosidase (N-DG)

Wild-type naringinase (N-WT) was deglycosylated using endoglycosidase-H (endo-H, 32 U/100 mg N-WT) for 6 h in 0.1 M orthophosphate buffer, pH 6.0, 37°C, and then purified by dialysis (50 kDa MWCO) to give deglycosylated naringinase (N-DG; Robinson et al., 2004).

Protein glycosylation to yield N-DG-Gal and N-WT-Gal

Proteins were glycosylated using the 2-imino-2-methoxyethyl (IME) 1-thioglycoside method (Stowell and Lee, 1980, 1982). An approximately 500:1 ratio of modification reagent to protein was used in each reaction. For example, N-DG-Gal was prepared as follows. Cyanomethyl-2,3,4,6-tetra-*O*-acetyl-β-D-thiogalactopyranoside (30 mg, 0.074 mmol) was dissolved in anhydrous methanol (3.0 ml) in a flask fitted with a magnetic stirrer under an inert atmosphere, treated with an anhydrous methanolic solution of sodium methoxide (30 μl, 1 M) and stirring continued at room temperature. After 36 h, all solvent was removed *in vacuo* yielding a white gum. N-DG (10 mg) was dissolved in an aqueous solution 0.25 M sodium tetraborate (2 ml) pH 8.5, added to the white gum, and stirred at room temperature for 24 h. The solution was dialyzed against deionised water (2 l, 4 changes) using Visking dialysis tubing (12-14 kDa MWCO), and then desalted using Sephadex G25 (PD10 column), eluting with deionised water. The resultant solution was freeze-dried yielding a white powder (Robinson et al., 2004).

Protein analysis

Proteins were analyzed by gel electrophoresis (10% SDS–PAGE, pH 8.8, Tris buffer; Vertical Slab Gel Kit, Atto Corporation), and by matrix-assisted laser desorption ionization mass spectrometry (MS, Ciphergen Biosystems PBS II, Ciphergen Biosystems; sinapinic acid matrix 10 mg/ml 3:2 water:acetonitrile, 0.2% TFA).

Prodrug stability study in blood

Rha-Dox (**11**) solutions (1, 10, or 100 μg/ml) were prepared in either 0.9% NaCl or rabbit blood and incubated at 37°C. For the samples incubated in 0.9% NaCl, aliquots (0.5 ml) collected at given time points were mixed with 20% TFA (15 μl), transferred into a Vivaspin 3000 tubes and centrifuged at 13,000*g* for 30 min. The filtrate was directly injected for HPLC determination. For blood samples, the blood was centrifuged at 13,000*g* for 10 min, 0.5 ml of supernatant was mixed with 20% TFA (15 μl), sonicated at 25°C for 5 min, transferred into Vivaspin 3000 tubes, and centrifuged at 13,000*g* for 30 min. The filtrate was analyzed by HPLC.

Similarly, Rha-5Fu (**8**) was incubated in 0.9% NaCl or rabbit blood at 37°C at 2, 20, or 200 μg/ml. For the samples incubated in 0.9% NaCl, aliquots (0.2 ml) collected at given time points were mixed with 20% TFA (12 μl) and deionised water (188 μl), sonicated at 25°C for 5 min, and centrifuged at 13,000*g* for 10 min. The supernatant was transferred into Vivaspin 3000 tubes, centrifuged at

13,000*g* for 30 min, and 50 µl of filtrate was used for HPLC analysis. For blood samples, the blood was centrifuged at 13,000*g* for 10 min, 0.2 ml of plasma was diluted with TFA and deionised water, and then sonicated and centrifuged as described earlier.

Analyses were performed on a reverse-phase HPLC system consisting of a Waters 2795 separations module (Waters Assoc. Milford, MA) coupled to a Jasco FP-920 Intelligent fluorescence detector (Waters Assoc.) for the Dox analysis (excitation wavelength set at 480 nm and emission at 560 nm, gain was set at ×100). For the 5Fu analysis, a Waters 2996 photodiode array detector was used (detection wavelength was set at 265 nm for 5Fu and 269 nm for Rha-5Fu). Chromatograms were acquired, stored, and processed using the Empower software program (Waters). Chromatographic separations were carried out using a Hypersil C8 analytical column (5 µm, 250 × 4.6 mm). The Dox samples were separated under isocratic conditions at 65% sodium phosphate buffer (20 mM, pH 2.8, containing 0.01% TFA) and 35% methanol at 1.0 ml/min. The mobile phases used for the 5Fu samples are summarized in Table 1.

Table 1. HPLC conditions for the 5Fu and Rha-5Fu samples.

	Time (min)	Mobile phase	Flow rate (ml/min)	Retention time (min)
5Fu	0–12	MeOH–H_2O (99:1)	0.5	9.8
	12–19	MeOH–H_2O (97:3)	2	
	19–26	MeOH–H_2O (99:1)	2	
Rha-5Fu	0–15	MeOH–H_2O (97:3)	1	11.9

Preparation of ^{123}I-labeled enzymes

The glycosylated enzymes were labeled with ^{123}I by using 1,3,4,6-tetrachloro-3α-6α-diphenylglycouril (IODO-Gen, Pierce) according to the manufacturer's instructions.

Gamma scintigraphy analysis

Biodistribution studies were performed in male New Zealand White rabbits. Hypnorm sedated animals ($n = 4$; average mass 1.0 kg) were injected intravenously with ^{123}I-labeled enzyme (2.5 mg/kg in < 1 ml; ~3 MBq) solution in PBS. Imaging was done on a Maxi Gamma Camera 406 (GE Medical Systems) at 1, 10, 30, 60, 90, and 120 min.

Results and discussion

Design and synthesis of the prodrugs

We hypothesized that rhamnose-capped Dox **11** would fragment according to the mechanism depicted in Scheme 2 on enzymatic hydrolysis by α-rhamnosidase to release Dox **9**; loss of rhamnosyl would lead to carbamic acid formation followed by spontaneous decarboxylation. An extended aminomethylcarbamate linker was used for 5Fu prodrug **8** (Scheme 2) based on the study by Monneret and co-workers for glucuronide-based prodrugs (Madec-Lougerstay et al., 1999). The mode of collapse of aminomethylcarbamate linker also involves rhamnosyl loss followed by decarboxylation and aminal hydrolysis.

The same L-rhamnosyl precursor **4**, containing a carbonate moiety as a precursor to carbamate formation, was used for the synthesis of both 5Fu and Dox prodrugs, **8** and **11,** respectively (Scheme 3). L-(+)-Rhamnose monohydrate (**1**) was peracetylated by reaction with acetic anhydride and pyridine (Fisher et al., 1920). Deprotection of the anomeric hydroxyl was achieved after treatment with benzylamine to afford compound **3** (Sim et al., 1993). Hemiacetal **3** was then reacted with 4-nitrophenyl chloroformate in the presence of pyridine to afford mixed carbonate **4** in 60% yield. Compound **4** was the key divergent intermediate used for the preparation of both the prodrugs.

Prodrug **8** was prepared from activated rhamnose precursor **4** as follows (Scheme 3). Coupling of compound **4** with glycine in DMF, to install the first portion of the aminal-carbamate linker, was initially unsuccessful. After further attempts at varying reaction conditions (with triethylamine or *N*-ethyldiisopropylamine, under phase transfer conditions, under reflux), glycinylcarbamate **5** was eventually obtained in 80% yield using sodium bicarbonate as base and a mixture of water and acetone as solvent. Oxidative decarboxylation (Gledhill et al., 1986; Madec-Lougerstay et al., 1999) of compound **5** with lead tetra-acetate (1.25 eq.) in the presence of pyridine (1 eq.) was achieved in 35% yield to afford acetate **6** after purification by flash chromatography on silica. Condensation of compound **6** with 5Fu in the presence of Et3N proceeded in 77% yield to afford compound **7**. We suspected the product to be unstable due to its hemiaminal-like structure, and indeed 2D-TLC on silica revealed partial degradation. Therefore, subsequently no attempt was made to purify compound **6** before coupling with 5Fu; decarboxylation of compound **5** was performed by heating lead tetra-acetate for 40 min under reflux; the reaction mixture was then cooled to room temperature and filtered through Celite®. Immediate coupling of the crude product with 5Fu yielded acetylated prodrug **7** in 97% yield over two steps. Finally, deacetylation under standard Zémplen conditions afforded 5Fu prodrug **8** in quantitative yield.

The Dox prodrug **11** was also prepared from compound **4** and doxorubicin hydrochloride (**9**) (Scheme 3). The reaction of the doxorubicin amine group with activated rhamnose **4** proceeded readily in the presence of 1.5 eq. of pyridine to afford carbamate **10**. Deacetylation of compound **10** required precise conditions. When carried out using 0.1 M sodium methoxide in dry MeOH at 0°C, the reaction proceeded almost quantitatively on a small scale (100 mg). However, during attempts to scale up (to >1.5 g), the reaction failed and formation of

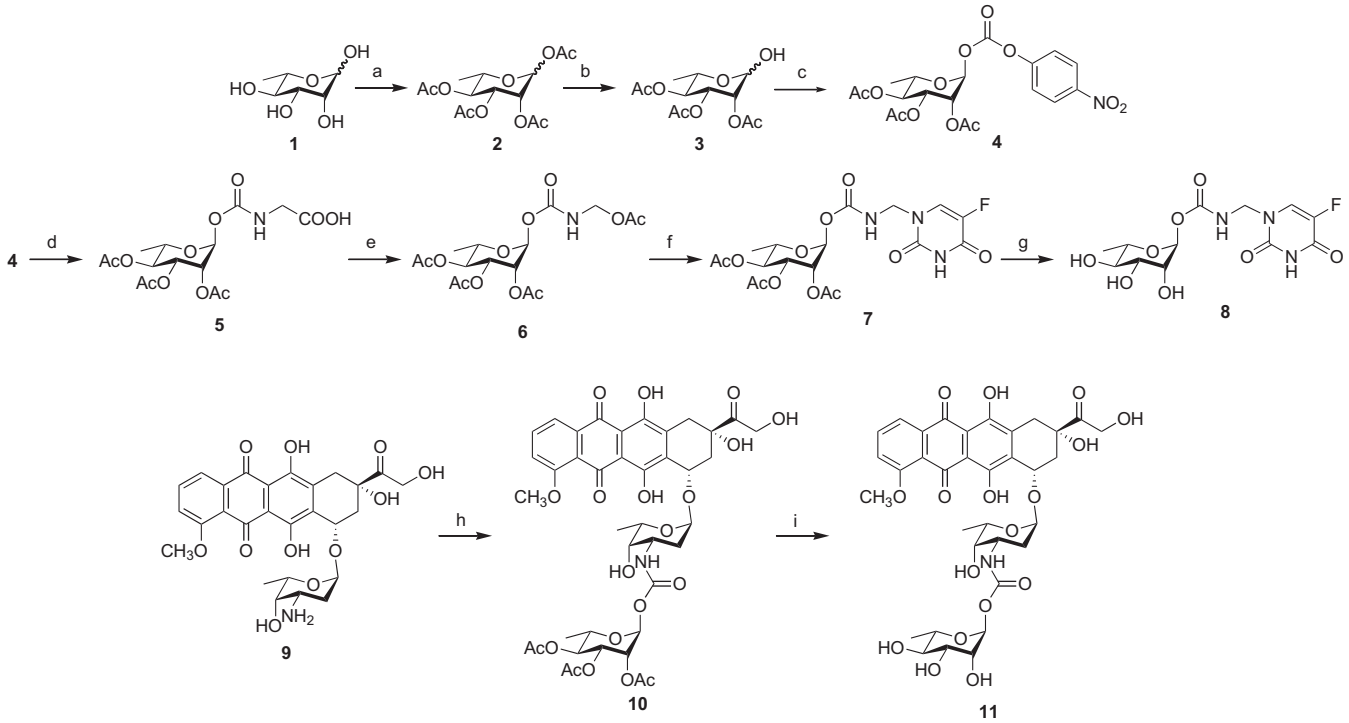
insoluble products was observed both during the reaction and after quenching with acidic Dowex® resin. Inspired by Florent et al. (1998), a mixture of MeOH/DMF/THF (100/20/5) was used as a solvent instead of neat MeOH (Scheme 3). In this new solvent system, no precipitation was observed during the reaction or after quenching with Dowex-H^+ resin and filtration. Overall, this last step was achieved in 94% yield, about twice the yield obtained in neat MeOH.

Enzyme glycoengineering

Naringinase, produced by the fungus *P. decumbens*, offers a ready source of α-L-rhamnosidase. Purified N-WT was obtained in four steps from the dialyzed naringinase. After dialysis into deionised water, the crude naringinase solution was separated by a short Bio-Gel P-100 column eluted with deionised water to remove most of the small molecular weight impurities. A second round of Bio-Gel P-100 chromatography separated α-L-rhamnosidase from fractions containing β-D-glucosidase. Finally, ion-exchange chromatography on a DEAE Sepharose column separated α-L-rhamnosidase from the remaining protein impurities. The yields (w/w) were 24, 45, and 54% for three steps respectively, with ~6% overall yield.

Naringinase produced by *P. decumbens* carries a mannose-rich motif (Richards and Leitch, 1989), which were removed to avoid any undesired ligand-mediated interaction. Treatment of N-WT with endo-β-*N*-acetylglucosaminidase H (endo-H) yielded N-DG.

The ASGPR, of which 50,000 to 500,000 copies (Eisenberg et al., 1991) are typically present on the surface of a hepatocyte cell, has been shown to provide an efficient target for carbohydrate-mediated drug delivery to the liver by endocytosis. Although individual carbohydrate ligand to receptor interactions are typically not strong enough to mediate efficient targeting, these can be greatly enhanced by taking advantage of the multivalent (or cluster) effect; this can be achieved by displaying multiple copies of a monosaccharide or branched saccharides at the surface of a macromolecule. Impressive enhancement of ASGPR binding of up to 1×10^6-fold have been obtained for tetra-antennary Gal-terminated ligands over monoantennary-Gal-terminated ones (Lee et al., 1983). Therefore, a strategy involving the attachment of multiple copies of Gal monosaccharides was selected for the glycoengineering of N-WT to target it to the ASGPR. Protein glycosylation can be carried out using a variety of chemical methods that offers key advantages such as versatility (choice of carbohydrate structure), selectivity (choice of glycosylation site), and ease of scale-up (Gamblin et al., 2009). For this study, we chose to use the IME method (Lee et al., 1976; Stowell and Lee, 1982) to modify lysine residues on N-DG. The enzymes N-WT and N-DG were incubated with monosaccharide Gal IME reagent to afford N-WT-Gal and N-DG-Gal, respectively. These synthetic glycoproteins were characterized by gel electrophoresis and mass spectrometry. Matrix-assisted laser desorption ionization MS analysis showed an average incorporation of 11–15 Gal residues per enzyme molecule: **N-WT** 76148;



Reaction conditions: a) Ac2O, pyridine, rt, quant.; b) BnNH2, THF, 91 %; c) 4-nitrophenyl chloroformate, pyridine (1 eq.), DCM, 60 %; d) glycine, NaHCO3, water/acetone (7/3), 80 %; e) Pb(OAc)4, pyridine, THF/toluene, 35 %; f) 5-fluorouracil, Et3N, DMF, 77 %; g) 0.1 M NaOMe, MeOH, quant.; h) 4, Et3N (1.5 eq.), DMF, 94 %; i) 0.016 M NaOMe, MeOH/DMF/THF (50/10/2.5), 94 %.

Scheme 3. Synthesis of 5-fluorouracil and doxorubicin prodrugs.

N-WT-Gal 79443 (N-WT + 3295 ~+14 Gal); **N-DG** 69341; **N-DG-Gal** 71892 (NDG + 2551 ~+11 Gal).

Blood stability and cap-release studies

Rha-Dox **11** was incubated at three different concentrations for 60 min in either 0.9% NaCl or rabbit blood to assess its stability as determined by HPLC analysis (Table 2). Although Rha-Dox **11** was, as expected, stable in 0.9% NaCl, a significant decrease was observed for blood samples of Rha-Dox after incubation at 37°C. However, no significant amount of Dox **9** was detected by HPLC, which suggests that the lower levels of Rha-Dox observed in blood cannot be attributed to hydrolysis of Rha-Dox by glycosidases present in blood. Other mechanisms, for example, binding with blood proteins or degradation by other pathways or absorption at the surface of blood cells, could account for the drop in Rha-Dox titres that were observed.

The Rha-5Fu prodrug **8** was shown to be very stable in both 0.9% NaCl and in blood, as shown in Table 3. Almost quantitative recovery of the intact prodrug was observed after 60 min incubation in rabbit blood.

Incubation of Rha-Dox **11** and Rha-5Fu **8** in both 0.9%NaCl and blood with both N-WT-Gal and N-DG-Gal showed ready release of Dox and 5Fu, respectively. This importantly confirmed both the activity of synthetically glycosylated enzymes and the liability of the prodrugs to these enzymes.

Effect of prodrugs on time course of distribution of synthetically glycosylated enzymes using gamma scintigraphy

Determination of the *in vivo* distribution of macromolecules can be achieved in a noninvasive manner by using gamma scintigraphy. Spatial distribution to the different organs can be determined quantitatively with ^{123}I labeling (Smedsrød et al., 1994). Male New Zealand white rabbits were dosed with N-DG-Gal, N-DG-Gal + Rha-Dox, or N-DG-Gal + Rha-5Fu. Images were acquired using a Maxi Gamma Camera 406 (GE Medical Systems, Slough, UK) at 1, 10, 30, 60, 90, and 120 min after administration. Analysis was carried out using a MICAS X image processing system (Bartec Technologies, Camberley, Surrey, UK). As can be seen on the time course distribution shown in Figure 1, strong localization of the ^{123}I-labeled N-DG-Gal was achieved in the liver, with a smaller amount detected in the kidney and bladder area. Importantly, coadministration of the rhamnose-capped prodrugs Rha-Dox or Rha-5Fu did not significantly affect enzyme localization. These observations are consistent with results obtained in our previous study for N-DG-Gal alone (Robinson et al., 2004), which had established that liver uptake is strongly dependent on its sugar coat. Taken together, these results suggest that dosing of the prodrugs together with N-DG-Gal does not lead to inhibition of N-DG-Gal uptake.

Table 2. Stability of Rha-Dox after 60 min in 0.9% NaCl or rabbit blood.

	Initial Rha-Dox concentration (μg/ml)	% Recovery (deviation)
0.9% NaCl	100	100.6 (1.8)
	10	100.5 (1.0)
	1	101.8 (3.4)
Rabbit blood	100	62.3 (1.7)
	10	65.6 (1.2)
	1	55.1 (1.2)

Table 3. Stability of Rha-5Fu after 60 min in 0.9% NaCl or rabbit blood.

	Initial Rha-5Fu concentration (μg/ml)	% Recovery
0.9% NaCl	200	99.9
	20	99.8
	2	101.5
Rabbit blood	200	97.2
	20	96.5
	2	94.6

Conclusions

Two rhamnose-capped prodrugs of the common cytotoxic drug Dox and 5Fu have been synthesized. Their demonstrated stability in blood makes them suitable for the LEAPT strategy as they do not undergo unwanted hydrolysis before reaching their target, in this case the hepatocytes. These findings complement the previously demonstrated enhancement in stability shown for the glycosylated enzyme N-DG-Gal (Robinson et al., 2004); chemical galactosylation of N-DG to form N-DG-Gal resulted in increased enzyme stability, extending its half-

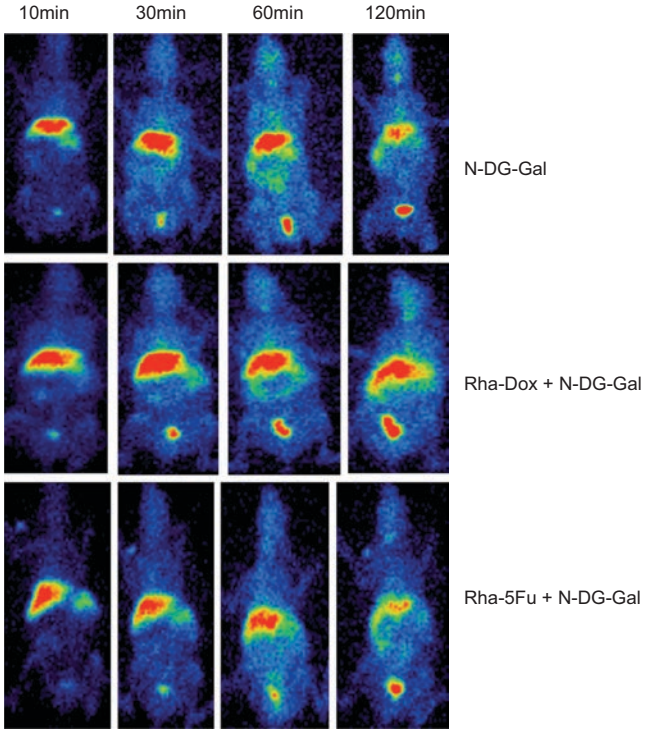


Figure 1. Gamma scintigraphy time-course determination of *in vivo* organ distribution.

life from 1 h for N-DG to more than 48 h for N-DG-Gal. Such results, suggesting increased stability of synthetically glycosylated enzymes, tally with those recently determined against proteolytic enzymes (Russell et al., 2009).

Rhamnosidase N-WT was glycoengineered to retune its binding specificity by alteration of its natural glycosylation pattern (to form N-DG) and subsequent modification by the attachment of multiple Gal moieties (to form N-DG-Gal) to specifically target the ASGPR and simultaneously take advantage of a multivalent effect to enhance binding. Time-course biodistribution studies described here show very clearly that N-DG-Gal is very quickly taken-up by the liver, and a smaller portion goes to the kidney and bladder. These results are consistent with the results obtained in a previous study, in which the *in vivo* distribution of the enzyme was assessed for each organ (liver, kidneys, bladder, heart/lung, brain by gamma scintigraphy) and showed rapid uptake for N-DG-Gal and rapid serum clearance (Robinson et al., 2004). Importantly, coadministration of prodrug (Rha-Dox or Rha-5Fu) does not inhibit N-DG-Gal uptake allowing uninterfered colocalization of both key components of the LEAPT system.

All the results presented here analyzing the synthesis and behavior of rhamnosyl prodrugs in the LEAPT system are consistent with results previously reported (Robinson et al., 2004) in which Rha-Dox prodrug was successfully evaluated in a disease model study of the LEAPT system. This prior study showed promising approximate halving in tumor burden and foci after 42 days of three-time weekly dosing compared with control groups of prodrug or N-DG-Gal alone. The efficacy of other prodrugs, such as Rha-5Fu, shown here for delivery of, for example, 5Fu suggests the modularity of the LEAPT approach. In principle, while using different drugs in this system, any suitable prodrug cap may also be employed (allowing use of many enzymes) and any sugar type may be used to decorate the releasing enzyme (allowing targeting to a wide range of cognate endogenous sugar-binding lectins).

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Declaration

B.G.D. and A.J.F. are shareholders in Glycoform, PG holds share options in Glycoform. None of the LEAPT technology or associated aspects is currently licensed or being evaluated by any commercial partner.

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