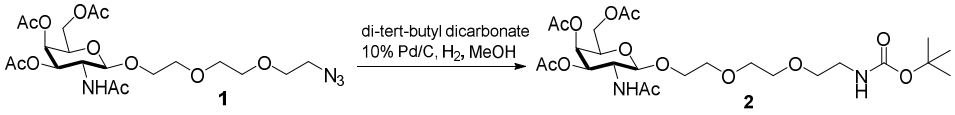
Supplementary material

Supplementary methods

Synthesis of triGalNAc

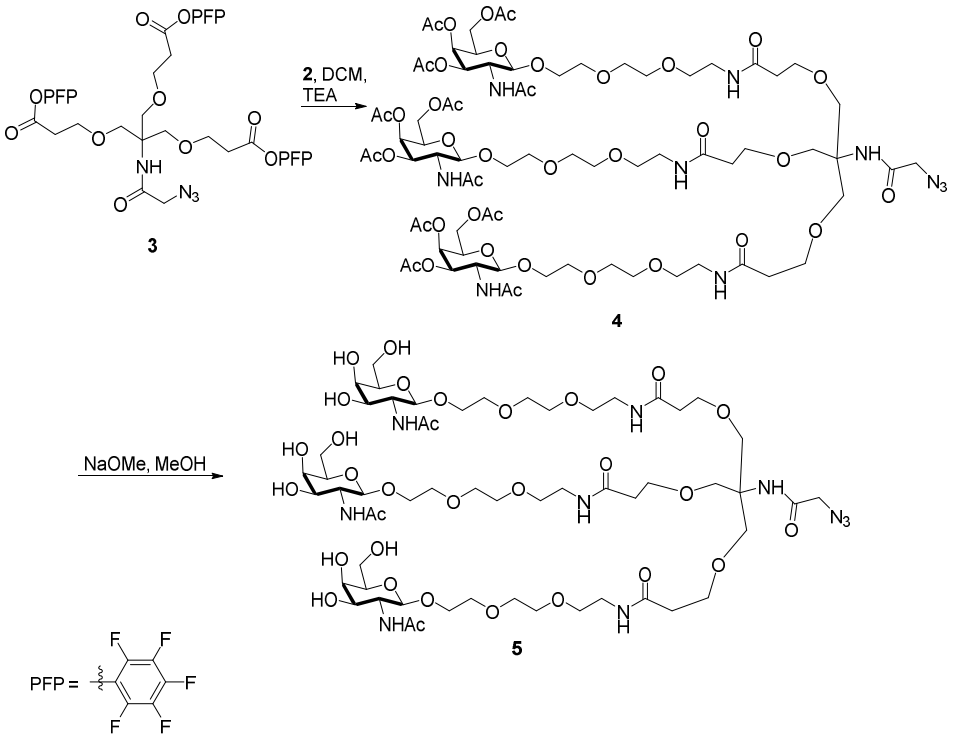
General considerations. All reagents were purchased from Sigma-Aldrich and used without further purification. Dichloromethane, *N*,*N*-diisopropylethylamine (DIPEA), THF, methanol, *N,N*-dimethylformamide (DMF) and pyridine were dried over activated 4Å molecular sieves, and petroleum ether and ethylacetate (EtOAc) were used as received. All reactions conducted in anhydrous solvents were carried out under an argon atmosphere. Silica gel column chromatography was performed using Merck Millipore silica gel 60 (0.040-0.063 mm). Thin layer chromatography (TLC) was performed using Merck silica gel 60 F254 (0.22 mm thickness, aluminium backed). Compounds were visualized at 254 nm or stained with 5% sulfuric acid in EtOH. ^{1}H-NMR spectra were measured at 400 MHz, ^{13}C-NMR spectra were measured at 101 MHz, and ^{19}F-NMR spectra were measured at 376 MHz, all on a Bruker AVANCE III 400 spectrometer. Chemical shifts are given in ppm and *J* values are given in Hz. All assignments for ^{1}H-NMR and ^{13}C-NMR have been confirmed by COSY, HSQC and HMBC. HRMS-ESI spectra were recorded on a Bruker APEX III FT-ICR mass spectrometer.



Scheme 1. Synthesis of sugar intermediate **2**.

*Synthesis of 2-(2-(2-tert-butyloxycarbonylamidoethoxy)ethoxy]ethoxy-3,4,6-tri-O-acetyl-2-(acetylamino)-2-deoxy-β-D-galactopyranoside (**2**).* Compound **1** was prepared according to a procedure described previously[44]. 2-(2-(2-Azidoethoxy)ethoxy]ethoxy-3,4,6-tri-*O*-acetyl-2-(acetylamino)-2-deoxy-β-D-galactopyranoside (**1**, 800 mg, 1.58 mmol) was dissolved in MeOH (25 mL) and 10% Pd/C (20 mg) and di-*tert*-butyl dicarbonate (520 mg, 2.37 mmol) were added. The reaction mixture was stirred at room temperature in an H_2 atmosphere for 4 h. The reaction mixture was passed through celite pad and concentrated to dryness under reduced pressure. The residue was purified by silica gel chromatography using 0-2.5 % MeOH in CH_2Cl_2 (v/v) as eluent to afford compound **2** as a viscous oil (750 mg) in 82% yield. R_f = 0.5 (MeOH/CH_2Cl_2, 5:95). ^{1}H NMR (400 MHz, DMSO): δ 7.79 (d, *J* = 9.2 Hz, 1H, NHAc),

6.76 (s, 1H, NHCO), 5.22 (d, J = 3.4 Hz, 1H, H-4,), 4.98 (dd, J = 11.2, 3.4 Hz, 1H, H-3), 4.56 (d, J = 8.5 Hz, 1H, H-1), 4.03 (m, 3H, H-2, H-6a, H-6b, CH_2), 3.93–3.73 (m, 2H, H-5, CH_2O), 3.63–3.45 (m, 6H, CH_2O), 3.37 (t, J = 6.1 Hz, 2H, CH_2O), 3.08-3.04 (m, 2H, CH_2NH), 2.11 (s, 3H, NHAc), 2.00 (s, 3H, OAc), 1.89 (s, 3H, OAc), 1.78 (s, 3H, OAc), 1.37 (s, 9H, t-Bu). ^{13}C NMR (101 MHz, DMSO): δ 169.89 (NH<u>C</u>OCH_3), 169.82, 169.52, 169.17 (3 × CH_3<u>C</u>OO), 155.48 (NHCO), 100.85 (C-1), 77.47 (<u>C</u>$(CH_3)_3$), 70.38(C-5), 69.81 (C-4), 69.67, 69.44, 69.35, 69.07, 68.21 ($(OCH_2CH_2)_2OCH_2$), 66.64 (C-3), 66.51(C-6), 61.38 (C-2), 49.23 (CH_2NH), 28.13 (C(<u>C</u>$H_3)_3$, t-Bu), 22.69 (NHCO<u>C</u>H_3), 20.42, 20.36, 20.33 (3 × <u>C</u>H_3COO). HRMS (ESI) calcd. for $[C_{25}H_{42}N_2O_{13}Na]^+$: 601.2579; found: 601.2590.



Scheme 2. Synthesis of triantennary azido GalNAc **5**.

*Synthesis of triantennary azido 3,4,6-tri-O-acetyl-2-(acetylamino)-2-deoxy-β-D-galactopyranoside (**4**).* Compound **3** was prepared according to a procedure described previously [Wengel et al., *Molecules*, 2017, 22, 852]. Compound **2** (705 mg, 1.22 mmol) was dissolved in CH_2Cl_2/trifluoroacetic acid (3:1, 8 mL) and stirred at room temperature for 1 h. The solvents were evaporated under reduced pressure and the resulting oil was dissolved in anhydrous CH_2Cl_2 (16 mL). To this mixture was added *N*-{tris[3-(pentafluorophenylcarboxylethoxy)methyl]methylamide}-2-azidoacetamide (**3**, 280 mg, 0.304

mmol) and the resulting mixture was adjusted to pH 8 with triethylamine (TEA) and stirred at room temperature for 24 h. The solvents were evaporated under reduced pressure and the residue was redissolved in CH_2Cl_2 (100 mL) and washed with H_2O (3 × 50 mL). The separated organic phase was dried (Na_2SO_4), filtered and evaporated to dryness under reduced pressure. The residue was purified by silica gel chromatography using 3-8 % MeOH/CH_2Cl_2 (v/v) as eluent to afford the acetyl protected triantennary GalNAc azide **4** as a white solid material (273 mg) in 49 % yield. R_f = 0.5 (MeOH/CH_2Cl_2, 10:90). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.05 (brs, 1H, NH), 6.89 (t, *J* = 5.3 Hz, 3H, NH), 6.72 (d, *J* = 8.8 Hz, 3H, NHAc), 5.34 (d, *J* = 3.0 Hz, 3H, Gal-H-4), 5.19 (dd, *J* = 11.2, 3.3 Hz, 3H, Gal-H-3), 4.79 (d, *J* = 8.5 Hz, 3H, Gal-H-1), 4.19–4.09 (m, 9H, Gal-H-2, H-6), 3.96–3.92 (m, 6H, Gal-C-5, CH_2O), 3.90 (s, 2H, CH_2N_3), 3.71–3.59 (m, 39H, CH_2O), 3.47–3.43 (m, 6H, CH_2NH,), 2.45 (t, *J* = 5.4 Hz, 6H, CH_2CO), 2.15 (s, 9H, NHAc), 2.05 (s, 9H, OAc), 1.99 (s, 9H, OAc), 1.95 (s, 9H, OAc). ^{13}C NMR (101 MHz, $CDCl_3$): δ 171.46 (NH<u>C</u>OCH_3), 170.67, 170.54, 170.45 (3 × CH_3<u>C</u>OO), 170.33 (CH_2<u>C</u>ONH), 167.43 (<u>C</u>OCH_2N_3), 101.57 (Gal-C-1), 70.77, 70.64, 70.53, 70.50 (4 × CH_2O), 70.27 (Gal-C-3), 69.81 (Gal-C-5), 69.27, 68.59, 67.45 (3 × CH_2O), 66.80 (Gal-C-4), 61.60 (Gal-C-6), 60.08 (<u>C</u>$(CH_2)_3$), 52.41 (CH_2N_3), 50.87 (Gal-C-2), 39.16 (CH_2NH), 36.49 (<u>C</u>H_2CO), 23.24 (NHCO<u>C</u>H_3), 20.70 (2 × CH_3COO), 20.68 (CH_3COO). HRMS (ESI) calcd. for $[C_{75}H_{120}N_{10}O_{40}Na]^+$: 1823.7555; found: 1823.7585.

*Synthesis of triantennary azido 2-(acetylamino)-2-deoxy-β-D-galactopyranoside (**5**).* Acetyl protected triantennary *N*-acetyl galactosamine azide **4** (209 mg, 0.116 mmol) was dissolved in dry MeOH (23 mL) and NaOMe (25 wt. % in methanol, 6.6 µL, 0.029 mmol) was added. The reaction mixture was stirred at room temperature for 1 h, neutralized with DOWEX-50WX2 (H^+ resin), filtered and evaporated to dryness under reduced pressure. The residue was redissolved in H_2O, dialyzed for two days using Spectra/Por Float-A-Lyzer G2 (MWCO: 100-500 D molecular weight cut-off) and lyophilized to furnish the desired product **5** as a white solid material (162 mg) in 98 % yield. R_f = 0.5 (CH_2Cl_2/MeOH/H_2O, 1.6:1.0:0.3). ^{1}H NMR (400 MHz, MeOD-*d*$_4$): δ 4.47 (d, *J* = 8.4 Hz, 3H, Gal-H-1), 3.98 (dd, *J* = 18.8, 9.7 Hz, 6H, Gal-H-3), 3.92-3.90 (m, CH_2, 5H), 3.80–3.52 (m, 53H, CH_2O), 3.43 (t, *J* = 5.6 Hz, 6H, CH_2NH), 2.49 (t, *J* = 5.5 Hz, 6H, CH_2CO), 2.04 (s, 9H, NHAc). ^{13}C NMR (101 MHz, MeOD-*d*$_4$): δ 174.35 (NH<u>C</u>OCH_3), 174.17 (CH_2<u>C</u>ONH), 169.90 (<u>C</u>OCH_2N_3), 103.40 (Gal-C-1), 76.66, 73.20, 71.49, 71.38, 71.34, 70.83, 70.15, 70.02, 69.79, 68.81, 62.63 (CH_2O, Gal-C-3, Gal-C-5, Gal-C-6), 61.78 (<u>C</u>$(CH_2)_3$), 54.14 (CH_2N_3), 53.23 (Gal-C-2), 40.41 (CH_2NH), 37.58 (<u>C</u>H_2CO),

23.34 ($NHCOCH_3$). HRMS (ESI) calcd. for $[C_{57}H_{102}N_{10}O_{31}Na]^+$: 1445.6605, found: 1445.6580.

Supplementary data

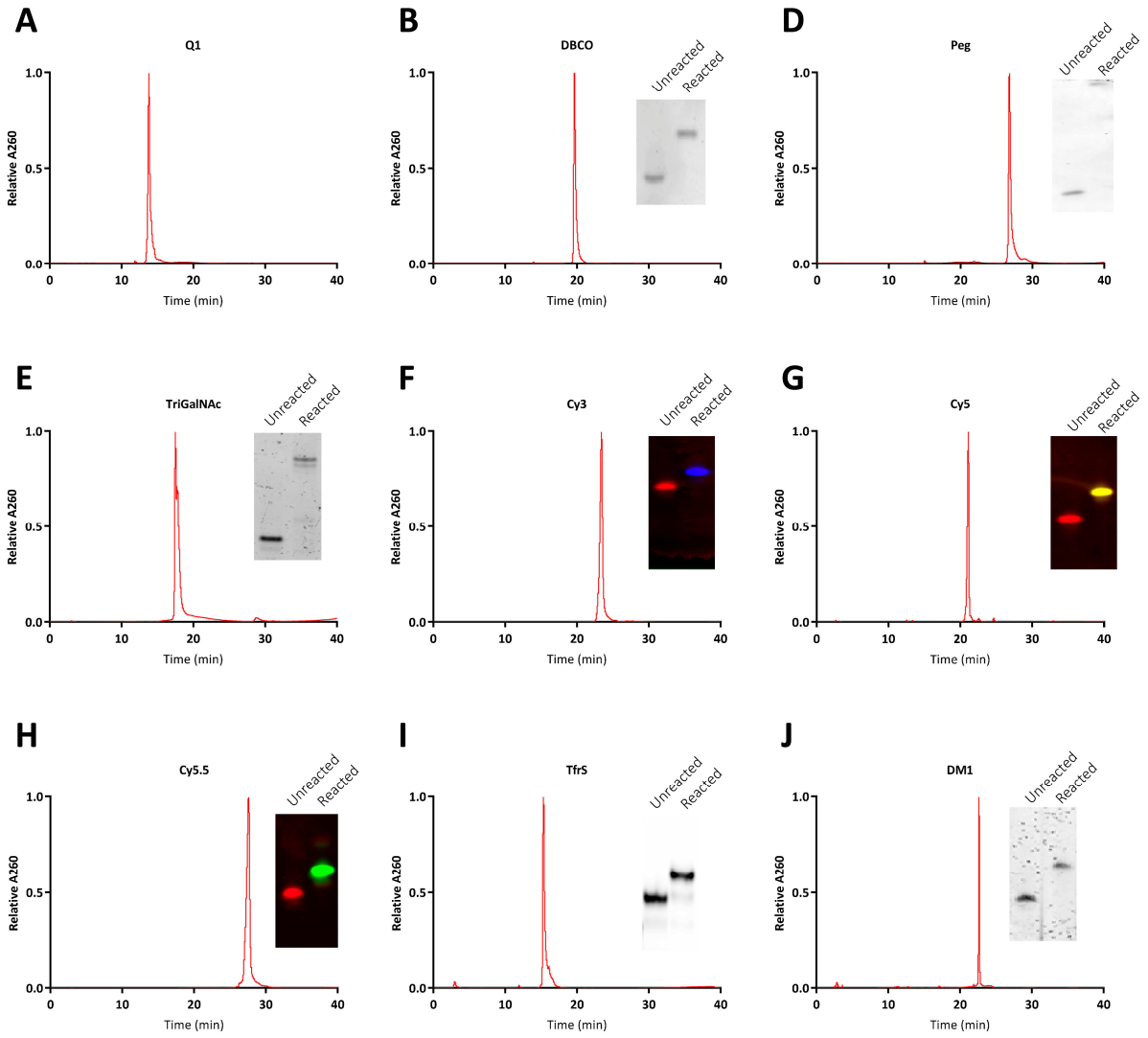


Figure S1. Representative analytical RP-HPLC chromatograms of purified bioconjugates. Each of the four strands of the HJ scaffold contains a 5'-amine group enabling conjugation to a number of different functionalities. This group was chosen because amine reactive NHS esters are available for a number of functional molecules, including fluorophores and PEG, as well as a range of bifunctional linkers. As a result, the 5'-amine provides a strong starting point for attachment of almost any type of molecule. Following conjugation to HJ oligos, conjugates were purified by RP-HPLC and subsequently freeze dried. The reacted oligos were reconstituted in RNase free water and analyzed by gel electrophoresis (shown in inserts) and analytical HPLC. For simplicity, only the results from Q1 are shown.

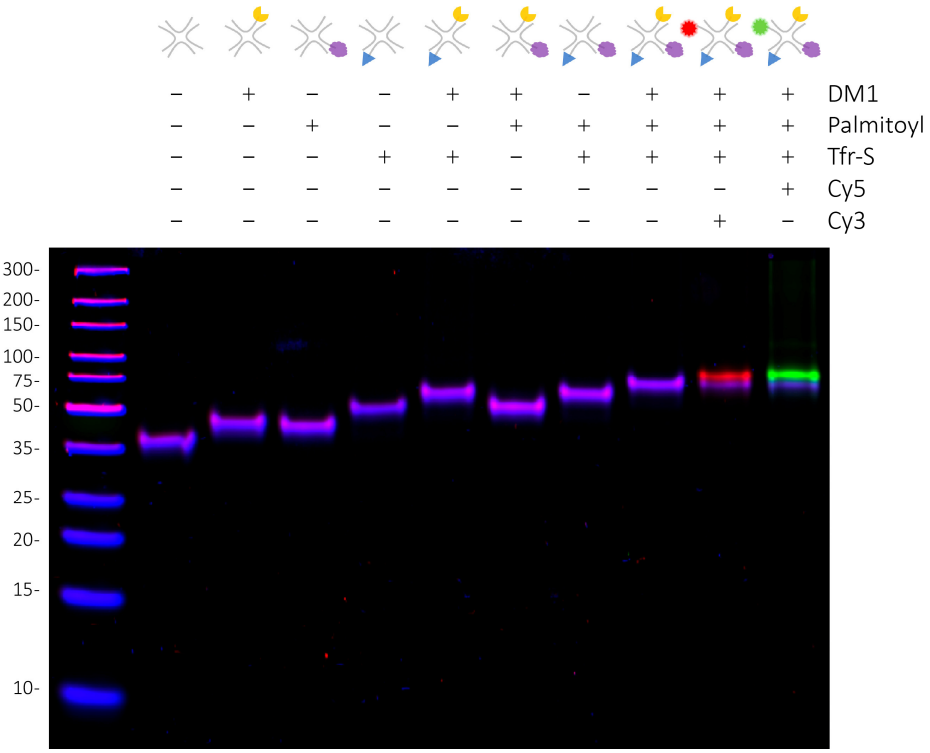


Figure S2. Assembly of HJs with up to four different functionalities. Native PAGE gel showing a stepwise assembly of HJs with a targeting agent (Tfr-S peptide, blue arrow), a pharmacokinetic modifier (palmitoyl, purple cloud), a cytotoxic drug (DM1, yellow pacman) and an imaging agent (Cy3 or Cy5, red and green sun). The image is an overlay of three scans: purple = SYBR Gold, red = Cy5, green = Cy3.

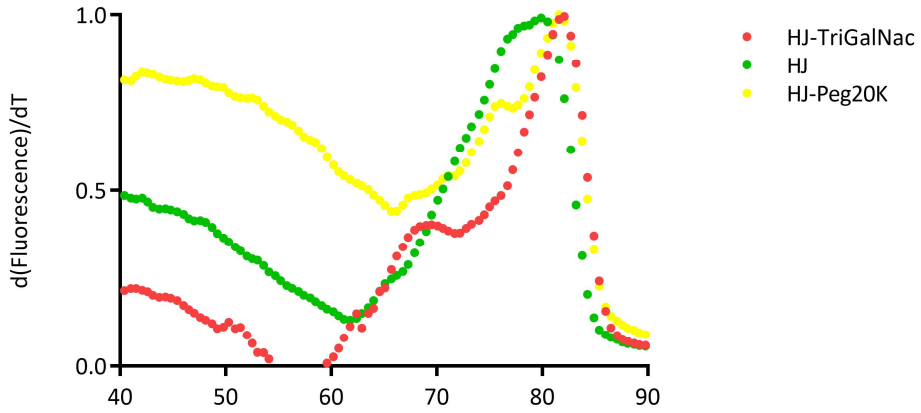


Figure S3. Melting curves of the HJ scaffold functionalized with triGalNAc or PEG20K, based on SYBR Gold binding. The measured T_m corresponds to that of the unfunctionalized HJ.

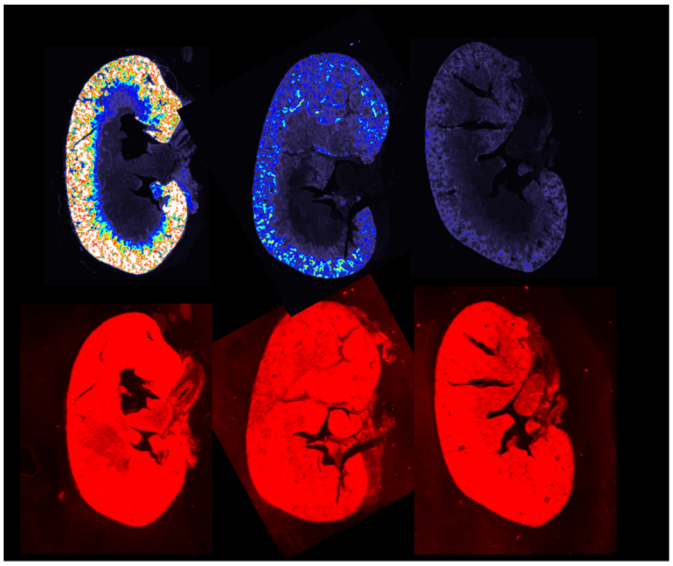


Figure S4. Fluorescent scan of paraffin-embedded mouse kidneys 2, 24 and 48 hours after I.V. injection. HJs were labeled with Cy5. In the upper panel, the Cy5 sigal is shown in rainbow color scale (red: high, blue: low). The lower panel shows the signal from Alexa488 membrane stain in red, showing the membranes of all cells in the tissue.

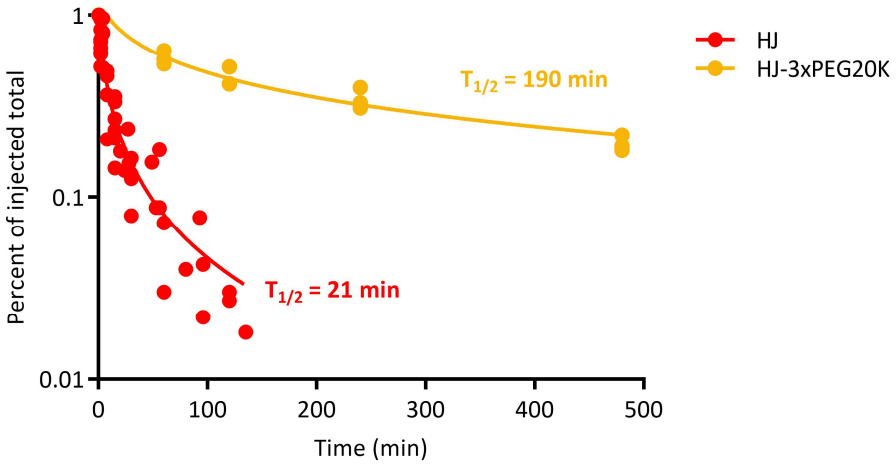


Figure S5. Pharmacokinetics of HJ-3xPEG20K. The HJ is the control also shown in figure 4B.

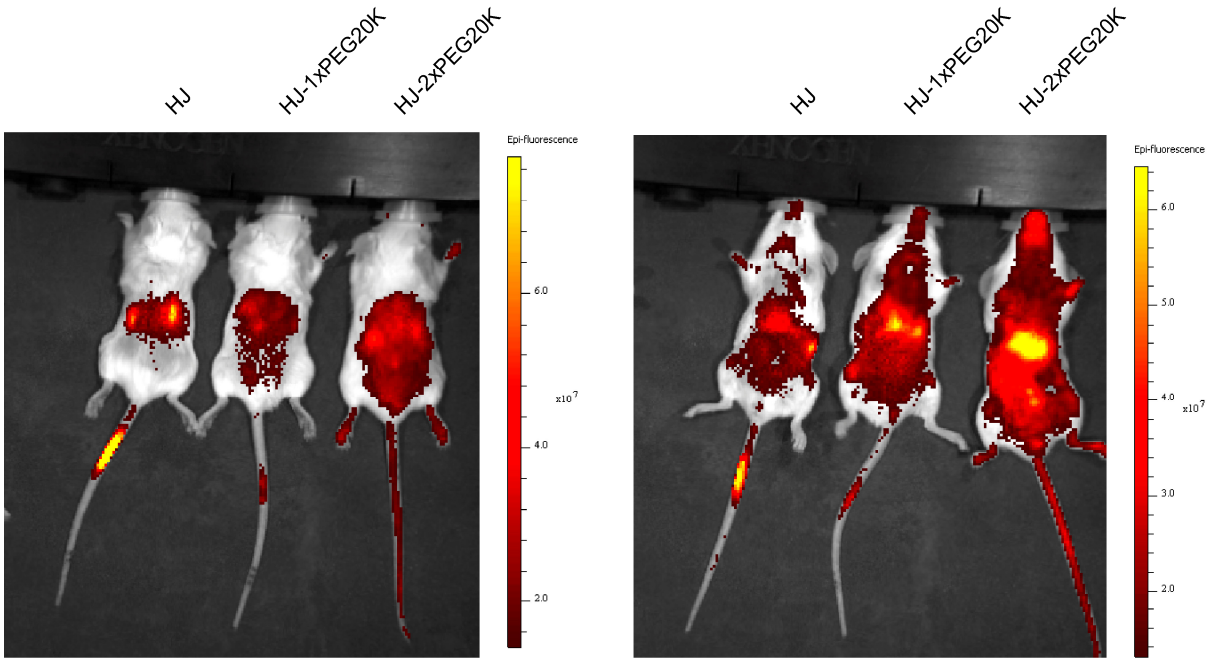


Figure S6. Whole animal scan of mice injected with Cy5.5-labeled HJ, HJ-1xPEG20K, HJ-2xPEG20K. Left panel shows mice with their back facing up, right panel shows mice lying on their back.

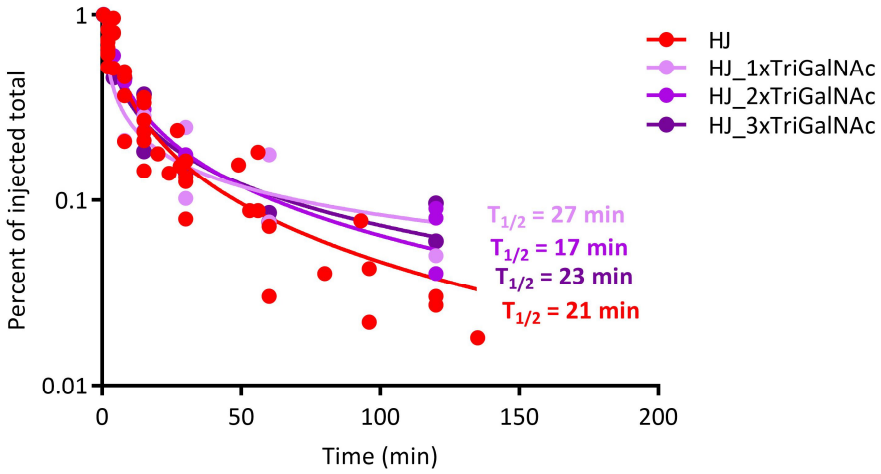


Figure S7. Pharmacokinetics of HJ-triGalNAc in mice.