Supporting Information

Targeting iron in colon cancer via glycoconjugation of thiosemicarbazone prochelators

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Contents

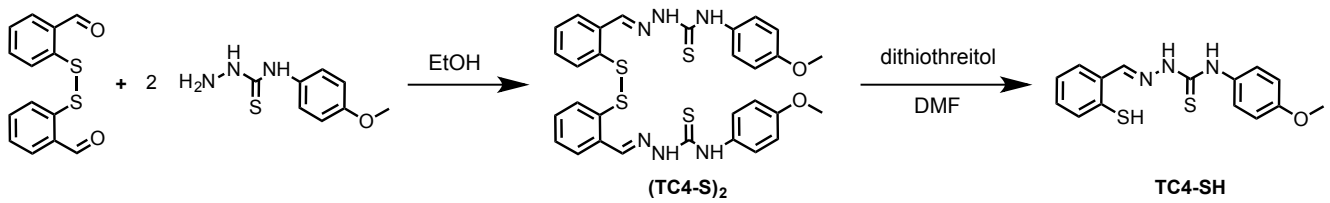
Materials and instruments

2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG),[1] 3-(2-pyridyldithio)propionic acid (**1**),[2] 4-(4-methoxyphenyl)-3-thiosemicarbazide,[3] 3,4,5,6-tetrakis((trimethylsilyl)oxy)glucopyranose and 3,4,5,6-tetrakis((trimethylsilyl)oxy)mannopyranose[4] were prepared as previously reported. Dichloromethane and *N,N*-dimethylformamide were dried by passage through a Vacuum Atmospheres solvent purifier. All other reagents were obtained commercially and used as received. AlexaFluor® 488 goat anti-rabbit IgG was purchased from Fisher and used and stored per manufacturer instructions. Rabbit polycolonal anti-GLUT1 antibody was purchased from VWR and used as specified.

Thin layer chromatography (TLC) was conducted on Silica Gel 60 F254 X plates. NMR spectra were recorded on Bruker AVIII 400 MHz and Bruker DRX-500 MHz NMR spectrometers. Chemical shifts are reported in parts per million (ppm, δ) with residual solvent peaks and/or TMS peak set as reference. Proton coupling patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br). High-resolution mass spectra (HRMS) were recorded on Bruker 9.4 T Apex-Qh hybrid Fourier transfer ion-cyclotron resonance (FT-ICR) spectrometer in the Mass Spectrometry Facility at the University of Arizona Department of Chemistry and Biochemistry. UV-visible absorption spectra were obtained on an Agilent 8453 spectrophotometer. Absorption assays in 96-well plates were recorded on a BioTek Synergy™ 2 microplate reader.

Flow cytometric analysis was performed at the University of Arizona Cytometry Core Facility (Arizona Cancer Center/Arizona Research Laboratories) using a FACSCanto II flow cytometer (BDBiosciences, San Jose, CA) equipped with an air-cooled 15-mW argon ion laser tuned to 488 nm. The emission fluorescence of 2-NBDG and AlexaFluor® 488 were detected and recorded through a 530/30 and band-pass filter in the FL1 channel. List mode data files consisting of 10,000 events gated on FSC (forward scatter) vs SSC (side scatter) were acquired and analysed using CellQuest PRO software (BD Biosciences, San Jose, CA). Appropriate electronic compensation was adjusted by acquiring cell populations stained with each dye/fluorophore individually, as well as an unstained control.

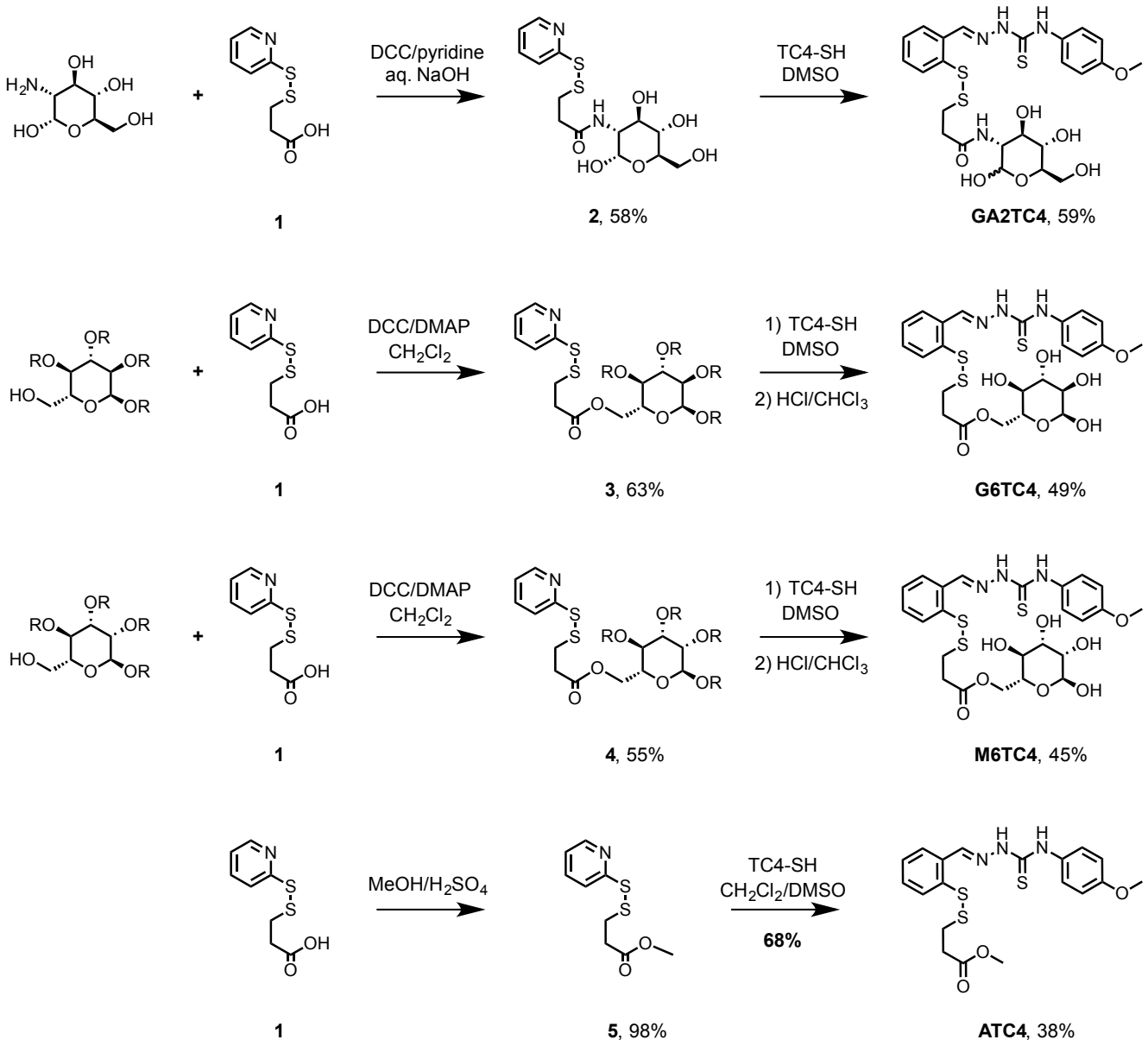
Synthetic procedures and chemical characterization



Scheme S1. Synthetic scheme for chelator TC4-SH.

Synthesis of (TC4-S)$_2$: 2,2'-Dithiobenzyldialdehyde (500 mg, 1.8 mmol) was combined with 4-(4-methoxyphenyl)-3-thiosemicarbazide (863 mg, 4.27 mmol) in ethanol (20 mL) and brought to reflux to dissolve the starting materials. The reaction progress was monitored by TLC, and the precipitated product was collected via filtration, washed with ethanol (3 x 6 mL) and dried under vacuum (1.2 g, 95%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.92 (s, 1H), 9.95 (s, 1H), 8.66 (s, 1H), 8.27 (dd, J = 5.9, 3.5 Hz, 1H), 7.69–7.54 (m, 1H), 7.48–7.30 (m, 4H), 6.93 (d, J = 8.9 Hz, 2H), 3.76 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 176.68, 157.41, 140.46, 135.37, 134.53, 132.27, 131.49, 130.84, 129.06, 128.68, 127.61, 113.83, 55.75. HRMS–ESI (*m/z*): $[M+H]^+$ calcd, 633.12293; found, 633.12291.

Synthesis of TC4-SH: (TC4-S)$_2$ (633 mg, 1.00 mmol) and dithiothreitol (DTT, 370 mg, 2.5 mmol) were dissolved in dry, degassed dimethylformamide (DMF, 16 mL) and allowed to stir for 2 hr under an argon atmosphere. The reaction progress was monitored by TLC, and solvent volume was reduced by rotary evaporation until ~1 mL DMF remained. Nanopure water (12 mL) was added and the precipitated product was collected via filtration, washed with nanopure water (3 x 6 mL) and dried under vacuum (631 mg, quantitative). ^{1}H NMR (499 MHz, DMSO-d_6): δ 11.88 (s, 1H), 9.90 (s, 1H), 8.46 (s, 1H), 8.10 (dd, J = 7.8, 1.6 Hz, 1H), 7.56–7.40 (m, 3H), 7.24 (dtd, J = 34.1, 7.4, 1.4 Hz, 2H), 7.04–6.91 (m, 2H), 5.75–5.57 (bs, 1H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 176.54, 157.35, 141.12, 133.43, 132.27, 131.68, 131.47, 130.44, 128.75, 127.43, 125.82, 113.84, 55.75. HRMS–ESI (*m/z*): $[M+H]^+$ calcd, 318.07293; found, 318.07289.



Scheme S2. Complete synthetic scheme for glycoconjugates and aglycone (R = trimethylsilyl).

Synthesis of 2: Compound **1** (1.00 g, 4.64 mmol) was dissolved in pyridine (4 mL) along with *N,N'*-dicyclohexylcarbodiimide (DCC, 1.15 g, 5.57 mmol) and stirred for 15 minutes. This solution was added slowly to a solution of D-glucosamine hydrochloride (3.01 g, 13.92 mmol) in aqueous NaOH (2.00 M, 2.30 mL). The reaction mixture was allowed to stir overnight then diluted with water (10 mL) to precipitate the urea product, which was removed by filtration. The resulting yellow solution was washed with diethyl ether, then the volume of the aqueous fraction was reduced by rotary evaporation with minimal heating (below 40 ^{o}C). The product precipitated as a white solid, which was collected on a fritted filter, washed with water and dried under vacuum (0.77 g, 58% yield). ^{1}H NMR (500 MHz, DMSO-d$_6$, δ): 8.46 (ddd, *J* = 4.8, 1.9, 0.9 Hz, 1H), 7.87–7.82 (m, 1H), 7.79 (tt, *J* = 7.0, 1.2 Hz, 2H), 7.24 (ddd, *J* = 7.3, 4.8, 1.2 Hz, 1H), 6.42 (dd, *J* = 4.6, 1.2 Hz, 1H), 4.95–4.86 (m, 2H), 4.61 (d, *J* = 5.5 Hz, 1H), 4.41 (dd, *J* = 6.4, 5.3 Hz, 1H), 3.59 (dddd, *J* = 15.7, 7.2, 4.8, 2.4 Hz, 3H), 3.52–3.44 (m, 2H), 3.11 (ddd, *J* = 9.8, 8.6, 5.3 Hz, 1H), 3.00 (t, *J* = 7.3 Hz, 2H), 2.55 (td, *J* = 7.2, 2.1 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d$_6$) δ 170.39, 159.72, 150.00, 138.35, 121.58, 119.57, 91.01, 72.53, 71.57, 70.90, 61.57, 54.87, 35.11, 34.77. HRMS–ESI (*m/z*): $[M+Na]^+$ calcd, 399.0655; found, 399.0654.

Synthesis of GA2TC4: A solution of **2** (300 mg, 1.08 mmol) in degassed DMSO (5 mL) was heated to 40 °C and a solution of TC4-SH (103 mg, 0.36 mmol) in degassed DMSO (2 mL) was added dropwise over 15 minutes. The solution was allowed to stir for 30 minutes and water (50 mL) was added to precipitate the product. The mixture was centrifuged to separate the precipitate, which was resuspended in water (20 mL) and collected on a fritted filter, washed with water and dried under vacuum (118 mg, 59% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.94 (s, 1H), 9.95 (d, *J* = 2.5 Hz, 1H), 8.66 (d, *J* = 3.8 Hz, 1H), 8.34–8.18 (m, 1H), 7.91–7.72 (m, 2H), 7.63–7.17 (m, 5H), 6.94 (dd, *J* = 7.7, 5.4 Hz, 2H), 6.58–6.37 (m, 1H), 5.02 – 4.77 (m, 2H), 4.60 (d, *J* = 5.6 Hz, 1H), 4.41 (s, 1H), 3.77 (d, *J* = 2.8 Hz, 3H), 3.64–3.55 (m, 2H), 3.53–3.40 (m, 2H), 3.16–3.04 (m, 1H), 2.95 (t, *J* = 7.3 Hz, 2H), 2.51–2.49 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 176.23, 170.40, 140.82, 139.37, 136.62, 133.54, 130.89, 130.11, 128.85, 128.66, 128.18, 125.78, 125.68, 91.01, 72.54, 71.58, 70.91, 61.58, 54.87, 35.24, 34.36. HRMS–ESI (*m/z*): $[M+Na]^+$ calcd, 605.1169; found, 605.1164.

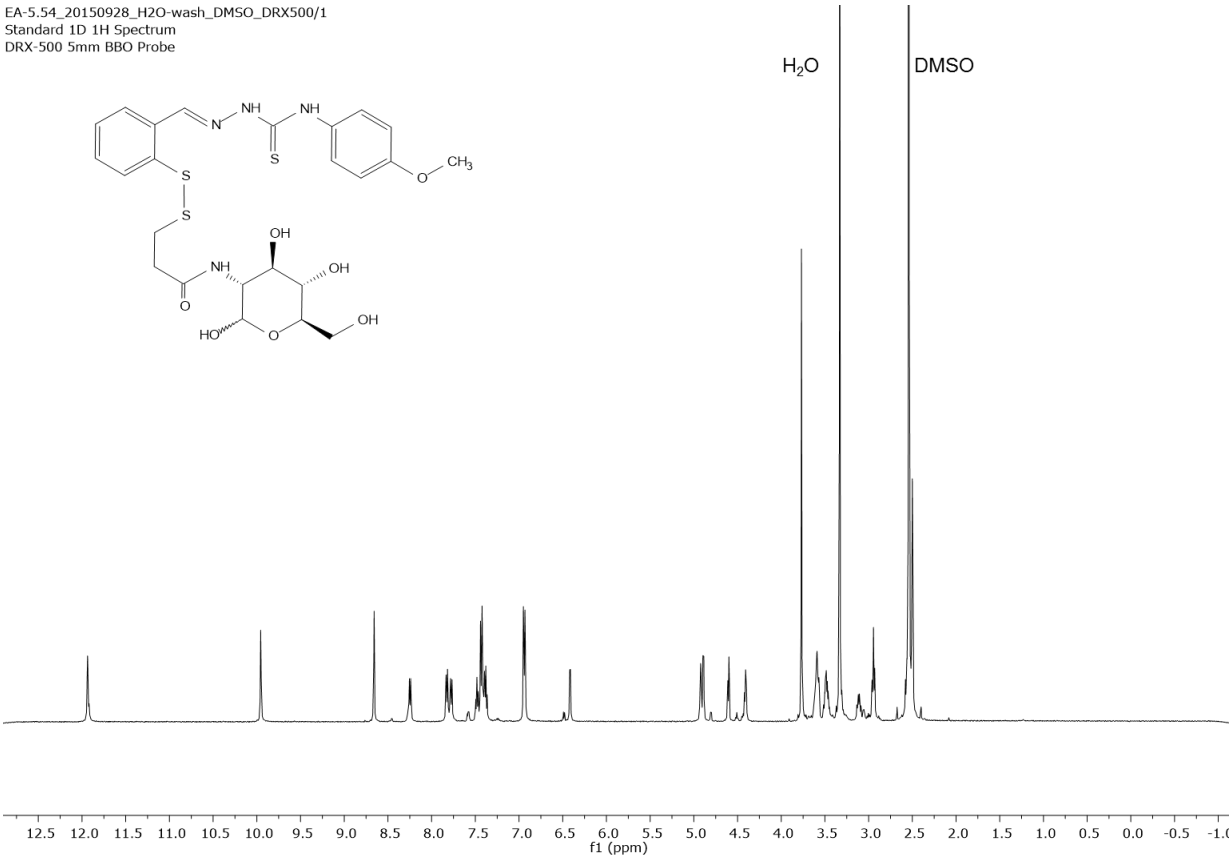


Figure S1. ^{1}H NMR spectrum of **GA2TC4** (500 MHz, DMSO-d_6).

Synthesis of 3: 3,4,5,6-tetrakis((trimethylsilyl)oxy)glucopyranose (1.74 g, 3.70 mmol), **1** (875 mg, 4.07 mmol), DCC (876 mg, 4.25 mmol) and dimethylaminopyridine (DMAP, 32.6 mg, 0.259 mmol) were combined in dry dichloromethane (12 mL) and allowed to stir under argon for 1.5 h. The reaction mixture was diluted with dichloromethane (40 mL) and filtered through a Celite plug to remove the urea side product. The resulting solution was concentrated under vacuum and loaded onto a silica column. Flash chromatography using a hexanes/ethyl acetate gradient (5-25% ethylacetate) resulted in precursor **3** as a clear oil (1.60 g, 63% yield). ^{1}H NMR (499 MHz, $CDCl_3$) δ 8.47 (d, *J* = 4.8, Hz, 1H), 7.74–7.56 (m, 2H), 7.09 (ddd, *J* = 7.2, 4.8, 1.2 Hz, 1H),

5.00 (d, J = 3.0 Hz, 1H), 4.38 (dd, J = 11.8, 2.2 Hz, 1H), 4.05 (dd, J = 11.8, 5.5 Hz, 1H), 3.90 (ddd, J = 9.8, 5.5, 2.3 Hz, 1H), 3.78 (t, J = 8.8 Hz, 1H), 3.48–3.29 (m, 2H), 3.04 (t, J = 7.3 Hz, 2H), 2.88–2.70 (m, 2H), 0.15 (s, 9H), 0.15 (s, 9H), 0.14 (s, 9H), 0.13 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.39, 159.70, 149.66, 137.06, 120.74, 119.70, 93.87, 73.89, 73.75, 72.38, 69.85, 64.23, 33.75, 33.18, 1.24, 0.97, 0.45, 0.18, 0.15. HRMS–ESI (*m/z*): $[M+Na]^+$ calcd, 688.2076; found, 688.2064.

Synthesis of G6TC4: Compound **3** (1.84 g, 2.69 mmol) was dissolved in dichloromethane (15 mL). TC4-SH (284 mg, 0.890 mmol) was dissolved in degassed dimethyl sulfoxide (5 mL) and added dropwise to the flask containing **3**. The reaction mixture was stirred for 1 h under argon, then the product was extracted using ethyl acetate (30 mL), and washed with water (3 x 15 mL) and brine (2 x 15 mL). The ethyl acetate layer was dried over sodium sulfate and evaporated under reduced pressure. The crude product was purified by flash chromatography using an ethyl acetate/hexanes gradient resulting in the TMS-protected product as a clear oil, which was susceptible to partial deprotection in NMR solvents. For full deprotection, the product was dissolved in chloroform (10 mL) and methanolic HCl (3 N, 25 μL) was added while stirring. The reaction progress was monitored by TLC and upon completion the solvent was removed by rotary evaporation. The resulting residue was dissolved in a minimal amount of methanol/acetone (5 mL), and the product was precipitated by addition of nanopure water (24 mL). The white precipitate was collected by centrifugation, washed with water (2 x 20 mL) and dried under vacuum resulting in an off-white sticky solid (254 mg, 49% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.95 (s, 1H), 9.96 (s, 1H), 8.67 (s, 1H), 8.36–7.71 (m, 2H), 7.48 (t, J = 7.7 Hz, 1H), 7.45–7.36 (m, 3H), 6.98–6.90 (m, 2H), 6.69–6.27 (m, 1H), 5.09 (dd, J = 29.6, 5.6 Hz, 1H), 4.98–4.71 (m, 2H), 4.57–4.26 (m, 2H), 4.01 (ddd, J = 15.8, 11.7, 6.5 Hz, 1H), 3.77 (s, 3H), 3.46–3.35 (m, 2H, partially hidden under H_2O peak), 3.16–3.10 (m, 1H), 3.08–3.00 (m, 1H), 2.99–2.87 (m, 2H), 2.82–2.71 (m, 2H). ^{13}C NMR (126 MHz, acetone-d_6) δ 176.66, 171.07, 157.47, 140.26, 136.52, 133.33, 131.93, 130.33, 130.31, 129.79, 128.66, 128.63, 127.69, 126.05, 113.43, 94.49, 94.37, 74.24, 74.00, 71.80, 71.59, 71.32, 70.32, 67.92, 67.61, 64.42, 64.38, 59.68, 54.86, 33.59, 33.56, 32.89, 32.84, 29.45, 29.30, 29.24, 29.15, 29.09, 28.99, 28.84, 28.77, 28.68, 28.53, 19.97. HRMS–ESI (*m/z*): $[M+Na]^+$ calcd, 606.1009; found: 606.1005.

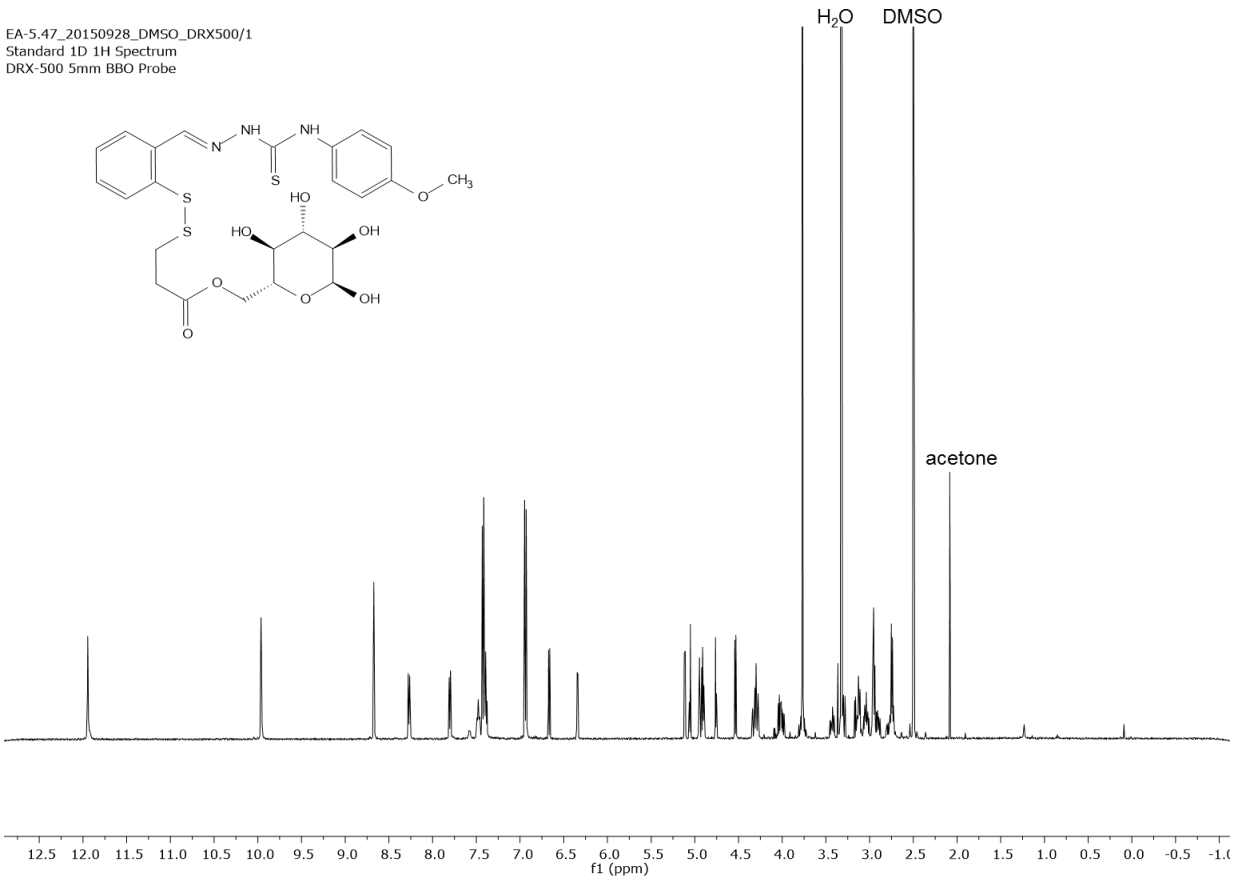


Figure S2. ^{1}H NMR spectrum of **G6TC4** (500 MHz, DMSO-d_6).

Synthesis of 4: 3,4,5,6-tetrakis((trimethylsilyl)oxy)mannopyranose (1.25 g, 2.70 mmol), **1** (630 mg, 2.90 mmol), DCC (630 mg, 4.25 mmol) and DMAP (22.7 mg, 0.186 mmol) were combined, purged with argon, and dissolved in dry dichloromethane (10 mL). The reaction mixture was allowed to stir for 1.5 h under argon, then diluted with dichloromethane (30 mL) and filtered through a Celite plug to remove the urea side product. The resulting solution was concentrated under reduced pressure and loaded onto a silica column. Flash chromatography using a hexanes/ethyl acetate gradient resulted in precursor **4** as a clear oil (1.01 g, 55% yield). ^{1}H NMR (499 MHz, chloroform-*d*) δ 8.48 (ddd, *J* = 4.9, 1.9, 1.0 Hz, 1H), 7.77–7.52 (m, 2H), 7.10 (ddd, *J* = 7.3, 4.8, 1.2 Hz, 1H), 4.92 (d, *J* = 2.1 Hz, 1H), 4.40 (dd, *J* = 11.6, 2.0 Hz, 1H), 4.08 (dd, *J* = 11.6, 5.8 Hz, 1H), 3.89–3.77 (m, 3H), 3.68–3.64 (m, 1H), 3.06 (t, *J* = 7.2 Hz, 2H), 2.81 (td, *J* = 7.2, 4.0 Hz, 2H), 0.17 (s, 9H), 0.15 (s, 9H), 0.14 (bs, 18H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.42, 149.69, 137.01, 120.69, 119.65, 95.53, 75.03, 71.97, 71.73, 68.44, 64.45, 33.84, 33.29, 0.74, 0.63, 0.33, -0.13. HRMS–ESI (*m/z*): $[M+Na]^+$ calcd, 688.2076; found, 688.2063.

Synthesis of M6TC4: Compound **4** (630 mg, 0.922 mmol, 3 equiv) was dissolved in dry dichloromethane (6 mL). TC4-SH (98 mg, 0.31 mmol) was dissolved in degassed dimethyl sulfoxide (2 mL) and added dropwise to the flask containing **4**. The reaction was stirred for 1 h under argon, then the product was extracted using ethyl acetate (15 mL), and washed with water (3 x 10 mL) and brine (2 x 10 mL). The ethyl acetate layer was dried with sodium sulfate and then evaporated under reduced pressure. The crude product was purified by flash chromatography using an ethyl acetate/hexanes gradient (0-25% ethyl acetate in hexanes) resulting in the TMS-

protected product as a clear oil. Deprotection was carried out in chloroform (4 mL) and methanolic HCl (3 N, 25 µL). The reaction progress was monitored by TLC and upon completion the solvent was removed by rotary evaporation. The resulting residue was dissolved in a minimal amount of methanol/acetone (1-2 mL), and the product was precipitated by addition of nanopure water (13 mL). The white precipitate was collected by centrifugation, washed with water (2 x 10 mL) and dried under vacuum resulting in an off-white sticky solid (82 mg, 45% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.95 (s, 1H), 9.97 (s, 1H), 8.67 (s, 1H), 8.27 (dd, J = 7.8, 1.5 Hz, 1H), 7.81 (dd, J = 8.0, 1.2 Hz, 1H), 7.53–7.46 (m, 1H), 7.45–7.37 (m, 3H), 6.94 (d, J = 9.0 Hz, 2H), 6.43–6.19 (m, 1H), 4.98–4.83 (m, 2H), 4.68–4.51 (m, 2H), 4.33 (ddd, J = 13.6, 11.6, 1.9 Hz, 1H), 4.11–3.94 (m, 1H), 3.77 (s, 3H), 3.71 (ddd, J = 9.3, 7.1, 2.2 Hz, 1H), 3.57–3.50 (m, 2H), 3.41–3.35 (m, 1H), 3.30 (s, 1H), 3.01–2.92 (m, 2H), 2.74 (dd, J = 7.2, 6.2 Hz, 2H). ^{13}C NMR (126 MHz, acetone-d_6) δ 176.66, 157.47, 140.26, 136.52, 133.33, 131.93, 130.33, 130.31, 129.79, 128.66, 128.63, 127.69, 126.05, 113.43, 94.49, 94.37, 74.24, 74.00, 71.80, 71.59, 71.32, 70.32, 67.92, 67.61, 64.42, 64.38, 54.86, 33.59, 32.89. HRMS–ESI (*m/z*): $[M+Na]^+$ calcd, 606.1009; found, 606.1006.

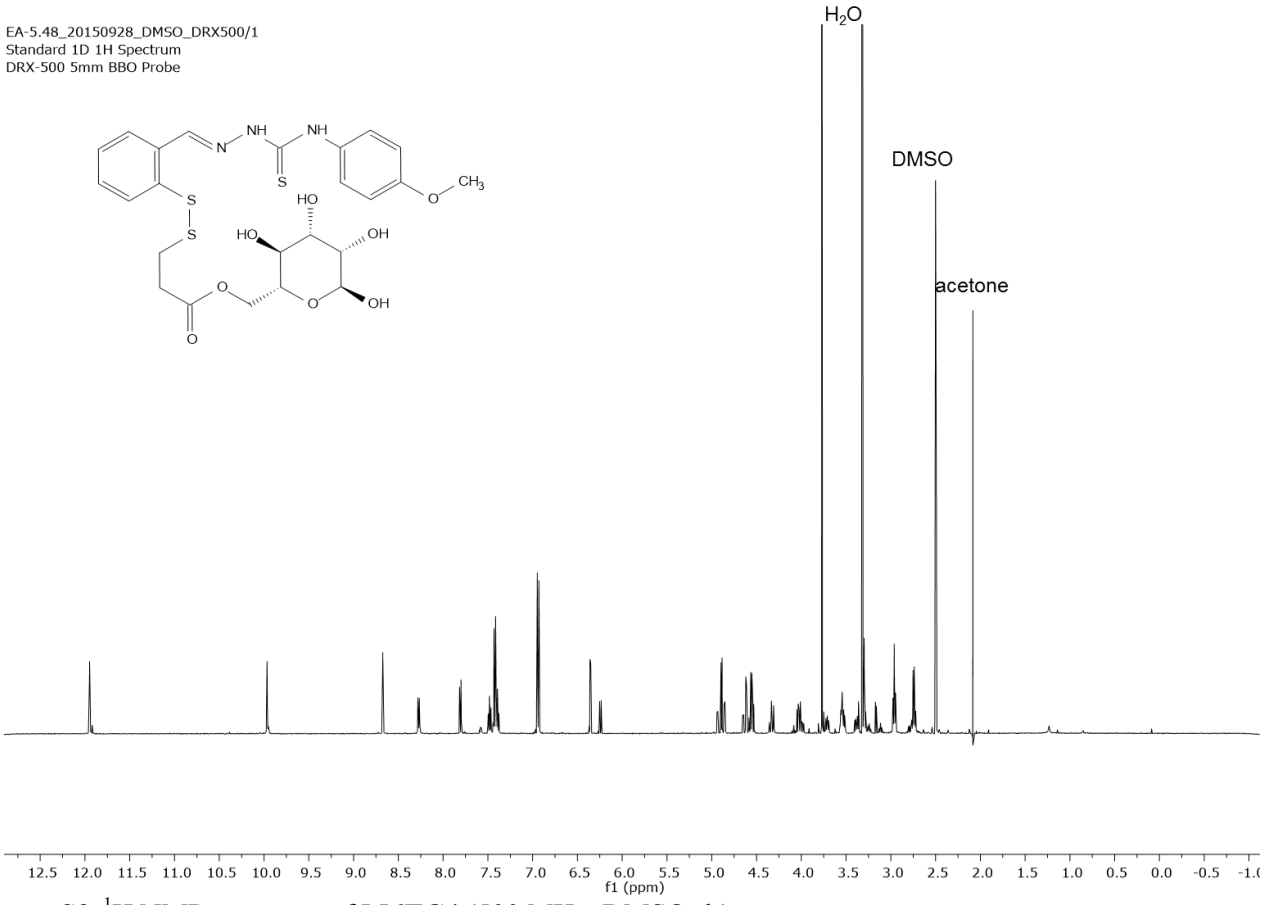


Figure S3. ^{1}H NMR spectrum of **M6TC4** (500 MHz, DMSO-d_6).

Synthesis of 5: Compound **1** (200 mg, 0.930 mmol) was dissolved in freshly distilled methanol (5 mL) along with concentrated sulfuric acid (100 µL). The solution was heated to reflux and stirred under argon for 1 h. The solvent was removed by rotary evaporation. The residue was dissolved in ethyl acetate (5 mL), washed with a saturated bicarbonate solution (2 x 5 mL) and dried over anhydrous sodium sulfate. Precursor **5** was obtained after rotary evaporation of the solvent as a yellow residue (209 mg, 98% yield). ^{1}H NMR (499 MHz, $CDCl_3$, matches data for reported compound from different procedure[5]): δ 8.49 (ddd, J = 4.8, 1.8, 1.0 Hz, 1H), 7.75–7.62 (m, 2H), 7.12 (ddd, J = 7.2, 4.8, 1.3 Hz, 1H), 3.71 (s, 3H), 3.07 (t, J = 7.2 Hz, 2H), 2.79 (t, J = 7.2 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 171.90, 159.72, 149.69, 137.01, 120.79, 119.76, 77.38, 77.13, 76.87, 51.88, 33.63, 33.40.

Synthesis of ATC4: Compound **5** (113 mg, 0.493 mmol) was dissolved in dichloromethane (2 mL). TC4-SH (78.4 mg, 0.246 mmol) was dissolved in degassed dimethyl sulfoxide (2 mL) and added dropwise to the flask containing **5**. The reaction mixture was stirred for 1 h under argon. The product was then extracted using ethyl acetate (10 mL), and washed with water (3 x10 mL) and brine (2 x 10 mL). The ethyl acetate layer was dried over anhydrous sodium sulfate and the solvent was removed by rotary evaporation. The resulting solid was loaded onto a silica column, and flash chromatography using ethyl acetate/hexanes gradient (0-25 % ethyl acetate in hexanes) afforded the desired product as an off-white powder (40 mg, 38%). ^{1}H NMR (499 MHz, DMSO-d_6): δ 11.96 (s, 1H), 9.97 (s, 1H), 8.68 (s, 1H), 8.28 (dd, J = 7.8, 1.6 Hz, 1H), 7.81 (dd, J = 8.0, 1.2 Hz, 1H), 7.57 – 7.31 (m, 4H), 7.00 – 6.85 (m, 2H), 3.78 (d, J = 2.8 Hz, 3H), 3.60 (s, 3H), 2.98 (t, J = 6.8 Hz, 2H), 2.76 (t, J = 6.7 Hz, 2H).^{13}C NMR (126 MHz, DMSO-d6) δ 176.64, 171.92, 157.39, 140.46, 136.36, 134.11, 132.27, 130.97, 130.80, 128.59, 128.53, 127.56, 113.83, 55.75, 52.08, 40.54, 40.46, 40.37, 40.29, 40.20, 40.13, 40.04, 39.96, 39.87, 39.79, 39.70, 39.54, 33.57, 33.15. HRMS–ESI (*m/z*): $[M+Na]^+$ calcd, 458.0637; found: 458.0635.

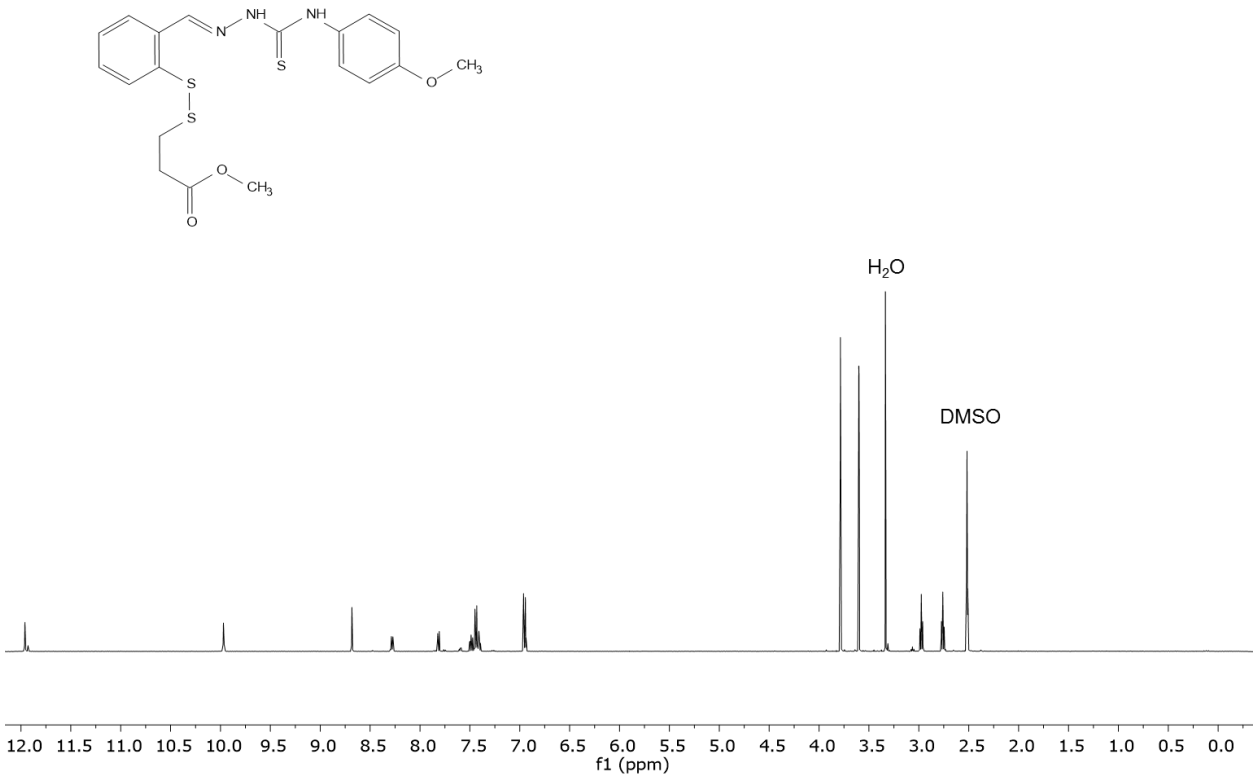


Figure S4. ^{1}H NMR spectrum of **ATC4** (500 MHz, DMSO-d_6).

Solubility measurements. Stock solutions were prepared in DMSO and diluted in buffered aqueous solutions (5.0 mM TRIS, pH 7.40). Buffer solutions containing identical amounts of DMSO (0.3% v/v) were used to obtain blank spectra. Molar extinction coefficients were obtained by recording UV-Vis spectra of a range of concentrations of the compounds (0.1−40 μM, depending on solubility) and then plotting the average of three absorbance values for each concentration at a specified wavelength. For solubility measurements, saturated solutions were centrifuged for 20 minutes at 3,200 rpm to remove any precipitate and concentrations were determined by UV-Vis spectroscopy. Absorbance values were found to be close to the linearity range for each compound therefore dilutions were not typically necessary. Measurements were conducted in triplicate sets.

Interactions of prochelators with Fe(II). Stock solutions of the prochelators were prepared in DMSO and a stock solution of $Fe(BF_4)_2$ was prepared (at ten-fold the concentrations of prochelators) in buffered water (degassed by three freeze-pump-thaw cycles, 5.0 mM TRIS, pH 7.40). Prochelator solutions were diluted in neutral aqueous buffer solutions (final concentrations: 10 μM, 0.1% DMSO in 5.0 mM TRIS, pH 7.40) and iron was added (1-2 μL increments) up to 3 equiv Fe(II) (typically 12 μL at the chosen concentrations). In all cases, negligible absorbance changes (and no indication of iron binding) were observed.

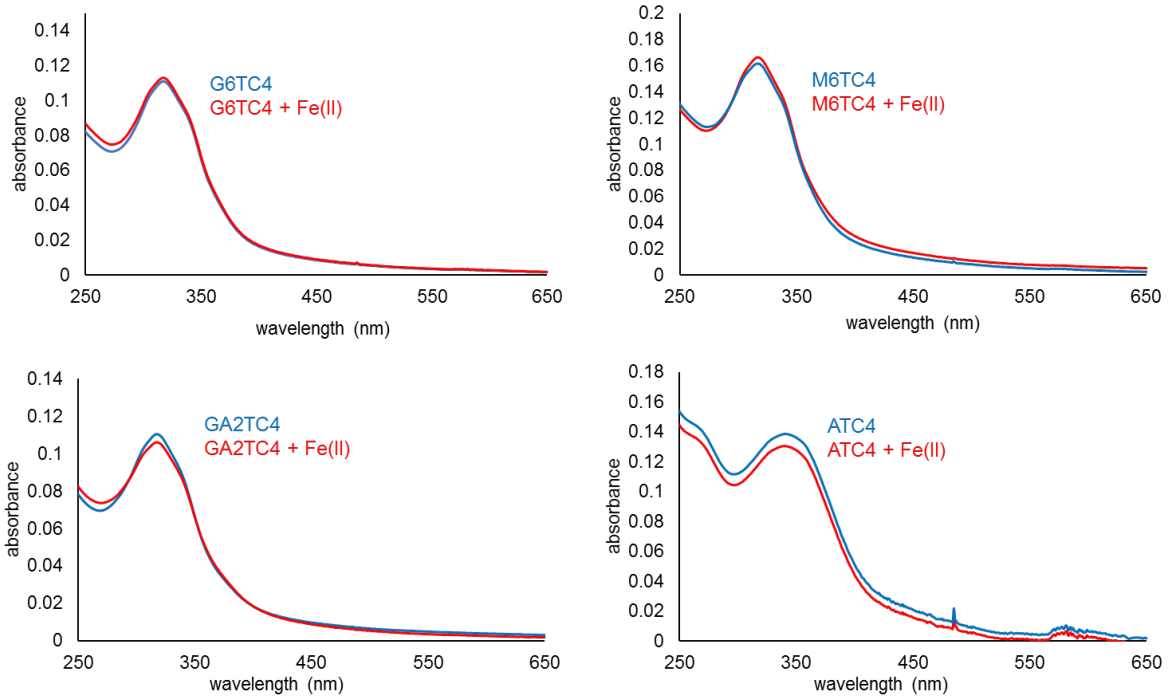


Figure S5. UV-Visible absorption spectra before (blue traces) and after (red traces) addition of $Fe(BF_4)_2$ (3 equiv.) to solutions of prochelators (10 μM) in air-free neutral aqueous solutions (5.0 mM TRIS, pH 7.40) under an argon atmosphere.

Cell culture and cell-based assays

Caco-2 (ATCC® HTB-37™) colorectal adenocarcinoma and CCD-18Co (ATCC® CRL-1459™) normal colon fibroblasts were cultured under a 5% CO_2 humidified atmosphere at 37 °C in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM), sodium pyruvate (1 mM), sodium bicarbonate (1.5 g/L), penicillin (100 units/mL), streptomycin (100 μg/mL) and human holo-transferrin (1.25 μM (1 mg/10 mL) prior to use. Flow cytometric analysis was performed at the University of Arizona Cytometry Core Facility (Arizona Cancer Center/Arizona Research Laboratories) using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) equipped with an air-cooled 15-mW argon ion laser tuned to 488 nm. List mode data files consisting of 10,000 events gated on FSC (forward scatter) vs SSC (side scatter) were acquired and analyzed using CellQuest PRO software (BD Biosciences, San Jose, CA). Appropriate electronic compensation was adjusted by acquiring cell populations stained with each dye/fluorophore individually, as well as an unstained control.

Competition assay of transporter-mediated uptake. Caco-2 cells were plated at 1×10^6 cells per well in 6-well plates and allowed to adhere for 36 hours. Growth media were then removed and cells were incubated for 12 hours in glucose-free EMEM. Cells were then treated with EMEM containing 1 g/L glucose (for unstained control), glucose containing EMEM with 2-NBDG (100μM) (for stained control) or a combination of 2-NBDG (100μM) and test compounds (50 μM) for 40 minutes. As a positive control, after starvation cells were treated with 100 μM phloretin for 30 minutes, then treated with EMEM containing 100 μM 2-NBDG for 40 minutes. Cells were washed with PBS (1 mL) and detached by addition of Trypsin-EDTA (400 μL) and incubation for 3 minutes at 37 ˚C. Following addition of EMEM (1 mL), the cell suspensions were centrifuged at 125 g for 10 minutes. The resulting pellets were suspended in PBS (0.5 mL) and transferred to a flow cytometry tube. Cells were stored on ice and analyzed by flow cytometry within 1 hour. Data are obtained as the average of three sets of geometric means of the flow cytometry histogram, and plotted as the percent difference from the control plus/minus the standard deviation between three values.

Cell-surface expression of GLUT1. Cells were plated at 1×10^6 cells per well in 6-well plates and allowed to adhere overnight. Cells were detached by addition of 0.25% Trypsin-EDTA (400 μL) and incubation for 3 minutes at 37 ˚C. Following addition of EMEM (1 mL), the cell suspensions were centrifuged at 125 g for 14 minutes. The resulting pellets were suspended in PBS (2 mL) containing bovine serum albumin (BSA, 1% w/v, to block non-specific binding of the antibody) then centrifuged at 125 g for another 14 minutes. The resulting pellet was suspended in 1% BSA (500 μL) and cells were counted. Cells were then treated with the primary antibody (at concentration determined from an optimized titration) and incubated on ice for 45 minutes. The cells were centrifuged for ten minutes at 125 g and washed with 1% BSA in PBS (1 mL) in order to remove excess antibody. The pellets were then resuspended in 1% BSA in PBS (500 μL) and the secondary antibody was added and incubated for 30 minutes on ice in the dark. Cells were centrifuged after addition of 1% BSA in PBS (300 μL) for 10 minutes at 125 g. The resulting pellet was then suspended in 300 μL of 1% BSA in PBS (300 μL), stored on ice and analyzed by flow cytometry within 1 hour.

Cytotoxicity assays. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assays were conducted by standard methods with slight modifications. Briefly, cells were seeded at 4000 cells per well for Caco-2 and 10,000 cells per well for CCD18-co and allowed to attach for 24 h. Test compounds dissolved in DMSO were diluted in EMEM to the specified concentration (with final DMSO concentration limited to 0.1%). Cells were incubated in the presence of the test compounds for 72 h, then the MTT solution (4 mg/mL, 10 μL) was added to each well and incubated for 4 h. Following media removal, DMSO (100 μL) was added to each well to dissolve the purple formazan crystals and the plates were incubated for an additional 30 minutes. Absorption at 560 nm was recorded and data were analyzed using logarithmic fits to obtain IC_{50} values. Each experiment was conducted in triplicate, and values are given as averages plus/minus standard deviation.

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