Supporting Information for:

A Strategy for the Selective Imaging of Glycans Using Caged Metabolic Precursors

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Experimental procedures

Materials and methods.

All chemical reagents were of analytical grade, obtained from commercial suppliers, and used without further purification unless otherwise noted. Moisture-sensitive reactions were performed in flame-dried flasks under a N₂ atmosphere. Organic extracts were dried over Na₂SO₄, and solvents were removed with a rotary evaporator at reduced pressure (20 torr), unless otherwise noted. Flash chromatography was performed using Silicycle Siliaflash P60 40-63Å 230-400 mesh silica gel. Analytical thin layer chromatography (TLC) was performed on glass-backed Analtech Uniplate GHLF silica gel plates, and compounds were visualized by staining with ceric ammonium molybdate, 5% H₂SO₄ in ethanol, ninhydrin, and/or the absorbance of UV light (λ = 254 nm). Reversed-phase HPLC was performed using a Rainin Instruments Dynamax SD-200 system equipped with a Varian UV-Vis detector (model 345) and a Microsorb C18 analytical column (4.6 x 250 mm) at a flow rate of 1 mL/min, a semipreparative column (10 x 250 mm) at a flow rate of 4 mL/min, or a preparative column (21.4 x 250 mm) at a flow rate of 20 mL/min. HPLC samples were filtered with a Pall Life Sciences Acrodisc CR 13 mm syringe filter equipped with a 0.2 μm PTFE membrane prior to injection. Dichloromethane (CH₂Cl₂) and acetonitrile (CH₃CN) were passed through activated alumina columns under N₂ before use. Pyridine was distilled over CaH₂ under N₂. Anhydrous N,N-dimethylformamide (DMF), N,N-diisopropylethylamine (DIPEA), triethylamine (TEA), and trifluoroacetic acid (TFA) were used from commercial sources without further purification. DIFO-biotin¹ and compounds ² and ³ were synthesized according to previously published procedures. Boc-tyrosine was purchased from EMD Chemicals (San Diego, CA). NMR spectra were acquired using a Bruker DRX-500, AV-500, AVQ-400, or AVB-400 spectrometer. ¹H NMR spectra were obtained at 400 or 500 MHz, and ¹³C NMR spectra were obtained at 75 or 125 MHz and are referenced to residual solvent peaks. Low- and high-resolution fast atom bombardment (FAB) and electrospray ionization (ESI) mass spectra were obtained at the UC-Berkeley Mass Spectrometry Laboratory.

Dulbecco’s phosphate-buffered saline pH 7.4 (PBS), fluorescein isothiocyanate-conjugated avidin (FITC-avidin), and RPMI-1640 media were purchased from Sigma Aldrich. Ham’s F-12 and OptiMEM I media, Hoechst 33342 nuclear stain, and quantum dot 605-streptavidin conjugate were obtained from Invitrogen Life Technologies, Inc. Fetal bovine serum (FBS) was obtained from HyClone Laboratories. Prostate-specific antigen (PSA) protease was purchased from EMD Biosciences. The colorimetric substrate (MeO-Suc-Arg-Pro-Tyr-PNA·TFA) was purchased from MP Biomedicals. Absorbance readings were collected with a Molecular Devices SpectraMax 190 UV-Vis absorbance plate reader. Microcon Ultracel YM-10 centrifugal filter devices were obtained from Millipore. Apoptosis Detection Kit I containing annexin V-phycocerythrin and 10X Binding Buffer was obtained from BD Biosciences. Tissue culture plates were obtained from Corning, and slides mounted with 8-tissue culture wells were obtained from Nunc. Flow cytometry analysis was performed on a BD FACSCalibur flow cytometer equipped with a 488 nm argon laser and 635 nm He-Ne red diode array laser. At least 10⁶ live cells were analyzed for each sample. Cell viability was ascertained by gating the samples on the basis of forward scatter (to sort by size) and side scatter (to sort by granularity). The average fluorescence intensity was calculated from three replicate samples to obtain a mean value in arbitrary units. The data points are representative of at least three separate experiments. Fluorescence microscopy was performed on a Zeiss Axiovert 200M inverted microscope...
equipped with a 63 × 1.4 numerical aperture Plan-Apochromat oil immersion lens was used for imaging. A 175 W Xenon lamp housed in a Sutter DG4 illuminator linked to the microscope by an optical fiber assured shuttering and illumination. Image stacks containing 20-40 sections spaced 0.5 μm apart were acquired by using a CoolSNAP HQ charged-coupled device camera (Roper Scientific). SLIDEBOOK software (Intelligent Imaging Innovations) was used to control the microscope and the camera. The image stacks were digitally deconvolved by using the nearest-neighbor algorithm of SLIDEBOOK.

Synthesis.

**Compound 3.** Compound 2\(^2\) (1.20 g, 4.58 mmol) was dissolved in a 1:1 solution of pyridine and CH\(_2\)Cl\(_2\) (46 mL). Tert-butyldimethylsilyl chloride (1.03 g, 6.87 mmol) and dimethylaminopyridine (30.2 mg, 0.247 mmol) were added. The reaction was stirred for 22 h. The crude product was purified by silica gel chromatography, eluting with 95:5 CH\(_2\)Cl\(_2\):CH\(_3\)OH to yield 1.15 g (67%) of an off-white solid as a mixture of anomers (3:1 \(\alpha:\beta\)). \(^1\)H NMR of \(\alpha\)-anomer (400 MHz, CH\(_3\)OD): \(\delta\) 5.04 (d, 1H, \(J = 1.6\)), 4.25 (dd, 1H, \(J = 1.6, 4.6\)), 3.78-4.05 (m, 6H), 3.54 (app t, 1H, \(J = 9.7\)), 0.94 (s, 9H), 0.11 (app t, 6H, \(J = 2.5\)). \(^{13}\)C NMR of \(\alpha\)-anomer (125 MHz, CH\(_3\)OD): \(\delta\) 169.0, 92.9, 72.3, 68.9, 67.2, 62.7, 53.9, 51.4, 25.0, 17.9. FAB-HRMS: Calcd. for C\(_{14}\)H\(_{29}\)N\(_4\)O\(_6\)Si\(^{+}\) (M+H\(^{+}\)) 377.1852, found 377.1848.

**Compound 4.** To a solution of 3 (9.20 g, 24.4 mmol) in pyridine (139 mL) was added acetic anhydride (69.3 mL, 0.733 mol). The reaction was stirred for 16 h and concentrated in vacuo. The crude product was then purified by silica gel chromatography using a gradient of 2:1 hexanes:ethyl acetate to afford 11.4 g (93%) of a yellow oil as a mixture of anomers (2:3 \(\alpha:\beta\)). \(^1\)H NMR of \(\beta\)-anomer (500 MHz, CDCl\(_3\)): \(\delta\) 6.56 (d, 1H, \(J = 9.5\)), 6.03 (s, 1H), 5.26 (app t, 1H, \(J = 9.9\)), 5.05 (dd, 1H, \(J = 3.9, 10.0\)), 4.60 (d, 1H, \(J = 9.6\)), 4.06 (m, 2H), 3.63-3.85 (m, 3H), 2.00-2.18 (m, 9H), 0.91 (s, 9H), 0.06 (app d, 6H, \(J = 9.1\)). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)): \(\delta\) 170.3, 169.10, 169.05, 168.2, 167.2, 166.6, 91.5, 90.2, 77.2, 77.0, 76.7, 75.7, 72.7, 71.9, 69.3, 64.91, 64.85, 61.1, 61.0, 52.5, 52.3, 49.8, 49.2, 25.61, 25.58, 20.82, 20.77, 20.72, 20.68, 20.6, 18.2, 18.1, -0.06, -5.58, -5.60. FAB-HRMS: Calcd. for C\(_{20}\)H\(_{34}\)N\(_4\)O\(_9\)SiLi\(^{+}\) (M+Li\(^{+}\)) 509.2247, found 509.2243.

**Compound 5.** Acetic acid (14 mL) was added to a solution of 4 (1.10 g, 2.20 mmol) in a 1:1 solution of THF and H\(_2\)O (9.6 mL). The resulting mixture was stirred for 24 h. The reaction was neutralized with sat. NaHCO\(_3\), and the crude product was extracted with ethyl acetate (3 x 25 mL). The organic layers were pooled, washed with brine (1 x 75 mL), dried with Na\(_2\)SO\(_4\), filtered, and concentrated in vacuo. Silica gel chromatography of the crude product, eluting with a gradient of 1:2 hexanes:ethyl acetate to 1:3 hexanes:ethyl acetate, yielded 800 mg (93%) of a white solid as a mixture of anomers (2:1 \(\alpha:\beta\)). \(^1\)H NMR of \(\alpha\)-anomer (500 MHz, CDCl\(_3\)): \(\delta\) 6.60 (d, 1H, \(J = 9.4\)), 6.06 (d, 1H, \(J = 1.8\)), 5.41 (dd, 1H, \(J = 4.4, 10.2\)), 5.16 (app t, 1H, \(J = 10.2\)), 4.62-4.65 (m, 1H), 4.04 (m, 2H), 3.59-3.84 (m, 3H), 2.02-2.21 (m, 9H). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)): \(\delta\) 170.9, 170.8, 170.2, 170.1, 168.6, 168.5, 167.3, 91.5, 90.3 77.3, 77.0, 76.7, 75.5, 72.5, 72.4, 71.2, 71.1, 68.6, 65.4, 65.3, 60.6, 52.4, 52.3, 52.0, 49.8, 49.3, 20.8, 20.72, 20.68, -0.1. FAB-HRMS: Calcd. for C\(_{14}\)H\(_{20}\)N\(_4\)O\(_9\)Li\(^{+}\) (M+Li\(^{+}\)) 395.1396, found 395.1399.
**Compound 7.** Compounds 5 (466 mg, 1.20 mmol) and 6 (963 mg, 1.75 mmol) were dried by azeotroping with toluene (3 x 10 mL). Pyridine (12 mL) was added, followed by dimethylaminopyridine (214 mg, 1.75 mmol). The resulting mixture was stirred for 18 h at 40 °C. The reaction was concentrated, and the residue was passed through a plug of silica gel using 9:1 toluene:acetone to elute. The crude product was concentrated to afford a white solid that was dissolved in CH$_2$Cl$_2$ (3 mL). TFA (1 mL) was added dropwise. The reaction was stirred for 30 min, diluted with toluene (10 mL), and concentrated in vacuo. The residual TFA was removed from the crude residue by azeotroping with toluene (3 x 10 mL). The compound was then dissolved in 1:1 ddH$_2$O:CH$_3$CN and purified by reversed-phase HPLC using a C18 column (75:25 ddH$_2$O:CH$_3$CN + 0.1 % TFA to 65:35 ddH$_2$O:CH$_3$CN + 0.1% TFA over 60 min) to yield 62.2 mg (62%) of a white solid as a mixture of anomers (2:1 α:β). 1H NMR of α-anomer (400 MHz, CH$_3$OD): δ 7.53 (d, 2H, J = 8.4), 7.37 (d, 2H, J = 8.4), 7.10 (d, 2H, J = 8.4), 6.75 (d, 2H, J = 8.4), 5.97 (d, 1H, J = 1.6), 5.24-5.31 (m, 2H), 5.13 (s, 2H), 4.60 (m, 1H), 4.34 (dd, 1H, J = 5.2, 12.0), 4.10-4.21 (m, 3H), 3.92 (d, 2H, J = 3.6), 3.18 (dd, 1H, J = 6.8, 14.0), 3.05 (dd, 1H, J = 14.0), 1.96-2.16 (m, 9H). 13 C NMR of α-anomer (75 MHz, CH$_3$OD): δ 171.8, 171.7, 171.0, 170.1, 168.2, 158.4, 156.4, 139.3, 133.3, 131.8, 130.4, 126.1, 121.4, 117.0, 93.2, 71.6, 70.64, 70.60, 67.4, 67.0, 56.8, 52.5, 50.8, 38.2, 20.82, 20.80, 20.75. ESI-HRMS: Calcd. for C$_{31}$H$_{37}$O$_{13}$N$_6$ + (M+H)$^+$ 701.2413, found 701.2425.

**Compound 8.** The peptide Mu-HSSK(Boc)L was synthesized on Fmoc-Leu Wang resin (100-200 mesh) using standard Fmoc solid-phase peptide synthesis with N$_\alpha$-Fmoc protected amino acids and DIC/HOBt ester activation in NMP. A five-fold excess of amino acid was used for the coupling steps, which involved gentle rotation for 1 h. Fmoc removal was achieved with 20 % piperidine in NMP (1 x 30 min). The peptide was capped at the N-terminus with a five-fold excess of 4-morpholine carbonyl chloride and TEA in NMP. The peptide was cleaved for 4 h using a solution of Reagent K$^4$ and triisopropylsilane (81.5:5:5:2.5:1 TFA:thioanisole:phenol:ddH$_2$O:ethanediol:triisopropylsilane), precipitated with methyl tert-butyl ether, and the crude product was dried and used without further purification. The crude peptide (308 mg, 0.450 mmol) was dissolved in DMF (4.4 mL), and TEA (0.0823 mL, 0.541 mmol) was added, followed by di-tert-butyldicarbonate (118 mg, 0.541 mmol). The resulting mixture was stirred for 29 h and concentrated in vacuo. The crude product was purified by reversed-phase HPLC using a C18 column (80:20 ddH$_2$O:CH$_3$CN + 0.1 % TFA to 50:50 ddH$_2$O:CH$_3$CN + 0.1% TFA over 40 min) to yield 351 mg (quant.) of a white solid. ESI-HRMS: Calcd. for C$_{34}$H$_{58}$O$_{12}$N$_9$ + (M+H)$^+$ 784.4199, found 784.4200.

**Compound 9.** Compounds 7 (20.5 mg, 0.0293 mmol) and 8 (45.9 mg, 0.0586 mmol) were dissolved in DMF (0.29 mL). (Benzotriazol-1-yl-oxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 28.5 mg, 0.0645 mmol) was added, followed by DIPEA (0.013 mL, 0.0733 mmol), dropwise. The reaction was stirred for 1 h, after which it was concentrated, and the resulting residue was purified by reversed-phase HPLC using a C18 column (65:35 ddH$_2$O:CH$_3$CN + 0.1 % TFA to 55:45 ddH$_2$O:CH$_3$CN + 0.1% TFA over 60 min) to yield 7.8 mg (18%) of a white solid. ESI-HRMS: Calcd. for C$_{68}$H$_{92}$O$_{24}$N$_{15}$ + (M+H)$^+$ 1466.6434, found 1466.6481.

**Compound 1.** TFA (0.25 mL) was added dropwise to a solution of 9 (10.7 mg, 0.0730 mmol) in CH$_3$CN (0.75 mL). The resulting mixture was stirred for 2 h, and the reaction was then diluted.
with toluene (10 mL). The reaction mixture was concentrated \textit{in vacuo}, and residual TFA was removed by azeotroping with toluene (3 x 10 mL). The crude product was purified by reversed-phase HPLC using a C18 column (75:25 ddH2O:CH3CN + 0.1 % TFA to 65:35 ddH2O:CH3CN + 0.1% TFA over 50 min) to yield 7.7 mg (76%) of a white solid. ESI-HRMS: Calcd. for C_{60}H_{85}O_{22}N_{15}^{2+} [(M + 2H)]^{2+} 683.7991, found 683.8005.

\textbf{PSA activity assay.}

Activity of PSA and heat-killed (HK) PSA (50 \mu g/mL) were verified according to a previously established protocol\textsuperscript{5} using a colorimetric substrate (MeO-Suc-Arg-Pro-Tyr-PNA·TFA, 500 \mu M) in 50 mM Tris, 0.1 M NaCl, pH 7.8. The enzymatic reactions were incubated at 37 °C and monitored at 405 nm for release of \textit{p}-nitroanilide every 10 min for 15 h. Buffer only and buffer with substrate were used as negative controls.

\textbf{In vitro enzymatic reactions.}

Compound 1 (500 \mu M) was incubated with PSA (50 \mu g/mL), no enzyme, or HK PSA (50 \mu g/mL) in 50 mM Tris, 0.1 M NaCl, pH 7.8, for 24 h at 37 °C. The samples were filtered with Microcon Ultracel YM-10 filter devices (10,000 MWCO) by centrifugation (10,621 x g for 20 min) to remove the protein. The cellulose membrane was rinsed (1 x 50 \mu L ddH2O), and the sample was centrifuged again (10,621 x g for 10 min). The wash step was repeated twice. The filtrates were then analyzed by reversed-phase HPLC using a C18 analytical column (100:0 ddH2O:CH3CN + 0.1 % TFA to 60:40 ddH2O:CH3CN + 0.1% TFA over 60 min), monitoring at 210 nm, and by mass spectrometry.

\textbf{Cell culture.}

Chinese hamster ovary (CHO) cells were cultured in Ham’s F-12 media supplemented with 10% FBS, 100 units/mL penicillin and 100 \mu g/mL streptomycin. PC-3 cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 units/mL penicillin and 100 \mu g/mL streptomycin. The cells were maintained at 37 °C and 5% CO\textsubscript{2} in a water-saturated incubator and counted using a hemocytometer. Cell densities were maintained between 1 x 10\textsuperscript{5} and 2 x 10\textsuperscript{6} cells per mL.

\textbf{Metabolism of Ac\textsubscript{3}ManNAz by CHO and PC-3 cells and analysis by flow cytometry.}

CHO or PC-3 cells were seeded in 12-well tissue culture plates in 1 mL media and grown until 80-90% confluency. The media was aspirated, and the cells were washed with OptiMEM I (3 x 1 mL). The cells were then treated with OptiMEM I (0.4 mL) containing Ac\textsubscript{3}ManNAz (0-100 \mu M). (For the experiment shown in Figure S1B, the cells were treated with Ac\textsubscript{3}ManNAz (0-100 \mu M) in their respective full media.) The cells were incubated for 0-12 h, washed with PBS + 1% FBS (FACS buffer) (3 x 1 mL), and then lifted by treatment with 1 mM EDTA in PBS (0.65 mL) for 20-40 min. The cells were then transferred to a 96-well V-bottom plate and pelleted by centrifugation (2536 x g for 3 min). The supernatants were decanted, and the cells were resuspended with FACS buffer (0.2 mL). The cells were pelleted again and after discarding the supernatant, they were resuspended with DIFO-biotin (100 \mu M from a 1 mM stock in 12%
DMF/PBS) in FACS buffer (0.1 mL) and incubated for 2 h at 37 °C. After the labeling reaction, the cells were washed with FACS buffer as described above (3 x 0.2 mL) and resuspended in 0.1 mL of FACS buffer containing FITC-avidin (1:200 dilution of a 1 mg/mL stock). The cells were incubated for 15 min on ice in the dark. The cells were washed once with FACS buffer (0.2 mL), stained with FITC-avidin for a second time, and washed with FACS buffer (3 x 0.2 mL). The cells were then resuspended in FACS buffer (0.4 mL) for analysis by flow cytometry.

Cell-selective labeling of CHO and PC-3 cell-surface glycans and cell viability assay.

CHO or PC-3 cells were seeded in 12-well tissue culture plates in 1 mL media and grown until 80-90% confluency. At this point, the media was aspirated, and the cells were washed with OptiMEM I (3 x 1 mL). The cells were then treated with OptiMEM I (0.4 mL) containing 1 (0-100 μM) and either PSA (0-50 μg/mL), no enzyme, or HK PSA (50 μg/mL). The cells were incubated for 12 h at 37 °C. Following incubation, the media was removed, and the cells were washed with PBS (3 x 1 mL). The cells were lifted by incubation with 1 mM EDTA in PBS (0.65 mL) for 20 min. The cells were then transferred to a 96-well V-bottom plate, labeled with DIFO-biotin, followed by FITC-avidin, and prepared for flow cytometry analysis as described above.

For the experiment shown in Figure S4, the cells were washed with FACS buffer (1 x 0.2 mL) and then PBS (2 x 0.2 mL) after the second FITC-avidin staining. Prior to flow cytometry analysis, the cells were resuspended in 1X annexin binding buffer (prepared according to the manufacturer’s protocol) containing annexin V-phycoerythrin (0.1 mL) and incubated for 15 min at rt in the dark. Additional 1X binding buffer (0.3 mL) was added, and the samples were analyzed by flow cytometry.

Imaging of cells by fluorescence microscopy.

CHO cells were seeded at a density of 1 x 10^5 cells per mL on slides mounted with 8-tissue culture wells in 0.4 mL media. After 2 d, the cells were washed with OptiMEM I (3 x 0.5 mL) and then treated with 1 (100 μM) and either PSA or HK PSA (50 μg/mL) in OptiMEM I (0.2 mL). The cells were incubated for 12 h at 37 °C. The cells were then washed with media (3 x 0.5 mL) and incubated with DIFO-biotin (100 μM) in media (0.125 mL) for 2 h at 37 °C. The cells were washed with FACS buffer (3 x 0.5 mL) and then labeled with a quantum dot 605-streptavidin conjugate (10 nM from a 1 μM stock in borate buffer, pH 8.3) in FACS buffer (0.1 mL) for 15 min at rt. The cells were washed with FACS buffer (3 x 0.5 mL) and stained with Hoechst 33342 (0.1 mL of a 1:1000 dilution from a 1 mg/mL DMSO stock) for 2 min in the dark. The cells were washed twice with media (0.5 mL) and imaged in media (0.2 mL). The images shown are maximum intensity z-projections of three z-planes acquired using a 63X objective.
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Supplemental data

Scheme S1. Synthesis of 1
Figure S1. Metabolic labeling of CHO and PC-3 cells with Ac₃ManNAz. (A) CHO cells were incubated with Ac₃ManNAz (0-100 μM) for 12 h at 37 °C. (B) CHO or PC-3 cells were incubated with (100 μM, black bars) or without (white bars) Ac₃ManNAz for 12 h at 37 °C. (C) CHO cells were incubated with Ac₃ManNAz (100 μM) for various amounts of time (0.5-6 h) at 37 °C. In all cases, the samples were subsequently labeled with DIFO-biotin (100 μM) for 2 h at 37 °C, followed by FITC-avidin, and analyzed by flow cytometry. *p < 0.006 (t-test). MFI = mean fluorescence intensity in arbitrary units (AU). Error bars represent the standard deviation of the average MFI from three replicate samples.

‡ The data shown in Figure S1C indicate that Ac₃ManNAz is taken up and metabolized to generate detectable levels of cell-surface azido glycans as early as 30 min after the treatment had commenced. In considering whether the caged azidosugar strategy might be feasible for in vivo imaging of PSA-expressing tissues, we noted that protease-activatable probes similar to the one that we designed persist in the vicinity of the target tissue in vivo for up to 24 h after delivery of the probe due to the formation of a concentration gradient surrounding the tumor.⁶⁷ Thus, while diffusion of Ac₃ManNAz away from the target tissue after uncaging by PSA would likely occur in vivo, we do not expect it to be problematic because the enzyme would be constantly turning over substrate such that the uncaged probe would be found at a higher concentration near the target cells for many hours. In our situation, higher levels of uncaged Ac₃ManNAz around the target tissue would result in higher levels of cell uptake compared to the surrounding tissue. Therefore, we believe that the data shown in Figure S1C demonstrates that diffusion is not problematic for implementation of the caged azidosugar strategy in vivo.
Figure S2. Flow cytometry analysis of CHO cells treated with 1 and PSA. CHO cells were incubated with or without 1 (25 μM) and PSA (0-50 μg/mL) for 12 h at 37 °C. The samples were then labeled with DIFO-biotin (100 μM) for 2 h at 37 °C, followed by FITC-avidin, and analyzed by flow cytometry. Error bars represent the standard deviation of the average MFI from three replicate samples.

Figure S3. Flow cytometry analysis of PC-3 cells treated with 1 and PSA. PC-3 cells were incubated with 1 (100 μM) and either no enzyme (–), HK PSA (50 μg/mL, HK), or PSA (50 μg/mL, +), for 12 h at 37 °C. The samples were then labeled with DIFO-biotin (100 μM) for 2 h at 37 °C, followed by FITC-avidin, and analyzed by flow cytometry. Error bars represent the standard deviation of the average MFI from three replicate samples.

Figure S4. Cell viability assay. CHO cells were incubated with 1 (0-100 μM) and either no enzyme (–), PSA (50 μg/mL, +), or HK PSA (50 μg/mL, HK), for 12 h at 37 °C. The samples were then labeled with DIFO-biotin (100 μM) for 2 h at 37 °C, followed by FITC-avidin and annexin V-phycoerythrin, and analyzed by flow cytometry. Percent live cells represent cells that stain weakly with annexin V. Error bars represent the standard deviation of the average % live cells for three replicate samples.