Supplementary information

Development of Triantennary N-Acetylgalactosamine Conjugates as Degraders for Extracellular Proteins
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Materials and Methods

Safety Statement
No unexpected or unusually high safety hazards were encountered.

General chemistry methods
Tri-GalNAc-biotin 1 and tri-GalNAc-CO₂H 2 were purchased from Sussex Research Laboratories Inc. Other reagents and solvents were purchased from Fisher Scientific and used as received. Tri-GalNAc-NHS ester 3 was prepared from tri-GalNAc-CO₂H 2 in the presence of N-hydroxysuccinimide and N,N′-dicyclohexylcarbodiimide in dimethylformamide (DMF) as used without further purification. The reaction was monitored by Agilent single quadrupole (SQ) LC/MS.

Cell culture
HepG2 and Huh7 cells were cultured in T75 flasks and maintained in low-glucose DMEM supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 1% L-glutamine and 1% penicillin/streptomycin under 5 % CO₂ at 37 °C. A549 cells were cultured in T75 flasks and maintained in RPMI supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% HEPES and 1% penicillin/streptomycin under 5 % CO₂ at 37 °C.

NeutraAvidin uptake experiments
Cells were seeded at 45,000 cells per well in 100 μL complete culture media in 96-well cell culture plates or plated at 250,000 cells per well in a 24-well plate. The next day, the medium was replaced followed by the sequential addition of NA-650 and tri-GalNAc-biotin 1 or tri-GalNAc-COOH 2 with various concentrations. The cells were incubated at 37 °C for different time periods and then washed twice with PBS to removed extracellular NA-650. The uptake in 96-well plate was determined by measuring the fluorescent intensity at 650 nm excitation/680 nm emission using the Synergy H1 microplate reader. Data was acquired using Gen5 software. The cells in 24-well plate were lysed for in gel fluorescence analysis.

Competition assay
HepG2 cells were plated and treated with 500 nM of NA-650 and 2 μM of tri-GalNAc-biotin 1 in the same manner as mentioned above. Extra tri-GalNAc-COOH 2 (1, 2, 5, 10, 20 μM) was added at the same time before incubation. After 4 h, cells were washed twice with PBS, and the uptake of NA-650 was read by the plate reader as mentioned above.

**Knockdown of ASGPR by siRNA**

HepG2 cells were seeded at 75,000 cells per well in a 24-well plate one day before transfection. Cells were then transfected with 20 pmol of ASGPR-specific or scramble siRNA and 1.5 μL transfection reagent RNAiMax for 48 h prior to the detection of ASGPR expression level or NA-650 uptake.

**Confocal microscopy**

HepG2 cells were seeded onto 8-well chamber slides at the density of 20,000 cells/well in 200 μL of complete culture medium. After adhesion, cells were treated with 500 nM of NA-650 and 2 μM of tri-GalNAc-biotin 1 for 18 h at 37 °C, followed by the 30-min incubation with LysoTracker Green DND26 (100 nM) at 37 °C. Hoechst 33342 (5 μg/ml) was added 10 min before the end of incubation. After three washes with PBS, the live cells were imaged using FluoView confocal microscope at 20x magnification with a 10x eyepiece. All images were acquired by FV10-ASW and analyzed by ImageJ.

**NeutrAvidin degradation analysis**

HepG2 cells were seeded at 250,000 cells per well in a 24-well plate. Next day, cells were incubated with 500 nM of NA-650 and 2 μM of tri-GalNAc-biotin 1 for 1 h followed by three washes with PBS. Cells were maintained subsequently in fresh media with or without 0.1 mg/mL leupeptin for another 3 h, 6 h, and 24 h before harvested for in gel fluorescence analysis.

**Antibody labeling**

The antibody solution was first loaded onto a 10 kDa Amicon Centrifugal Filter to remove the preservative and concentrate the antibody before labeling. To label the antibody with tri-GalNAc, 50 μL of the antibody (concentration above 1 mg/mL) in PBS was mixed with tri-GalNAc-NHS ester 3 at 1:3, 1:12 or 1:25 molar ratio. The reaction was incubated overnight at room
temperature on a rotator, followed by filtration with 500 μL of PBS for 5 times using 10 kDa Amicon Centrifugal Filter.

**Tri-GalNAc-antibody uptake experiment**

HepG2 cells were plated at 45,000 cells per well in 100 μL complete culture media in a 96-well cell culture plate 8 h prior to the treatment. Cells were then incubated with 25 nM Ab-647-GN with different modification levels for 16 h before measuring the fluorescence intensity in the cells.

**Mouse IgG uptake experiment**

HepG2, Huh7 and A549 cells were plated at 250,000 cells per well in a 24-well plate. Complete growth media supplemented with 50 nM of mouse anti-biotin-IgG-647 (protein target in Fig. S4) or mouse anti-rabbit-IgG-647 (protein target in Fig. 4B) and 25 nM of tri-GalNAc labelled goat anti-mouse IgG or goat anti-mouse IgG Fab was sequentially added. The cells were incubated at 37 °C for 6 h and then lysed for in gel fluorescence analysis.

**Mouse IgG degradation analysis**

HepG2 cells were seed at 250,000 cells per well in a 24-well plate. Next day, cells were incubated with 50 nM of mouse anti-biotin IgG-647 and 25 nM of tri-GalNAc goat-anti-mouse IgG Fab for 3 h followed by three washes with PBS. Cells were maintained subsequently in fresh media with or without 0.1 mg/mL leupeptin for another 3 h before harvested for in gel fluorescence analysis. For the co-treatment assay, HepG2 or Huh7 cells were seed at 250,000 cells per well in a 24-well plate. Next day, cells were incubated with 50 nM of mouse anti-biotin IgG-647, 25 nM of tri-GalNAc goat-anti-mouse IgG Fab, together with 10 μM chloroquine or 0.1 mg/mL leupeptin for 6 h followed by the collection of samples for in gel fluorescence analysis.

**NeutrAvidin and IgG complex uptake experiment**

HepG2 cells were plated at 250,000 cells per well in a 24-well plate. Next day, 50 nM mouse anti-biotin IgG-647 was mixed with 200 nM goat anti-mouse IgG or goat anti-mouse IgG Fab on a rotator at room temperature for 1 h before treatment. Cells were then respectively incubated with mouse anti-biotin IgG-647, anti-biotin IgG-647/goat anti-mouse IgG Fab and anti-biotin IgG-
647/goat anti-mouse IgG complex together with 200 nM tri-GalNAc-COOH 2 or tri-GalNAc-biotin 1 for 6 h. The cells were then harvested and lysed for in gel fluorescence analysis.

**MALDI-MS**

α-Cyano-4-hydroxycinnamic acid (HCCA) was dissolved in 50% Acetonitrile/H2O to give a 10 mg/mL solution as the matrix solution. The sample was absorbed on Omix C4 pipette tips, washed by 0.1% TFA for three times and then eluted with 20 μL 75% Acetonitrile/H2O. 1 μL sample solution and 1 μL HCCA solution were spotted on the MALDI target plate and mixed thoroughly before the spot was allowed to dry under room temperature. MALDI-MS spectra were acquired on Bruker UltraFlex MALDI-TOF/TOF mass spectrometer operated in linear positive ion mode. Masses were calculated from windowed raw data in Sigmaplot 13.0 by fitting to gaussian curves, with constant baseline as an additional free parameter. Parameter starting values were the default values of the program, and were automatically iterated 200 times to obtain fits. Plots were made in Origin 2020, where high-frequency noise was removed using 100 points windowed FFT filter.

**EGFR degradation analysis**

HepG2 cells were seeded at 100,000 cells per well and Huh7 cells were seeded at 75,000 cells per well in a 24-well plate. Next day, cells were treated with 30 nM Ctx or Ctx-GN for 48 h before collection for western blot analysis.

**Western blotting**

Cells were lysed with 1X RIPA lysis buffer containing 25 mM Tris, pH 7–8, 150 mM NaCl, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% (v/v) Triton X-100, protease inhibitor cocktail (Roche, one tablet per 10 mL) and 1 mM phenylmethylsulfonyl fluoride on ice for 10 min. The lysates were then centrifuged at 14 000g at 4 °C for 15 min and the supernatant was collected followed by measuring the protein concentration using BCA assay. Lysates were adjusted to the equal amount before mixed with the 4x Laemmli Loading Dye and heated at 95–100 °C for 5 min. After cooling down, samples were loaded onto 7.5% SDS–polyacrylamide gel electrophoresis and transferred to PVDF membrane. The membrane was first blocked in 5% (w/v) non-fat milk in the TBS-T washing buffer (137 mM NaCl, 20 mM Tris, 0.1% (v/v) Tween)
and then incubated with primary antibodies at 4 °C overnight. After 3 washes with TBST, the membrane was incubated with secondary HRP-linked antibodies for 1 h, and then washed 3 times with TBST. Then the membrane was incubated in the Clarity ECL substrate for 3-5 min before acquiring the immunoblot by ChemiDoc MP Imaging Systems.

Sources of key reagents and antibodies

| Reagents and antibodies                                      | Vendor                  | Catalog #     |
|--------------------------------------------------------------|-------------------------|---------------|
| Trivalent GalNAc-biotin 1                                   | Sussex Research         | BT000130      |
| Trivalent GalNAc-COOH 2                                     | Sussex Research         | PE000130      |
| NeutrAvidin protein, DyLight 650                            | Fisher Scientific       | 84607         |
| LysoTracker Green DND26                                     | Fisher Scientific       | L7526         |
| Hoechst 33342                                                | Fisher Scientific       | H3570         |
| Leupeptin                                                    | Sigma-Aldrich           | L2884-5MG     |
| Chloroquine diphosphate salt                                | Sigma-Aldrich           | C6628-25G     |
| AffiniPure Goat Anti-Mouse IgG                              | Jackson ImmunoResearch  | 115-005-062   |
| Goat anti-mouse IgG, Alexa Fluor®647                        | Invitrogen              | A-21235       |
| Affinipure Fab Fragment Goat anti-mouse IgG                 | Jackson ImmunoResearch  | 115-007-003   |
| Anti-Biotin Mouse Monoclonal Antibody (Alexa Fluor®647)     | Jackson ImmunoResearch  | 200-602-211   |
| Mouse Anti-Rabbit IgG Antibody (Alexa Fluor® 647)           | Jackson ImmunoResearch  | 211-605-109   |
| Cetuximab                                                    | Selleckchem             | A2000         |
| ASGPR1 siRNA (h)                                             | Santa Cruz Biotechnology| sc-29746       |
| Control siRNA                                                | Santa Cruz Biotechnology| Sc-37007       |
Figure S1. Tri-GalNAc-biotin mediates the uptake of NA-650 through ASGPR in a time- and dose- dependent manner. A. NA-650 uptake in HepG2 cells treated with NA-650 (500 nM) and increasing concentrations of compound 1 for 4 h. B. Cellular uptake of NA-650 (500 nM) in HepG2 cells in the presence of compound 1 (2 μM) within 24 h. C. ASGPR expression levels in HepG2, Huh7 and A549 cell lines (exp. = exposure time). D. Uptake of NA-650 (500 nM) in the presence of 1 (2 μM) in HepG2 cells treated with control or ASGPR siRNA.

Figure S2. Tri-GalNAc Antibody (Ab-647-GN) with higher degree of modification exhibits greater uptake efficiency. A. Goat anti-mouse-647 antibody labeled with various amounts of tri-GalNAc. UL: unlabeled; 3x: 3 molar equivalent; 12x: 12 molar equivalent; 25x: 25
molar equivalent. B. Cellular uptake of Ab-647-GN (25 nM) with different degrees of labeling in 16 h. Data presented as Mean±SD, n=3. Ns: not significant, **p<0.01.

**Figure S3. Characterization of antibody-based degraders.** MALDI-TOF-MS spectra of goat anti-mouse IgG or anti-mouse IgG Fab fragment with or without tri-GalNAc labeling. The average numbers of tri-GalNAc labelled on each antibody were listed in the table.

**Figure S4. Tri-GalNAc labeled antibody Fab fragment mediates the highest endocytosis of mouse anti-biotin IgG-647.** HepG2 and Huh7 cells were treated with mouse anti-biotin IgG-647 (50 nM) in the presence of 25 nM of goat anti-mouse IgG and goat anti-mouse IgG Fab with or without tri-GalNAc labeling. The uptake was measured and compared after 6 h treatment.
Figure S5. Leupeptin and chloroquine rescue mouse anti-biotin IgG-647 degradation. Increased amount of internalized mouse anti-biotin IgG-647 in HepG2 and Huh7 cells in the presence of 10 μM chloroquine or 0.1 mg/mL leupeptin for 6 h.

Figure S6. Characterization of Ctx-GN. MALDI-TOF-MS spectra of Cetuximab with or without tri-GalNAc labeling and the number of tri-GalNAc labelled per Cetuximab.