Supporting Information for:

Site-Specific Conjugation of the Indolinobenzodiazepine DGN549 to Antibodies Affords Antibody-Drug Conjugates with an Improved Therapeutic Index As Compared With Lysine Conjugation

Chen Bai[1], Emily Reid[1], Alan Wilhelm[1], Manami Shizuka[1,2], Erin K. Maloney[1], Rassol Laleau[1], Lauren Harvey[1,3], Katie E. Archer[1], Dilrukshi Vitharana[1,4], Sharlene Adams[1], Yelena Kovtun[1], Michael L. Miller[1], Ravi Chari[1], Thomas A. Keating[1], Nicholas C. Yoder[1]*

*Correspondence to: palmitoylated@gmail.com

1. Science, Technology, and Translation; ImmunoGen, Inc.; 830 Winter Street, Waltham MA 02451
2. Current address: Pharmaron; 404 Wyman Street; Waltham, MA 02451
3. Current address: Kaleido Biosciences; 65 Hayden Ave., Lexington MA 02421
4. Current address: Anokion; 50 Hampshire Street; Cambridge, MA 02139

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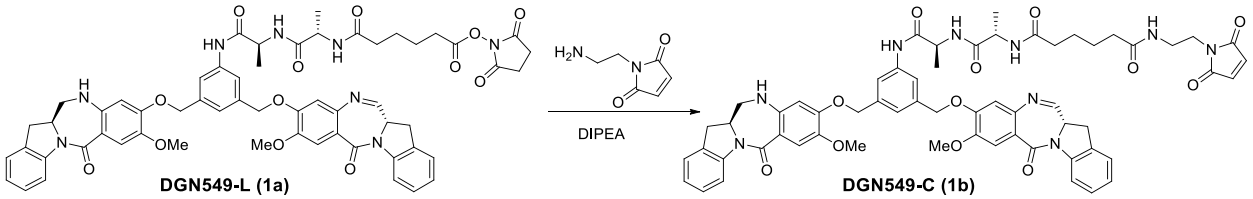
Supplementary Methods: Synthesis and Characterization of DGN549-C (Compound **1b**)



Supplementary Figure 1. Synthesis of compound DGN549-C (**1b**) from DGN549-L (**1a**).

Methods:

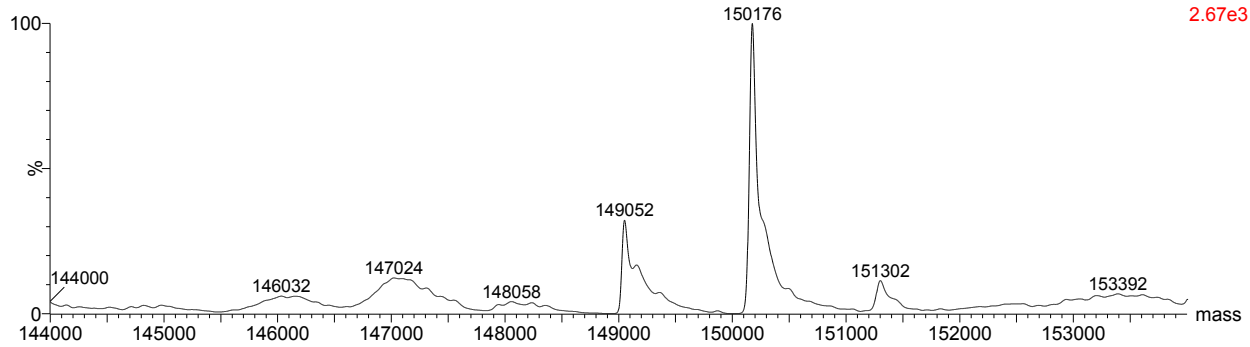
Compound **1a**[1] (80 mg, 0.074 mmol) and N-(2-aminoethyl)maleimide hydrochloride (2.17 mg, 0.011 mmol) were dissolved in anhydrous dichloromethane (2976 µl). *N,N*-diisopropylethylamine (DIPEA, 25.9 µl, 0.149 mmol) was added and the reaction stirred for 4 hours at room temperature. The crude reaction was checked by LC-MS and there was no remaining starting material. The reaction was concentrated under nitrogen and redissolved in 3:1:1 anhydrous THF/ACN/water and purified by reverse phase semi-prep HPLC (C18 column, ACN/H_2O). Fractions containing the desired product were frozen and lyophilized to obtain compound **1b** (18 mg, y=21%, 97% pure). HRMS (ESI^+): calc. for C60H61N9O12 (M + H) 1100.4440, found 1100.4449. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.01 (s, 1H), 8.14 (d, J = 8.0 Hz, 1H), 8.05 (d, J = 8.0 Hz, 1H), 7.96 – 7.89 (m, 2H), 7.77 (t, J = 6.0 Hz, 1H), 7.65 (m, 2H), 7.36 (s, 1H), 7.28 – 7.03 (m, 7H), 7.00– 6.91 (m, 4H), 6.36 (s, 1H), 6.25 (d, J = 6 Hz, 1H), 5.69 (s, 2H), 5.13 (m, 2H), 4.99 (s, 2H), 4.50 – 4.21 (m, 4H), 3.80 (s, 2H), 3.60 (s, 3H), 3.68 – 3.40 (m, 5H), 3.25 – 2.94 (m, 4H), 2.75 (dd, J = 17.1, 4.3 Hz, 1H), 2.03 (m, 2H), 1.85 (m, 2H), 1.35 (m, 4H), 1.24 (d, J = 6.8 Hz, 3H), 1.13 (d, J = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.3, 172.2, 172.0, 171.4, 171.0, 165.6, 164.9, 163.1, 152.0, 150.5, 147.4, 142.9, 142.1, 141.8, 140.8, 140.0, 139.4, 137.5, 137.4, 134.4, 130.8, 130.3, 127.3, 127.0, 125.1, 124.7, 124.4, 123.3, 121.7, 120.0, 117.9, 116.3, 115.7, 115.1, 111.8, 110.8, 109.7, 102.3, 70.0, 69.5, 57.4, 56.0, 55.8, 54.9, 53.2, 49.0, 47.9, 37.2, 36.7, 35.1, 34.8, 32.8, 31.7, 24.8, 24.7, 18.0, 17.9.

Supplementary Methods: Affinity Capture of ADCs from Plasma Samples

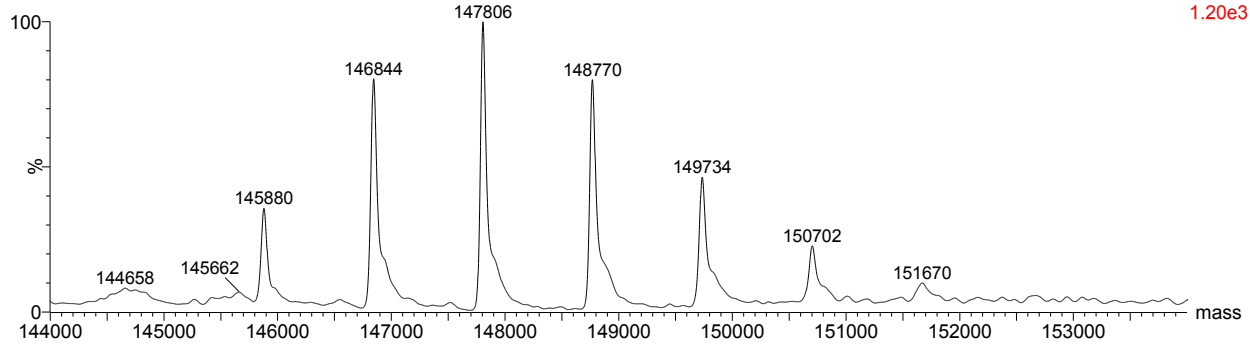
To affinity purify ADCs from plasma samples, xMag-Streptavidin Microparticles (Biochain, CA) were washed with 50 mM tris(hydroxymethyl)aminomethane (Tris), 0.15 M sodium chloride pH 8.0 washing buffer twice and resuspended in the same washing buffer to their original volume. ~100 ug of biotinylated FRα-Fc (ImmunoGen, Inc.) was then added to 200 µL of streptavidin particles and rotated at room temperature for 2 h. The beads were washed 3 times with washing buffer and re-suspended to their original volume in washing buffer with 0.4% Tween-20. 200 µL plasma sample containing ADC was then added to the FRα-Fc coated particles along with 40 µL washing buffer and Tween-20 to a final concentration of 0.2%. After gentle shaking at room temperature for 2 h, the particles were washed 3 times with 1 mL washing buffer and eluted using 50 µL of 0.1 M sodium citrate pH 3.0 buffer with 50% ethylene glycol. The eluent was immediately neutralized with 9 µL of 1 M tris(hydroxymethyl)aminomethane pH 8.5, and then analyzed by SEC-MS as previously described.[2]

Supplementary Figure 2. Intact LC-MS Characterization of ADCs (A) **2c**; (B) **3a**, and (C) **3b**

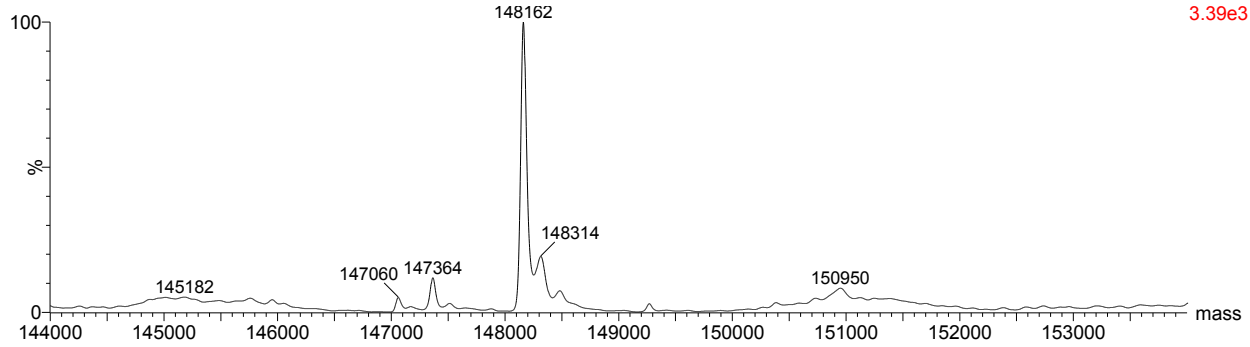
(A)



(B)



(C)

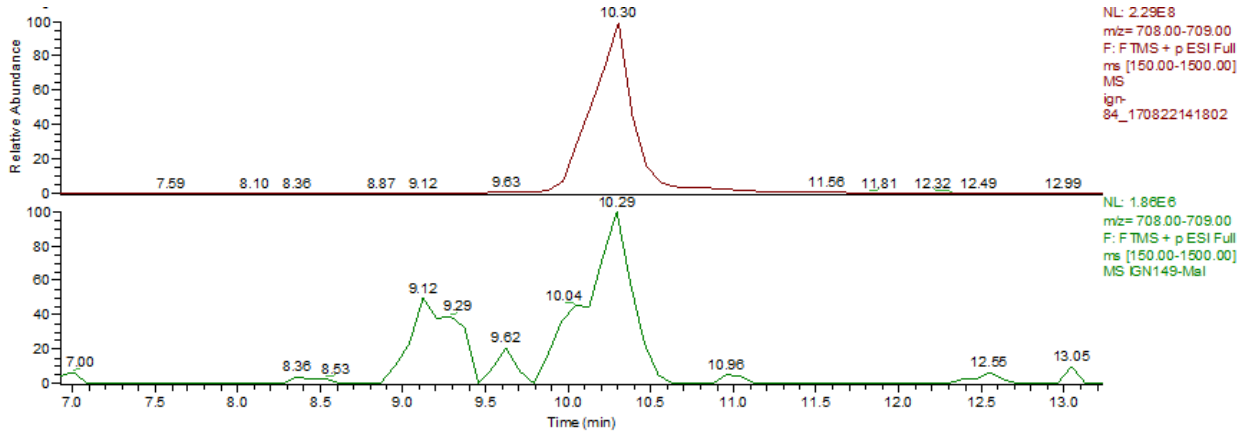


Supplementary Table 1. Characterization of ADCs

ADC	DAR	Monomer (%)	Free Drug (%)	Yield (%)
2a	2.5	99.5	0.5	77
2b	1.9	97.2	0.2	94
2c	3.5	98.5	0.8	84
3a	2.6	99.0	<1.0	80
3b	2.0	98.1	2.8	80

Supplementary Figure 3. Identification of FGN849 as the catabolite released from ADC **2b**. (A) Extracted ion chromatograms for ADC **2b** catabolite (bottom) compared to a FGN849 standard (top) are shown. (B) Structures and masses of other plausible catabolites not detected.

(A)



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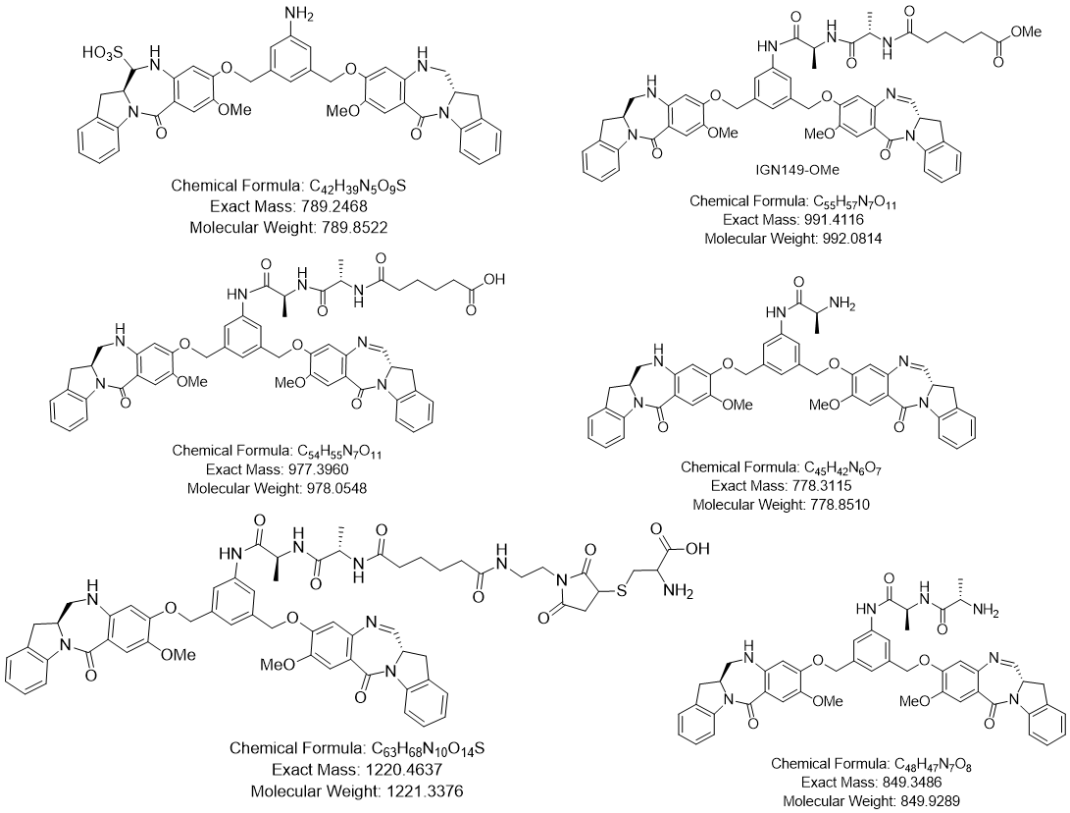
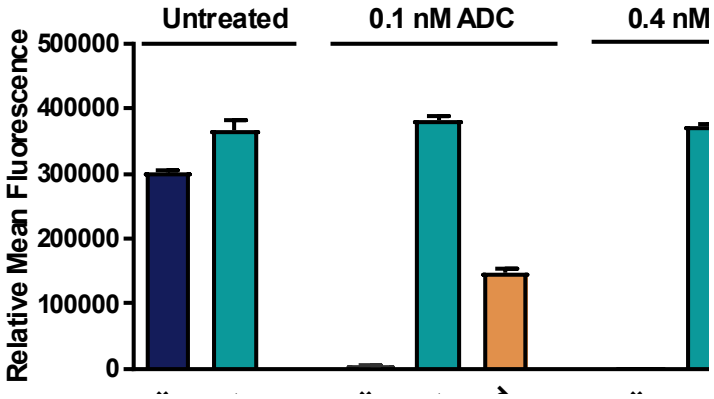
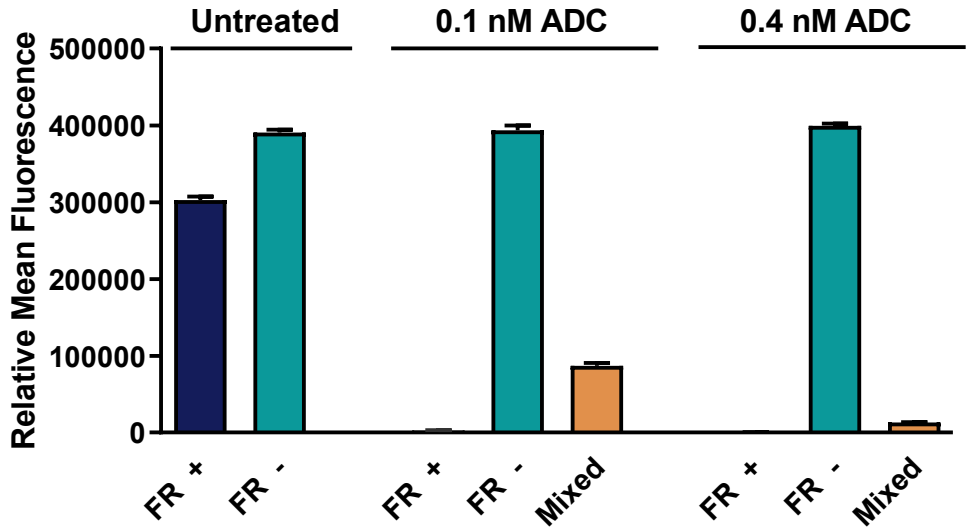


Supplementary Figure 4. Bystander cytotoxicity of mAb1 ADCs on FRα-negative 300.19 cells in the presence of FRα+ 300.19 cells transfected with the gene encoding human FRα: (A) ADC **2a**, (B) ADC **2b**.

(A)



(B)

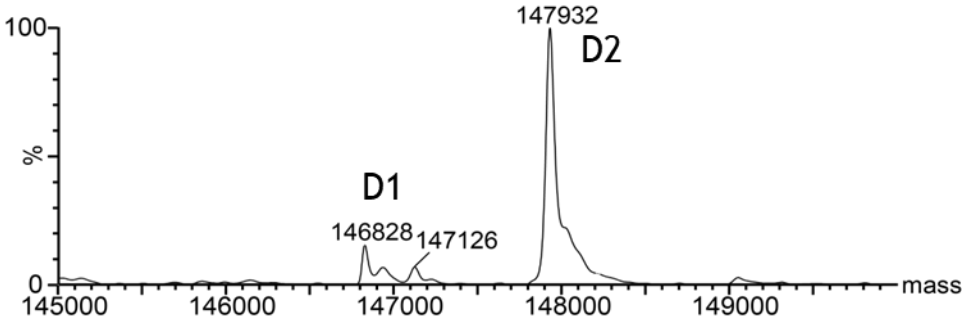


Supplementary Table 2. Binding of mAb1 ADCs to T47D cells expressing FRα and of mAb2 ADCs to HNT-34 cells expressing CD123 assayed by flow cytometry.

ADC or Ab	**EC_{50} (M)**	**Cell Line**
mAb1	2×10^{-10}	T47D
2a	7×10^{-10}	T47D
2b	2×10^{-10}	T47D
2c	1×10^{-10}	T47D
mAb2	2×10^{-10}	HNT-34
3a	1×10^{10}	HNT-34
3b	8×10^{-11}	HNT-34

Supplementary Figure 5. Affinity capture of ADC **2b** from mouse plasma; (A) pre-injection; (B) three days post-dose.

(A)



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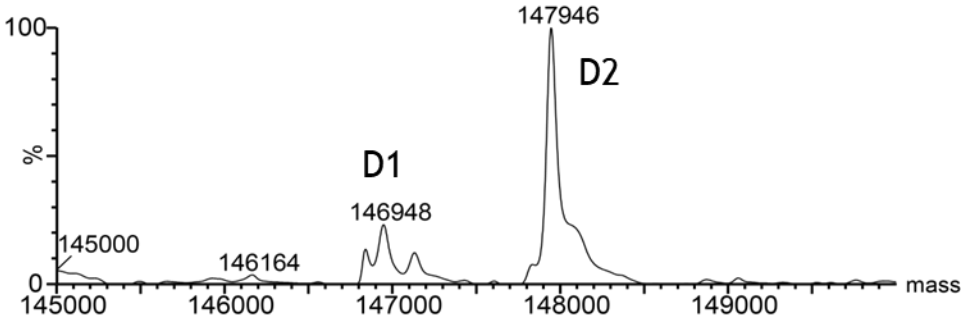

References

(1) Reid, E. E., Archer, K. E., Shizuka, M., Wilhelm, A., Yoder, N. C., Bai, C., Fishkin, N. E., Harris, L., Maloney, E. K., E., H. *et al.* (2019) Effect of linker stereochemistry on the antitumor activity of anti-body-drug conjugates (ADCs) containing indolinobenzodiazepine payloads. *ACS Medicinal Chemistry Letters* 10 (8), 1193-1197.

(2) Lazar, A. C., Wang, L., Blättler, W. A., Amphlett, G., Lambert, J. M. & Zhang, W. (2005) Analysis of the composition of immunoconjugates using size-exclusion chromatography coupled to mass spectrometry. *Rapid Communications in Mass Spectrometry* 19 (13), 1806-1814.